



pH-Dependent Modulation of Structural and Functional Properties of Safflower (*Carthamus tinctorius* L.) Protein Induced by Ultrasonic Treatment

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Abstract

This study aimed to evaluate the effects of ultrasonic and pH shift treatments (pH 2 and 12), applied both individually and in combination, on the structural and functional properties of safflower protein. Ultrasonic treatment at pH 7 significantly reduced particle size (from 457.60 to 144.14 nm) and surface hydrophobicity (from 38.93 to 33.93 μg), while enhancing solubility (from 15.32 to 26.04%) and foaming capacity (from 22.50 to 26.67%). Acidic pH shift (pH 2) increased particle size by up to 78%, accompanied by marked improvements in water holding capacity (up to 114%) and foaming capacity (up to 59%). Conversely, alkaline pH shift (pH 12) led to a substantial reduction in particle size (up to 74%) and a pronounced increase in surface hydrophobicity (up to 50%), resulting in the highest emulsion activity (14.62 m^2/g) and emulsion stability (5.50 min) indexes. The combined application of pH shift with ultrasonic treatments reduced particle size by up to 40% while increasing the absolute ζ -potential and surface hydrophobicity by 24% and 20%, respectively. These changes contributed to increases of up to 45% in solubility, 28% in water holding capacity, and 30% in foaming stability. However, reductions in emulsifying properties were observed, with the emulsion activity and emulsion stability indexes decreasing by up to 3.64 m^2/g and 0.88 min, respectively. Overall, the findings demonstrate that safflower protein functionality is highly pH-dependent and that the strategic combination of ultrasonic and pH shift treatments represents an effective approach to modulate its structural and functional properties for food applications.

Keywords Plant-based proteins · Protein modification · Foaming properties · Emulsifying properties · Solubility · Particle size

Introduction

The replacement of animal-based proteins with plant-based proteins has gained considerable momentum in the food industry, driven by economic, sustainability, dietary, allergenicity, and health concerns [1]. This increasing interest has also stimulated extensive research into the identification and utilization of novel and sustainable protein sources. Among these, safflower (*Carthamus tinctorius* L.), one of

the earliest cultivated oilseed crops, stands out due to its high oil yield and notable agronomic advantages, including tolerance to salinity, drought, and heat. Although safflower is primarily cultivated for oil production, its high essential amino acid content and favorable functional properties make the residual protein-rich meal a promising alternative protein source for producing protein concentrates and isolates [2]. Accordingly, previous studies have reported that safflower protein isolates can be effectively used in a wide range of food applications, including dough-based systems, bakery products, desserts, ice cream, meat analogs, soups, and beverages [3]. Despite this potential, the successful application of plant-based proteins as alternatives in food systems largely depends on improving their functional properties, including solubility, water and oil binding capacities, foaming and emulsifying properties, and gelation behavior [4]. For this purpose, various modification techniques, such

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as ultrasonic, microwave, heat, enzymatic, and pH shift treatments, have been widely investigated to enhance the functional performance of protein isolates [5].

Among these approaches, ultrasonic treatment has attracted considerable attention as a low-cost and environmentally friendly physical modification method to enhance protein functionality [6]. Its effects are primarily attributed to cavitation-induced shear forces, which disrupt intermolecular interactions and induce structural rearrangements, including subunit dissociation and aggregation [7]. In addition, pH shift is an effective chemical modification strategy that enhances protein functionality by exposing proteins to extremely acidic or alkaline conditions, thereby promoting extensive unfolding through the disruption of intermolecular forces. Subsequent readjustment to neutral pH results in partial refolding into more flexible conformations [8]. However, both ultrasonic and pH shift treatments have certain limitations when applied individually. Ultrasonic treatment may involve high equipment costs and potential quality deterioration under excessive treatment conditions [9]. Similarly, pH shift treatment may present limitations due to the exposure of proteins to extremely acidic or alkaline conditions, which can lead to protein denaturation and impaired biological activity. To overcome these limitations, the combination of pH shift and ultrasonic treatment has gained increasing attention as an effective strategy for enhancing protein structural modification and functionality [10].

Consistent with this, numerous studies have reported that the combined application of pH shift and ultrasonic treatments markedly improves the functional properties of various plant proteins, including soybean [11], faba bean [12], pea [13], rice [1], amaranth [6], sesame [14], rapeseed [15], peanut [16], melon seed [7], and pumpkin seed [17]. However, despite growing interest in safflower protein as a sustainable plant protein source, studies investigating the combined effects of pH shift and ultrasonic treatments on its structural and functional properties remain limited. To the best of our knowledge, only one study has investigated this approach in safflower protein, focusing exclusively on alkaline pH shift combined with ultrasonic treatment at different power levels [18]. Consequently, the effects of acidic pH shift, as well as the comparative and combined impacts of acidic and alkaline pH shift with ultrasonic treatment, remain largely unexplored. Therefore, the present study systematically evaluated the effects of acidic and alkaline pH shift, applied individually and in combination with ultrasonic treatment, on the structural and functional properties of safflower protein. By addressing this knowledge gap, this study provides new insights into the structure-function relationships of safflower protein under combined pH shift and ultrasonic treatments, highlighting its potential as a functional plant protein ingredient.

Materials and Methods

Materials

Safflower seed meal (Askon variety) was sourced from a commercial safflower oil processing facility (Ripsa-Özşahin Tarımcılık, Kayseri, Turkey). The meal was ground to pass through a 1-mm sieve and stored at 4 °C until protein extraction.

