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Application of high-intensity ultrasound to *Coccoloba uvifera* seeds proteins and its effect on the amino acid profile, techno-functional properties, and antioxidant capacity

Aplicación de ultrasonido de alta intensidad a proteínas de semillas de *Coccoloba uvifera* y su efecto sobre el perfil de aminoácidos, propiedades tecno-funcionales y capacidad antioxidante

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ABSTRACT

Applying high-intensity ultrasound (HIU) to plant proteins improves the techno-functionality and antioxidant activity. Then, this research examined the impact of HIU at different power levels (600, 840, and 1080 W) and durations (10, 15, and 20 min) on the amino acid composition, hydrosolubility, foaming, emulsification, and antioxidant capacity of *Coccoloba uvifera* seed protein (CUSP). Compared with the control (untreated protein), CUSP subjected to HIU presented increased levels of glutamic acid, aspartic acid, proline, glycine, and serine. The hydrosolubility of samples treated with HIU significantly improved, ranging from 79.90 to 87.53 %. Treatment with HIU at 600 W for 10 min enhanced the foaming properties, while exposing CUSP to HIU at 600 and 1080 W for 15 min improved the emulsifying properties. Compared with the control (52.12 ± 1.85 %), the 840 W treatment for 15 min enhanced the antioxidant properties (96.82 ± 0.16 %). The application of HIU in CUSP enhanced the functionality. However, the degree of improvement depends on the HIU level and the treatment duration. This study demonstrated the feasibility of employing HIU to increase the functional attributes of plant proteins for potential utilization in food products.

KEY WORDS: Plant protein, Protein modification, Particle size distribution, Emulsions, Techno-functional properties.

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RESUMEN

La aplicación de ultrasonido de alta intensidad (HIU) a proteínas vegetales mejora la tecno-funcionalidad y actividad antioxidante. Por ello, esta investigación examinó el impacto del HIU a diferentes niveles de potencia (600, 840 y 1080 W) y duraciones (10, 15 y 20 min) sobre la composición de aminoácidos, hidrosolubilidad, formación de espuma, emulsificación y capacidad antioxidante de la proteína de la semilla de *Coccoloba uvifera* (CUSP). En comparación con el control (proteína no tratada), la CUSP sometida a HIU presentó mayores niveles de ácido glutámico, ácido aspártico, prolina, glicina y serina. La hidrosolubilidad de las muestras tratadas con HIU mejoró significativamente en un rango de 79.90 a 87.53 %. El tratamiento con HIU a 600 W durante 10 min mejoró las propiedades espumantes, mientras que la exposición a 600 y 1080 W durante 15 min mejoró las propiedades emulsionantes. En comparación con el control (52.12 ± 1.85 %), el tratamiento de 840 W durante 15 min mostró alta propiedad antioxidante (96.82 ± 0.16 %). La aplicación de HIU a la CUSP mejoró la funcionalidad. Sin embargo, el grado de mejora dependió del nivel de HIU y de la duración del tratamiento, demostrando la viabilidad de HIU para aumentar atributos funcionales de proteínas vegetales para posible utilización en productos alimenticios.

PALABRAS CLAVE : Proteína vegetal, Modificación de proteínas, Distribución del tamaño de partículas, Emulsiones, Propiedades tecno-funcionales.

Introduction

The food industry mainly depends on animal-derived proteins. However, the rising global population has increased the demand for these proteins, which has caused considerable environmental damage, including greenhouse gas emissions and biodiversity loss. Therefore, exploring alternative protein sources like seed fruits is essential, as they offer promising protein options (Yu *et al*., 2021). *Coccoloba uvifera L.*, commonly called seagrape, is a tree native to tropical America and the Caribbean. *C. uvifera* mainly grows in shrubby areas along the Atlantic and Pacific coasts of Mexico (Collantes-Chávez-Costa et al., 2019). This species has been used in traditional Mexican medicine for a long time. Recently, interest in *C. uvifera* has grown, as research has shown that its fruit contains compounds that demonstrate significant antioxidant activity. The seeds of *C. uvifera* comprise about 80–85% of the fruit's weight, containing a considerable amount of carbohydrates and a viable protein fraction for extraction (Segura-Campos et al., 2015). The potential for extracting protein from *C. uvifera* seeds for food industry applications remains unexplored, highlighting the critical need for further research.

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Plant-based proteins extracted through conventional methods have compact structures and limited functional properties, necessitating structural modification. High-intensity ultrasound (HIU, 10–1000 W/cm² or 20–100 kHz) has been utilized for protein modification because it generates higher production yields, enhances the quality of processed foods, is user-friendly, and is environmentally sustainable (Flores-Jiménez *et al*., 2023). Ultrasound alters proteins primarily through cavitation, which includes shock waves, microcurrents, microjets, turbulence, shear forces, and elevated temperature and pressure. These effects modify food materials by breaking down intermolecular interactions such as hydrogen bonds, electrostatic forces, disulfide bridges, van der Waals forces, and hydrophobic interactions (Flores-Jiménez *et al*., 2023; Xue *et al*., 2018).