Protein Extraction

Safflower protein was extracted according to the protocol of Korkmaz [19] with some modifications. For defatting, safflower seed meal was mixed with hexane at a 1:3 (w/v) ratio and shaken at 200 rpm for 1 h at room temperature using an orbital shaker (Jeio Tech, OS-4000, Korea). This process was repeated three times, after which the solid residue was air-dried overnight at room temperature. The defatted meal was dispersed in distilled water at a 1:33 (g/mL) ratio and adjusted to pH 11.0 with 2 M NaOH, followed by shaking in a water bath (Nüve, ST 30, Turkey) at 23 °C and 125 rpm for 31 min. The mixture was then centrifuged (Nüve, NF 800, Turkey) at 4,000×g for 5 min, filtered through filter paper, and the supernatant was collected. Protein precipitation was achieved by adjusting the pH of the supernatant to 5.0 with 2 M HCl, followed by centrifugation at 4,000×g for 5 min. The resulting protein precipitate was re-suspended in distilled water to obtain approximately 10% (w/v) total solids, corresponding to an approximate protein concentration of 9.23% (w/v), and the pH was adjusted to 7.0 using 2 M NaOH.

pH Shift and/or Ultrasonic Treatments

Safflower proteins were modified using ultrasonic, pH shift, or a combination of both treatments. The safflower protein dispersion at neutral pH, without any treatment, served as the control and was labeled “pH 7”. For ultrasonic treatment, a safflower protein dispersion (10% w/v, 100 mL) at pH 7 was sonicated for 10 min in pulse mode (3 s on, 2 s off) at 100% amplitude using an ultrasonic processor (200 W, 20 kHz, Sonopuls HD 2200.2, Bandelin, Germany) equipped with a cylindrical probe (13.0 cm diameter). To control temperature rise during sonication, the beaker was placed in an ice-water bath. For the pH shift treatment, protein dispersions were adjusted to pH 2 or 12 by controlled addition of HCl or NaOH and stirred at room temperature for 1 h, after which the pH was readjusted to 7.0, and the samples were further stirred for an additional 1 h. The ultrasonic-assisted pH shift treatment was carried out by combining the pH shift and ultrasonic treatment described above. Briefly, protein

dispersions were first adjusted to pH 2 or 12 by controlled addition of HCl or NaOH and stirred for 1 h, followed by ultrasonic treatment for 10 min under the same conditions. The pH was subsequently readjusted to 7.0, and the samples were stirred for an additional 1 h. All treatments, including ultrasonic, pH shift, and combined processes, were independently performed in triplicate. The protein dispersions were freeze-dried in a lyophilizer (Teknosem, TRST 4/4 DS, Turkey) for further analysis. The moisture content of the powders ranged between 3.05 and 3.58%.

The absolute power input into the samples at maximum power was determined using the calorimetric method of Alavi et al. [12] as follows:

$$P = m C_p \frac{\Delta T}{\Delta t}$$

Where m is the sample mass (g), C_p is the specific heat capacity of the medium (4.18 kJ/g K), $\Delta T/\Delta t$ is the temperature rise rate (K/s). Power density was calculated by dividing P by the sample volume, yielding a constant value of 0.36 W/mL for all treatments.

The samples were coded as pH 7, pH 7-US, pH 2, pH 2-US, pH 12, and pH 12-US, where pH 7 represents the control, pH 7-US denotes the sample processed with ultrasonic treatment at pH 7, pH 2 and pH 12 correspond to samples modified by pH shift alone, and pH 2-US and pH 12-US refer to samples subjected to the combined pH shift and ultrasonic treatments.

Scanning Electron Microscopy

The surface morphology of protein samples was examined by Scanning Electron Microscopy (SEM, Jeol, JCM-5000 NeoScope, Japan) at an accelerating voltage of 15 kV. Samples were sputter-coated with gold prior to analysis. Micrographs were recorded at a magnification of $\times 1500$ [2].

Color

The color characteristics of powders were determined using a colorimeter (3nh, ST50, China) in the CIELAB colour space, providing L^* (lightness), a^* (red–green), and b^* (yellow–blue) values.

Particle Size and ζ -Potential

Protein samples were dispersed in distilled water to obtain a final concentration of 3.0 mg/mL, and their particle size and ζ -potential were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The prepared dispersions were

analyzed under the following conditions: a protein refractive index of 1.450, an absorbance of 0.001, and a measurement temperature of 25 °C [20].

Turbidity

Protein dispersions were prepared at a concentration of 1 mg/mL in distilled water and mixed for 30 min to achieve complete hydration, while distilled water served as the blank. Turbidity was evaluated by recording absorbance value at 600 nm using a UV-Vis spectrophotometer (Shimadzu, UV- 1280, Japan) [20].

Surface Hydrophobicity

Surface hydrophilicity of samples was evaluated using a bromophenol blue (BPB) binding assay based on the method of Zhang et al. [21], with minor modifications. Protein dispersion (5 mg/mL, 2 mL) was reacted with 400 μ L of BPB solution (1 mg/mL) and incubated at room temperature for 10 min. The mixture was then centrifuged at $2,000\times g$ for 5 min. A blank was prepared using phosphate buffer instead of protein solution while keeping the BPB volume constant. After appropriate dilution, the absorbance of the supernatant was measured at 595 nm, and surface hydrophobicity was calculated using the following equation.

$$\text{Bound BPB } (\mu\text{g}) = \frac{\text{Absorbance}_{\text{blank}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{blank}}} \times 200$$

Protein Solubility

Protein samples were dispersed in distilled water at a concentration of 10 mg/mL and centrifuged at $6,000\times g$ for 15 min. The supernatant was carefully collected, and the protein content was determined using the Lowry method [22]. The total protein content of protein samples (92.28%, dry basis; $N\times 6.25$) was determined according to the AACC standard method [23]. Protein solubility (PS) was calculated as the percentage of the protein content in the supernatant relative to the total protein content of the protein sample [5].

Water Holding Capacity

The water holding capacity (WHC) of samples was measured following the method of Huang et al. [8]. Briefly, protein dispersions (50 mg/mL) were centrifuged at $5,500\times g$ for 10 min, after which the supernatant was decanted, and the hydrated residue was weighed. WHC was calculated using the following equation.

$$\text{WHC (g/g)} = \frac{\text{weight of residue} - \text{weight of sample}}{\text{weight of sample}}$$

Foaming Properties

The foaming properties of samples were evaluated in terms of foaming capacity (FC) and foam stability (FS) according to the method described by Kang et al. [24]. A specified volume (V_0) of protein dispersion (10 mg/mL) was homogenized at 10,000 rpm for 2 min using a homogenizer (Witeg, HG-15D, Germany), and the total volume (V_1) was recorded. The dispersion was then left at room temperature for 30 min, and the total volume was measured again as V_2 . FC and FS were calculated using the measured volumes and the following equations [25].

$$\text{FC (\%)} = \frac{V_0 - V_1}{V_0}$$

$$\text{FS (\%)} = \frac{V_2 - V_0}{V_1 - V_0}$$

Emulsion Properties

The emulsifying properties of the samples were determined following a previously reported method [7]. Protein dispersions were prepared at a concentration of 5 mg/mL, and sunflower oil was added at a water to oil ratio of 3:1. The mixture was emulsified using a high-speed homogenizer at 10,000 rpm for 1 min. Aliquots (30 μ L) of the emulsion were collected from the bottom of the container at 0 and 10 min, and diluted with 3 mL of 0.1% SDS solution. Absorbance was measured at 500 nm and recorded as A_0 and A_{10} , with distilled water used as the blank. The samples were replaced with distilled water as a blank control. The emulsion activity index (EAI) and emulsion stability index (ESI) were calculated following equations.

$$\text{EAI (m}^2\text{/g)} = \frac{4606 \times A_0 \times D}{C \times \varphi \times L \times 10^4}$$

$$\text{ESI (min)} = \frac{A_0}{A_0 - A_{10}} \times 10$$

where D is the dilution factor (100), C is the soluble protein concentration (g/mL), φ is the oil volume fraction in the emulsion, and L is the optical length of the cuvette (1 cm).

SDS-PAGE

SDS-PAGE of protein samples was conducted as described by Sun et al. [20], and a standard protein ladder with a

Fig. 1 SEM images of safflower protein samples

molecular weight range of 11–245 kDa was used. Protein dispersion (5 mg/mL) was prepared in deionized water and centrifuged at 6,000 $\times$ g for 10 min. The supernatant was mixed with loading buffer (0.5 M Tris-HCl, pH 6.8; 1% (v/v) SDS; 2.5% (v/v) glycerol; 0.1% (v/v) bromophenol blue; 0.5% (v/v) 2-mercaptoethanol) and heated at 100 $^{\circ}$ C for 10 min to denature the proteins. Running was performed at 80 V for separating and 100 V for stacking gels using a polyacrylamide gel electrophoresis system (Bio Rad, Mini Protean, USA). Following electrophoresis, the gel was fixed and stained with Coomassie Brilliant Blue R-250 solution (50% methanol, 10% acetic acid), then destained with deionized water and imaged.

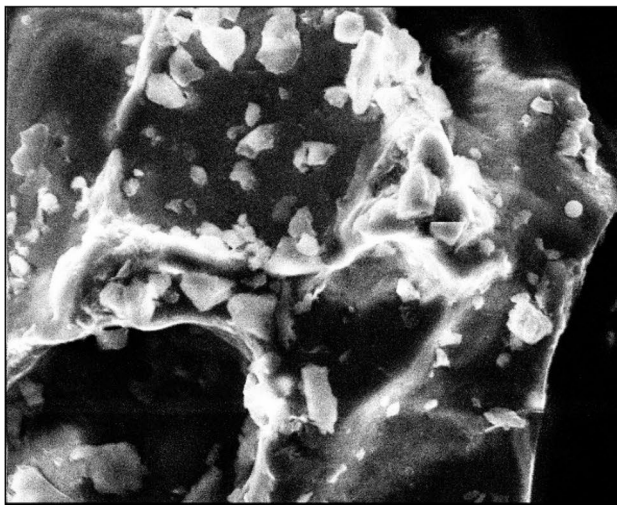
Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) in MINITAB software (version 17.0, USA), and differences among means were evaluated using Tukey's post hoc test. All measurements were conducted in triplicate and expressed on a dry-weight basis. Data are presented as mean $\pm$ standard error.

Result and Discussion

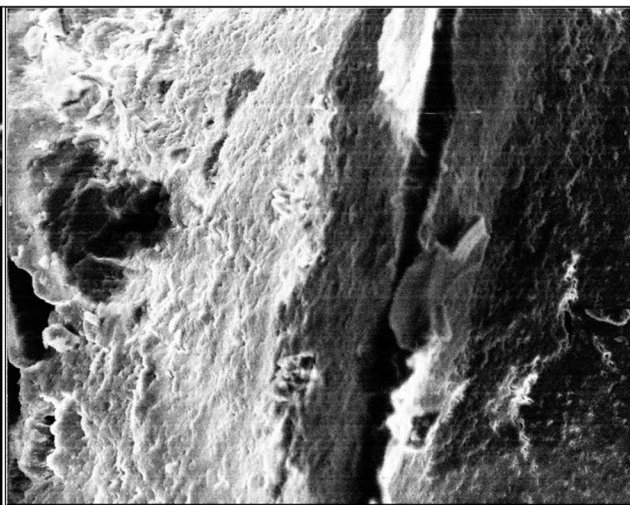
SEM and Color

Surface morphology, evaluated by SEM, provides critical information on microstructural changes during protein modification, enabling the visualization of the effects of physical treatments on particle structure, porosity, and surface characteristics [26]. As shown in Fig. 1, the control sample exhibited a relatively compact and dense structure with smooth and continuous surfaces. However, after ultrasonic treatment (pH 7-US), the surface structure became more fragmented and irregular, with noticeable surface disruptions. Similar observations were reported in previous studies, indicating that ultrasonic treatment can increase surface damage in rapeseed and pea proteins due to its strong mechanical effects [15, 26]. pH shift treatment at pH 2 resulted in the formation of larger, more porous, and sponge-like aggregates compared to the control. This behavior may be attributed to protein denaturation under acidic conditions, which promotes the exposure of hydrophobic clusters and reactive groups, thereby enhancing intermolecular interactions and leading to the formation of larger aggregates [27, 28]. Moreover, during pH readjustment from 2 to 7, passing through the isoelectric point ($\approx$ pH 5) may lead to a reduction in electrostatic repulsion, which



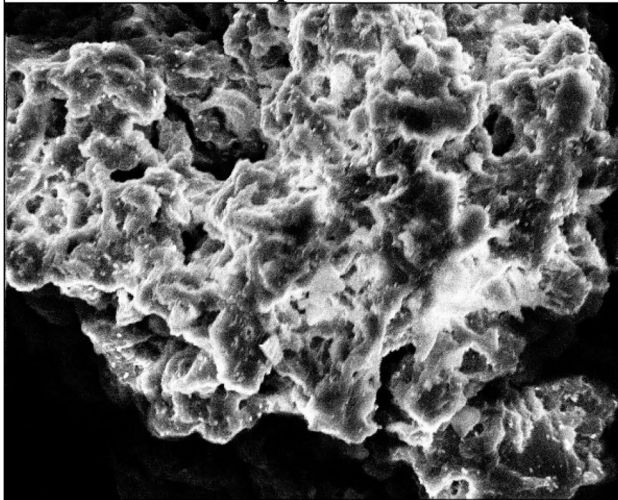
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Bilim ve Teknoloji Uygulama ve Araştırma Merkezi, Balıkesir Üniversitesi

pH 7



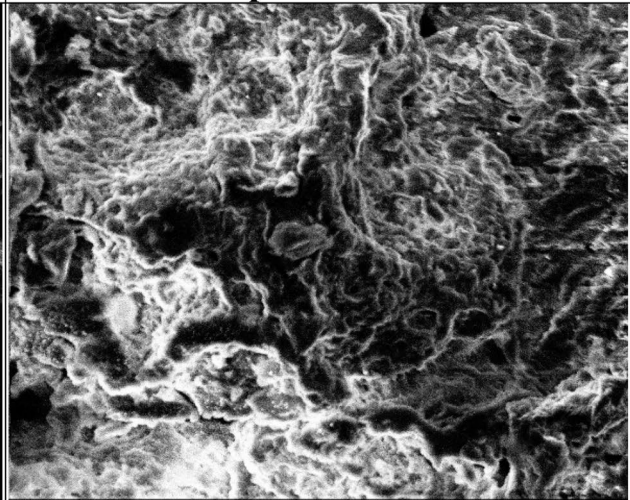
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pH 7-US



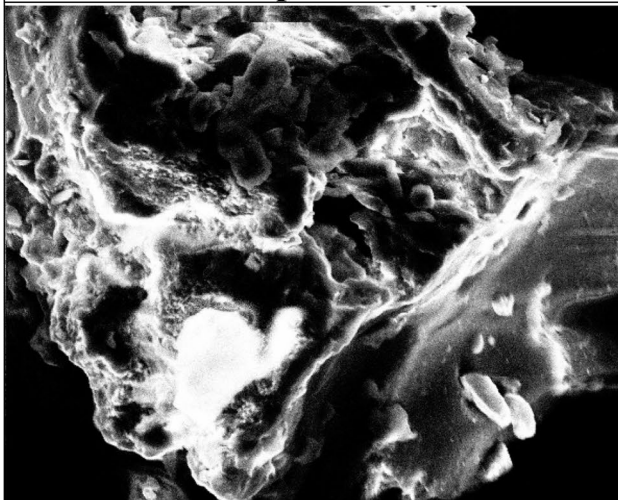
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pH 2



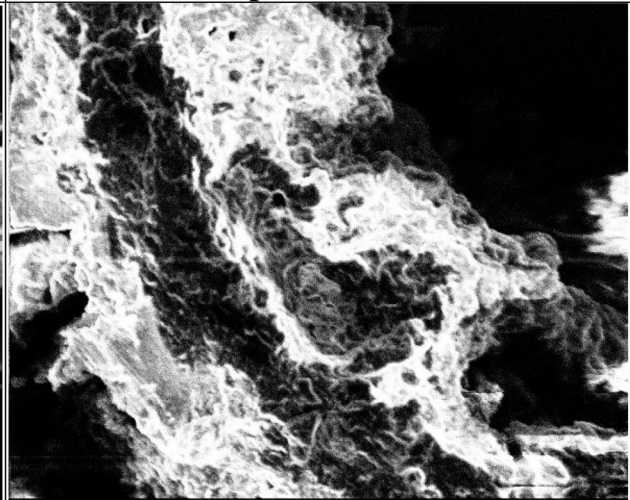
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Bilim ve Teknoloji Uygulama ve Araştırma Merkezi, Balıkesir Üniversitesi

pH 2-US



Vac-High PC-Std. 15 kV x 1500 20 μm 003134
Bilim ve Teknoloji Uygulama ve Araştırma Merkezi, Balıkesir Üniversitesi

pH 12



Vac-High PC-Std. 10 kV x 1500 20 μm 003160
Bilim ve Teknoloji Uygulama ve Araştırma Merkezi, Balıkesir Üniversitesi

pH 12-US

could promote protein aggregation and the development of porous, sponge-like structures [28]. On the other hand, the sample treated pH shift at pH 12 exhibited larger lamellar-like structure, which may be attributed to the exposure of hydrophobic and sulfhydryl groups and the formation of large aggregates under extreme alkaline conditions [5, 11]. The combined application of ultrasonic and pH shift treatments at both pH 2 and pH 12 produced highly irregular and fragmented structure, indicating substantial disruption of protein aggregates. These structural alterations may be attributed to the cavitation effect generated during ultrasonication, which can increase protein surface charge and promote the exposure of nonpolar groups [21, 29]. Moreover, the intense physical forces associated with ultrasound, including microjets, shear forces, shock waves, and turbulence, can disrupt hydrogen bonding within protein structures, thereby contributing to the formation of cracks and surface fractures observed in the SEM images [21].

Color is an important factor in evaluating the quality and acceptability of food products; therefore, the color characteristics of the samples were determined, and the results are presented in Table 1. The L^* values of the samples ranged from 51.61 to 54.88, and ultrasonic treatment resulted in a slight but significant decrease in L^* value, indicating a minor darkening ($p < 0.05$). This effect may be attributed to ultrasonic treatment-induced protein unfolding, which increases the exposure of molecular regions previously embedded within the macromolecular structure, thereby reducing lightness [30]. The a^* and b^* color parameters were significantly affected by pH shift treatments ($p < 0.05$). pH shift treatment at pH 12 resulted in increased a^* value, indicating enhanced redness. This effect may be associated with the increased solubility of carotenoprotein complexes under strongly alkaline conditions. Moreover, exposure to high pH can induce carotenoprotein denaturation, leading to the release of free carotenoids and, consequently, a more pronounced red appearance [31]. In contrast, pH shift treatment at pH 2 caused a reduction in both a^* and b^* values, suggesting decreased redness and yellowness. This phenomenon was likely due to a greater reduction in pigment intensity during acid processing, resulting in lighter and less intensely colored protein isolates [32].

Table 1 The color characteristics of powders

	L^*	a^*	b^*
pH7	52.99 ± 0.54 ^{ab}	2.59 ± 0.09 ^d	27.85 ± 0.40 ^b
pH7-US	51.61 ± 0.66 ^b	3.37 ± 0.14 ^c	29.20 ± 0.23 ^a
pH2	54.88 ± 0.20 ^a	2.11 ± 0.05 ^e	24.08 ± 0.10 ^c
pH2-US	53.55 ± 0.51 ^{ab}	1.93 ± 0.01 ^e	23.34 ± 0.17 ^c
pH12	53.60 ± 0.06 ^{ab}	3.80 ± 0.05 ^b	27.94 ± 0.23 ^b
pH12-US	52.12 ± 0.23 ^b	4.76 ± 0.02 ^a	29.91 ± 0.11 ^a

*Means followed by different letters in each column are significantly different ($p < 0.05$)

Particle size, ζ -Potential, Turbidity, and Surface Hydrophobicity

Particle size is a key physical characteristic that significantly affects functional properties, including solubility, foaming, and emulsion properties [8, 11]. The particle size of the control sample (pH 7) was 457.60 nm and decreased significantly to 144.14 nm after ultrasonic treatment (pH 7-US) ($p < 0.05$) (Fig. 2a). These results were consistent with previous studies reporting that ultrasonic treatment significantly decreases the particle sizes of soy [20], pea [13, 20], faba bean [12], and rapeseed [15] proteins. The authors attributed this decrease to the mechanical effects of ultrasonic cavitation, which generate strong shear forces that disrupt hydrogen bonding as well as the hydrophobic and electrostatic interactions in large protein aggregates [12, 13, 15, 20]. pH shift treatment at pH 2 caused a significant increase in the particle size of safflower protein, reaching 813.70 nm ($p < 0.05$). Similarly, Kang et al. [24] reported an increase in the particle size of soybean protein after acidic pH shift treatment, which was attributed to enhanced non-covalent interactions leading to protein aggregation. On the other hand, pH shift treatment at pH 12 resulted in a decrease in the particle size of safflower protein up to 119.48 nm ($p < 0.05$). This decrease in particle size under highly alkaline conditions can also be attributed to increased ionic strength [11]. When the pH is far from the isoelectric point of protein, electrostatic repulsion is enhanced, leading to a reduction in protein particle size [24]. Moreover, combining pH shift at both pH 2 and pH 12 with ultrasonic treatment reduced the particle size of safflower protein ($p < 0.05$). The combination of pH shift and ultrasonic treatment may disrupt intermolecular disulfide bonds, leading to protein depolymerization. Consequently, large aggregates can dissociate into smaller subunits or monomers, resulting in a reduced particle size [8].

The surface charge of proteins, as indicated by the ζ -potential, determines system stability, with higher absolute ζ -potential values leading to stronger electrostatic repulsion and reduced aggregation [24]. As illustrated in Fig. 2b, ultrasonic treatment significantly increased the absolute ζ -potential of safflower protein at pH 7 ($p < 0.05$). This increase can be attributed to structural disruption induced by ultrasonic treatment, which may expose additional charged groups along the protein chains [15]. The effect of pH shift treatment at pH 2 on the absolute ζ -potential of safflower protein was insignificant ($p > 0.05$). However, the absolute ζ -potential of safflower protein exhibited a significant increase after pH shift treatment at pH 12 ($p < 0.05$). This phenomenon can be explained by protein unfolding structure and the resulting enhancement in surface charge after alkaline pH shift, which was consistent with the findings

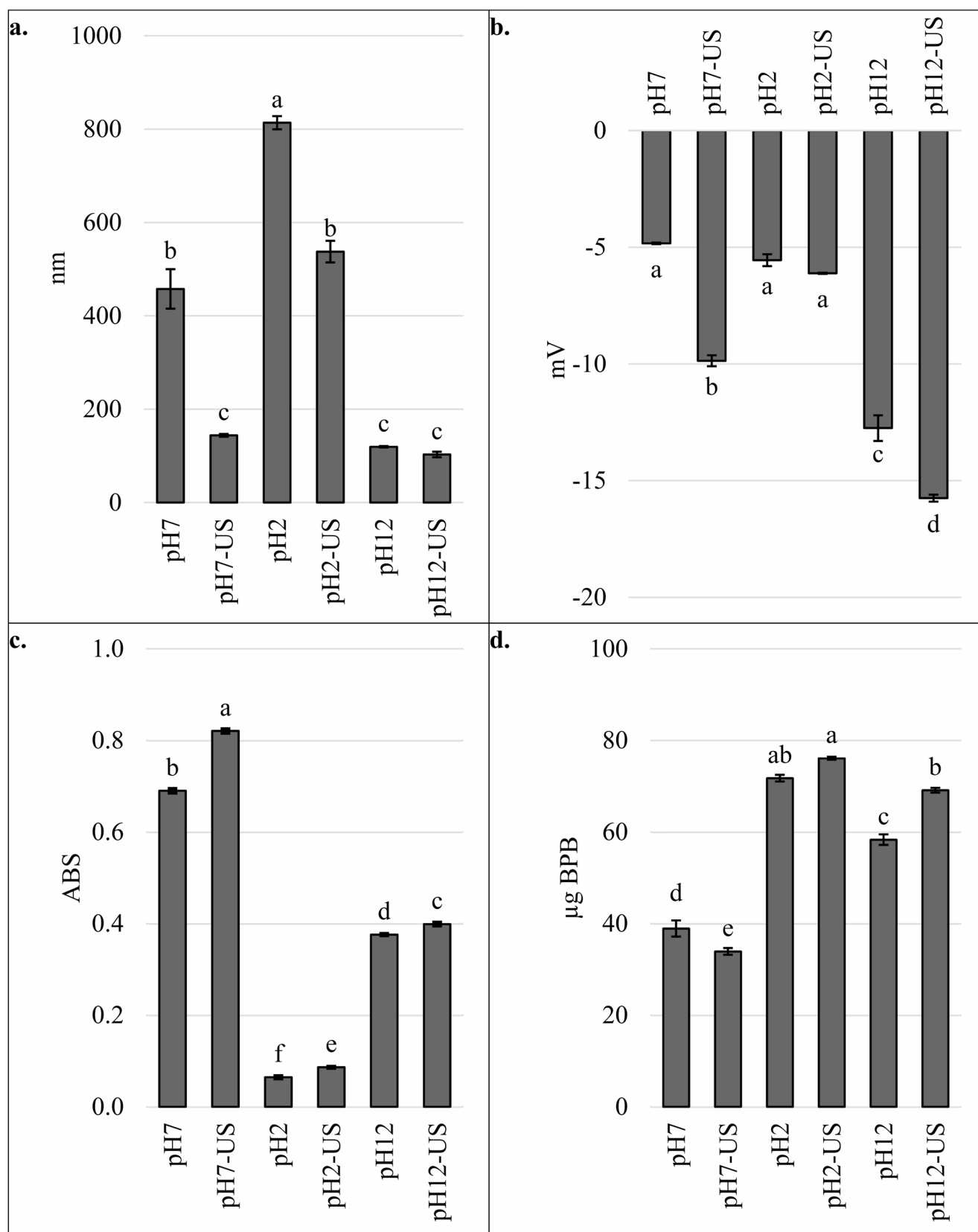


Fig. 2 Effects of pH shift and ultrasonic treatments on the average particle size (a), zeta potential (b), turbidity (c), and surface hydrophobicity (d) of safflower protein samples

reported for soybean protein by Kang et al. [24]. Furthermore, the combination of pH shift at pH 12 and ultrasonic treatment significantly increased the absolute ζ -potential of safflower protein ($p < 0.05$). This suggested that the combined treatment more effectively disrupted the protein structure, promoting the exposure and migration of charged groups on the protein surface, and thereby enhancing system stability [24].

The turbidity of the control sample (pH 7) increased from 0.690 to 0.821 after ultrasonic treatment (pH 7-US) ($p < 0.05$) (Fig. 2c). In contrast, pH shift treatments dramatically decreased the turbidity of safflower protein, with a more pronounced reduction observed in samples processed at pH 2 ($p < 0.05$). However, the combined application of pH shift and ultrasonic treatments resulted in a significant increase in turbidity ($p < 0.05$). Since turbidity was an indicator of protein aggregation [8], higher turbidity values were associated with increased soluble protein levels [13].

Surface hydrophobicity represents the exposure of hydrophobic residues on the protein surface and is strongly linked to its foaming, emulsifying, and solubility properties [7]. Ultrasonic treatment significantly decreased the surface hydrophobicity of safflower protein at pH 7 ($p < 0.05$) (Fig. 2d). This decrease may be attributed to ultrasonic energy-induced partial protein denaturation, which can enhance intermolecular interactions and binding affinity [24]. Moreover, ultrasonic treatment induced unfolding of the molecular structure of proteins, leading to the formation of additional non-covalent bonds that may mask the hydrophobic regions of proteins, reducing their surface hydrophobicity [21]. A significant increase in surface hydrophobicity was observed in safflower protein when subjected to pH shift at both pH 2 and pH 12 ($p < 0.05$). These findings agreed with earlier studies showing that pH shift treatment increased the surface hydrophobicity of quinoa [25], faba bean [12], and soybean [24] proteins by promoting structural unfolding and the exposure of previously buried hydrophobic residues [25]. The combination of pH shift and ultrasonic treatments resulted in a greater increase in surface hydrophobicity than pH shift alone ($p < 0.05$). This result indicated that the combined action of ultrasonic cavitation and the polarity of acidic and alkaline conditions induced

protein unfolding and disrupted hydrophobic interactions, thereby exposing more hydrophobic residues [15].

Functional Properties

Protein Solubility

PS is a critical functional property of proteins, as it strongly influences other characteristics, including WHC, foaming, and emulsifying properties [5]. PS was significantly affected by all treatments (Table 2). The control sample (pH 7) exhibited a PS of 15.32%, whereas ultrasonic treatment markedly increased solubility to 26.04% ($p < 0.05$). Several studies were also reported an increase in the solubility of plant proteins after ultrasonic treatment [12, 13, 15, 20]. This improvement was mainly attributed to ultrasonic cavitation, which disrupts intermolecular interactions, including hydrogen bonding, van der Waals forces, and dipole-dipole interactions, thereby promoting protein-water interactions and increasing solubility [15]. Moreover, the reduction in the particle size of safflower protein after ultrasonic treatment may have contributed to the increased PS. A decrease in particle size increased the specific surface area available for hydration, thereby promoting protein-water interactions and enhancing PS [7, 12]. On the other hand, pH shift at pH 2 caused a dramatic decrease in the PS of safflower protein ($p < 0.05$), indicating extensive aggregation and precipitation under strongly acidic conditions. Under such conditions, proteins were highly susceptible to denaturation, and the formation of disulfide bond cross-links, along with hydrophobic aggregation, results in a pronounced reduction in PS [24]. The PS of the sample treated with pH shift at pH 12 was significantly higher than at pH 2, but still lower than the control ($p < 0.05$), reflecting partial unfolding accompanied by moderate aggregation. The PS observed after alkaline pH shift can be attributed to protein unfolding at high pH due to electrostatic repulsion and deamidation, followed by partial refolding after neutralization [20]. Additionally, the changes in PS observed in this study were generally consistent with the corresponding variations in surface hydrophobicity. The exposure of hydrophobic groups during pH shift treatment may promote protein-protein interactions, thereby reducing solubility [12]. Consistent with previous

Table 2 The techno-functional properties of powders

	PS (%)	WHC (g/g)	FC (%)	FS (%)	EAI (m ² /g)	ESI (min)
pH7	15.32 ± 0.33 ^b	1.08 ± 0.04 ^{de}	22.50 ± 1.44 ^c	81.76 ± 2.94 ^a	9.26 ± 0.15 ^c	4.06 ± 0.07 ^c
pH7-US	26.04 ± 0.44 ^a	0.93 ± 0.02 ^e	26.67 ± 1.67 ^{bc}	78.33 ± 1.67 ^{ab}	7.97 ± 0.06 ^d	3.11 ± 0.10 ^d
pH2	1.33 ± 0.04 ^e	2.31 ± 0.04 ^b	35.83 ± 0.83 ^a	44.13 ± 1.27 ^c	3.46 ± 0.04 ^e	1.28 ± 0.06 ^e
pH2-US	1.62 ± 0.11 ^e	2.95 ± 0.04 ^a	36.67 ± 1.67 ^a	68.45 ± 2.98 ^b	3.00 ± 0.03 ^e	0.74 ± 0.01 ^f
pH12	8.97 ± 0.50 ^d	1.26 ± 0.04 ^d	34.17 ± 2.20 ^a	53.89 ± 2.42 ^c	14.62 ± 0.05 ^a	5.50 ± 0.06 ^a
pH12-US	13.00 ± 0.32 ^c	1.51 ± 0.05 ^c	30.83 ± 0.83 ^{ab}	70.30 ± 2.46 ^b	10.98 ± 0.25 ^b	4.62 ± 0.04 ^b

^aMeans followed by different letters in each column are significantly different ($p < 0.05$)

studies on soy [24], pea [13], rice [1], and rapeseed [15] proteins, the combination of ultrasonic and pH shift treatments exhibited a synergistic effect. This effect led to enhanced PS through protein unfolding, aggregate disruption, and increased protein-water interactions [13, 15, 24].

Water Holding Capacity

WHC is a critical functional property for protein isolates incorporated into aqueous food systems, as it directly influences viscosity, consistency, and textural properties [5, 11]. The WHC of the control sample (pH 7) was 1.08 g/g, whereas that of the ultrasonic-treated sample was 0.93 g/g, corresponding to a decrease of 13.89% ($p < 0.05$) (Table 2). The decrease in WHC after ultrasonic treatment can be attributed to changes in the PS of safflower protein. Increased PS led to a higher fraction of proteins remaining in the supernatant during centrifugation, thereby reducing the amount of protein available to retain water within the system [2]. In contrast, pH shift treatments at both pH 2 and pH 12 significantly increased WHC of safflower protein ($p < 0.05$), indicating that exposure to extreme pH conditions promoted protein unfolding and structural rearrangement, thereby enhancing the availability of hydrophilic groups for water binding [8]. Furthermore, the combination treatment further enhanced WHC at both pH levels, indicating a synergistic interaction between pH shift and ultrasonic treatments. Ultrasonic cavitation likely intensified pH-induced protein unfolding, thereby strengthening protein-water interactions [11].

Foaming Properties

FC reflects the ability of proteins to form air-water dispersions, whereas FS refers to the proportion of foam retained after a given period and is a key indicator of the structural stability of whipped food systems [6]. As shown in Table 2, ultrasonic treatment significantly increased the FC of safflower protein at pH 7 ($p < 0.05$). A similar result was reported by Alavi et al. [12], who observed that the FC of faba bean protein significantly increased from 93.3% to 193.3–265.3%. The authors attributed this increase to the reduction in particle size and the enhancement of protein solubility induced by ultrasonic treatment [12], which was consistent with the results obtained for safflower protein. Additionally, pH shift treatments at both pH 2 and pH 12 markedly increased FC ($p < 0.05$). The highest FC values were observed for the pH 2 and pH 2-US samples ($p < 0.05$), indicating that acidic pH conditions enhanced protein adsorption at the air-water interface [6]. This behavior was consistent with increased surface hydrophobicity, which promotes protein-protein interactions and favors the

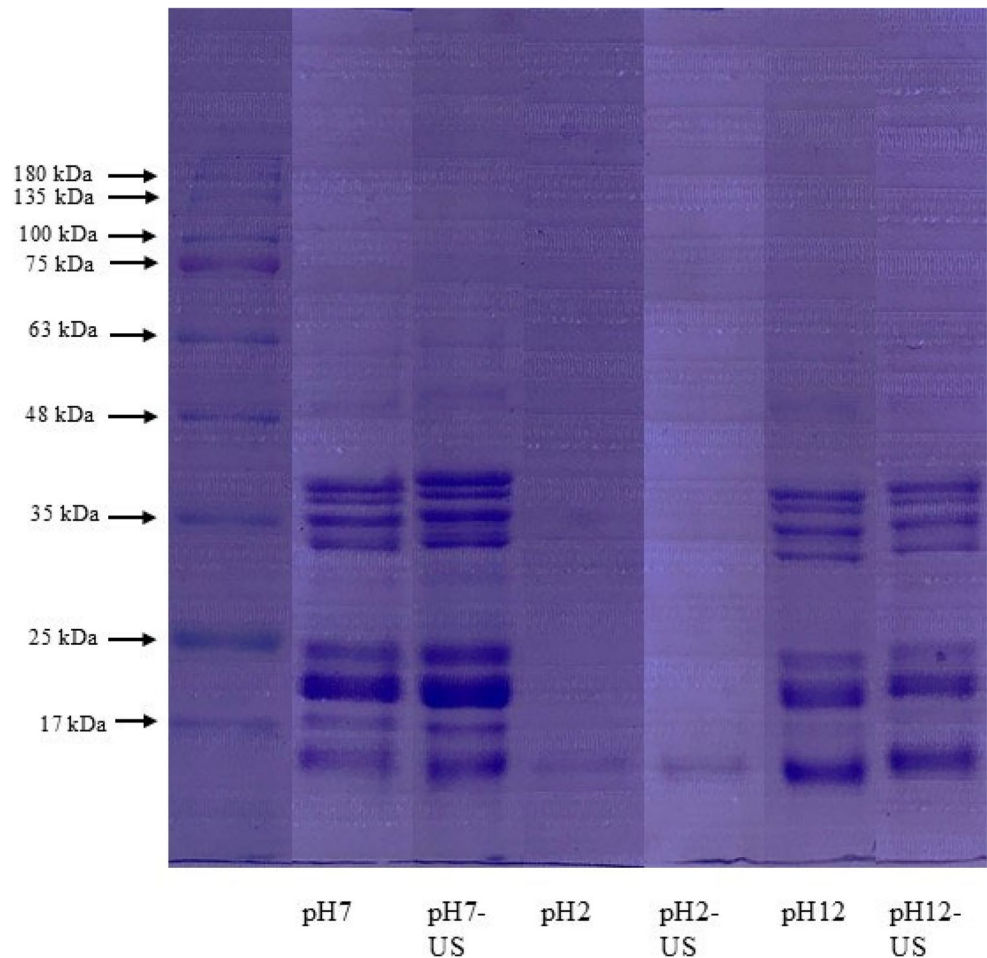
formation of more viscous and cohesive interfacial films [6]. Ultrasonic treatment exerted a pH-dependent effect on FC, slightly improving FC at pH 2 while leading to a decrease at pH 12. This pH-dependent effect of ultrasonication on FC may be related to variations in protein conformational flexibility. In particular, reduced protein flexibility induced by ultrasonication may impair protein rearrangement at the air-water interface, thereby decreasing foaming capacity [33].

In contrast to FC, FS showed a different trend. The control sample at pH 7 exhibited the highest FS, whereas a pronounced reduction in FS was observed at pH 2, suggesting that although acidic conditions favored foam formation, the resulting foam was less stable due to the formation of less viscoelastic and cohesive interfacial protein films [25]. Notably, ultrasonic treatment significantly improved FS at both pH 2 and pH 12, consistent with previous studies on soybean proteins, where Kang et al. [24] reported similar improvements in FS under both acidic and alkaline pH shift conditions. The increased FS observed in the pH 2-US and pH 12-US samples may be attributed to the increased surface hydrophobicity of samples. This promotes the formation of a stronger and more elastic protein membrane at the interfacial layer, thereby delaying liquid drainage between bubbles and improving FS [24].

Emulsion Properties

The emulsifying properties of proteins describe their ability to form and stabilize oil-water systems by adsorbing at the interface [14]. These properties are commonly characterized by two parameters: the EAI, which reflects the effectiveness of proteins in forming emulsions, and the ESI, which indicates the ability of the emulsion to remain stable over time [17]. The control sample (pH 7) demonstrated moderate emulsifying properties, with 9.26 m²/g EAI and 4.06 min ESI (Table 2). Ultrasonic treatment at pH 7 resulted in a significant decrease in both EAI and ESI ($p < 0.05$). This reduction indicated that ultrasonic treatment-induced changes in surface hydrophobicity may have impaired the ability of proteins to adsorb at the oil-water interface and form a stable interfacial film [2, 7]. Similarly, pH shift treatments at both pH 2 and pH 12 significantly affected the emulsifying performance of safflower protein ($p < 0.05$), and this pH-dependent effect was mainly attributed to variations in PS of samples [28]. pH shift treatment at pH 2 caused a significant decrease in both EAI and ESI, indicating impaired emulsifying performance under acidic conditions. This was consistent with previous findings reporting that emulsifying properties were strongly dependent on protein solubility, with lower solubility leading to diminished emulsifying performance [34]. In particular, the decreased emulsifying properties at pH 2 can be attributed to reduced protein solubility

Fig. 3 SDS-PAGE patterns of safflower protein samples



under acidic conditions, which limits protein adsorption at the oil–water interface. In contrast, pH shift treatment at pH 12 significantly enhanced emulsifying properties, yielding the highest EA (14.62 m²/g) and ES (5.50 min) among all samples ($p < 0.05$). Similar improvements in emulsifying performance following alkaline pH shift were reported for various plant proteins, including soybean [24], rapeseed [34], sesame [14], peanut [21], and quinoa [25] proteins. The enhanced emulsifying performance under alkaline conditions was primarily attributed to protein unfolding and increased random-coil formation. These structural changes provided more effective adsorption of protein molecules at the oil–water interface [14, 24, 25]. Moreover, the decreased particle size of samples likely reduced interfacial tension, thereby enhancing interfacial activity and overall emulsion performance [25]. The impaired emulsifying performance observed after the combination of ultrasonic treatment with acidic and alkaline pH shift may be attributed to altered protein structure, which makes them more susceptible to aggregation or reduces their hydrophilicity [16, 21]. Consequently, protein adsorption at the oil–water interface decreased, resulting in reduced emulsifying performance.

SDS-PAGE

The molecular weight distribution of safflower protein samples was analyzed using SDS-PAGE (Fig. 3), which demonstrated that the various treatments significantly altered the molecular weight profiles. The control sample (pH 7) exhibited several distinct protein bands, mostly with molecular weights lower than 75 kDa. After ultrasonic treatment (pH 7-US), the overall band pattern remained similar; however, band intensity slightly increased, suggesting that ultrasonication enhances protein solubility by disrupting weak intermolecular interactions and releasing protein molecules from aggregates [24, 33]. In contrast, the protein profile was markedly altered after pH shift at pH 2. The bands became extremely weak or nearly disappeared, indicating extensive protein aggregation and precipitation under strongly acidic conditions, which reduced protein solubility and limited their migration into the gel [25]. Alkaline pH shift caused an increase in the intensity of low-molecular-weight bands (<17 kDa) compared with the control. This indicated that exposure to strong alkaline conditions induced protein unfolding and dissociation of large protein aggregates into

smaller subunits [24]. It should be noted that SDS-PAGE reflects the distribution of soluble protein fractions under denaturing conditions, while variations in band intensity may also be influenced by changes in protein solubility and electrophoretic mobility. Accordingly, the observed differences may be interpreted as reflecting treatment-induced alterations in protein structure and solubility rather than a single, well-defined mechanism of aggregation or dissociation.

Conclusions

In this study, safflower protein was modified using pH shift and ultrasonic treatments (pH 2 and 12), applied both individually and in combination, to investigate their effects on structural and functional properties. Ultrasonic treatment induced cavitation-driven shear forces that disrupted protein aggregates, leading to a marked reduction in particle size. These structural changes enhanced PS and FC, while the accompanying decrease in surface hydrophobicity limited protein adsorption at the oil-water interface, resulting in reduced emulsifying performance. pH shift treatment at pH 2 caused extensive protein aggregation and precipitation due to strong intermolecular interactions and partial denaturation. This structural rearrangement increased particle size and promoted higher WHC and FC values; however, the reduced PS and impaired interfacial activity under acidic conditions led to a pronounced decrease in emulsifying properties. In contrast, pH shift at pH 12 promoted protein unfolding and enhanced electrostatic repulsion, resulting in a smaller particle size, higher surface charge, and increased exposure of hydrophobic residues. These changes significantly improved EAI and ESI, highlighting the favorable impact of alkaline treatment on interfacial functionality. The combined application of pH shift and ultrasonic treatments further intensified protein structural modification, as evidenced by reduced particle size, increased absolute ζ -potential, and elevated surface hydrophobicity. While this synergistic treatment improved solubility, water-holding capacity, and foaming stability, excessive structural disruption appeared to negatively affect emulsifying performance. Overall, the results demonstrate that the functional behavior of safflower protein is highly dependent on pH conditions. Controlled pH shift combined with ultrasonic treatment represents an effective strategy for tailoring the structure-function relationships of safflower protein for targeted food applications.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical Approval This article contains no studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

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