Previous research has shown that HIU treatment can reduce the particle size of protein molecules, alter amino acid profiles and thermal/rheological properties, and enhance the hydrosolubility and surface hydrophobicity of various plant proteins, such as those from fava beans (Adal, 2024), potato (Zhao *et al*., 2022), and millet (Nazari *et al*., 2018). These modifications enhance water and oil retention, foaming, gelation, and emulsification by altering the secondary and tertiary structures of the proteins. However, to our knowledge, no studies have concentrated on extracting proteins from *C. uvifera* seeds to showcase their potential use in the food industry. Therefore, this research aims to extract *C. uvifera* seed protein (CUSP), followed by applying high-intensity ultrasound to evaluate their effects on the amino acid profile, techno-functional properties, and antioxidant capacity. This research could have a significant impact, as it may produce CUSP with acceptable technology properties. The extraction of antioxidants and proteins from *C. uvifera* can encourage expanding cultivation practices and highlight the importance of coastal dune flora for various applications in the food and pharmaceutical industries. This initiative is set to promote agricultural development, thereby supporting the economic growth of the region where the fruit originates.

Material and methods

Reagents chemicals

N-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, >97 %), potassium persulfate, acetonitrile (HPLC grade, >99.8 %), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$), bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) L-Norleucine and Bradford reagent, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Additionally, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were acquired from Jalmek (Nuevo León, México).

Vegetal material

C. uvifera fruits were collected from the coastal region of Tecolutla, Veracruz, Mexico (20° 23′17″ N 97° 01′31″ W) during the period from September to October 2023. The seeds were extracted from the fruit and subjected to a 24-h drying process at 60 °C in a convection oven. (Novatech HS60-AID, Tlaquepaque, Jalisco, Mexico). The seeds were ground to obtain *C. uvifera*

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seed flour (CUSF). The CUSF was vacuum-sealed and stored under ambient conditions to ensure its preservation and quality until use. The CUSF had a protein content of 11.54 ± 0.50 %.

C. uvifera seed protein concentrate (CUSP) extraction

The CUSP was prepared using alkaline solubilization and isoelectric precipitation with ultrasonic assistance. Leaf flour (30 g) was placed in a 1 L beaker with 563 mL of distilled water and 188 mL of a 0.2 M NaOH solution. The mixture was stirred on a magnetic stirring plate for 10 min and subjected to ultrasound-assisted extraction using a digital ultrasonic cleaner (model CD-4820, Guangdong, CHN) operating at 42 kHz for 20 min. The mixture was centrifuged (Sorvall X Pro series, Thermo Scientific™, Waltham, MA.) at 4,000 rpm for 20 min at 25 °C. The supernatant was then collected, the pH was adjusted to 4.0 (1 M HCl), and the mixture was precipitated for 24 h. The resulting precipitate was retrieved via centrifugation at 4,000 rpm for 15 min (25 °C) (Vera-Salgado *et al*., 2022). The resulting CUSP was lyophilized and stored at 4 °C for further analysis. The protein content of the CUSP was determined via the 920.87 method (AOAC, 1990).

High-intensity ultrasound treatment

CUSP solutions at 5 % (w/v) in distilled water were stirred for 10 min for homogenization. An ultrasonic homogenizer (Hanchen Integrated Ultrasonic Homogenizer Sonicator) with a 0.636 cm diameter titanium probe was used to sonicate 50 mL of the CUSP dispersions in 100 mL beakers, which were placed in a water bath (22–25 °C). The protein dispersions were treated at 20 kHz at different power output levels (0, 600, 840, and 1080 W) for 10, 15, and 20 min. The HIU-treated samples were centrifuged at 4,000 rpm for 20 min at 25 °C. The recovered supernatant was lyophilized and stored for further analysis.

Amino acid profile of HIU-treated and untreated CUSP

The method described by Brion-Espinoza *et al*. (2021) was utilized for acid hydrolysis of samples and their subsequent GC–MS analysis. Briefly, acid hydrolysis was conducted on the CUSP samples (treated and untreated with HIU) using 6 M HCl at 110 °C (24 h). The hydrolysates were filtered and centrifuged at 15,000 rpm for 15 min (Hettich MIKRO 200/200R, DEU). Subsequently, 100 µL of hydrolysate and 10 µL of L-Norleucine (internal standard at 0.2 mg/mL) were mixed within a vial. The resulting mixture was subjected to evaporation under a stream of N_2 until a dry residue was obtained. Then, 200 µL of acetonitrile and 200 µL of MTBSTFA were added to the precipitate remaining in the vial. The vial was incubated at 100 °C for 2.5 h in a glycerol bath. The derivatized sample (1 µL) was introduced in split mode (20:1) at 260 °C using helium (2 mL/min) on a GC 7890A instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to a mass spectrometer (MS) 240 Ion Trap. Amino acids in the treatments were identified via amino acid standards and NIST software (v. 2.3). Quantification was performed by adjusting the response of the internal standard for each amino acid. The amino acid profile was expressed as g of amino acids/100 g of dry material.

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Techno-functional properties

Hydrosolubility of HIU-treated and untreated CUSP

The hydrosolubility of the samples was determined following the method described by Klompong *et al*. (2012). HIU-treated and untreated samples (10 mg) were dissolved in distilled water (1 mL) at pH 7.0. The dispersions were stirred (100 rpm) for 30 min. The samples were centrifuged at 15,000 rpm (15 min) (MIKRO 220 R centrifuge, Hettich, Tuttlingen, Germany). The supernatant was gathered, and the protein concentration was measured via Bradford's method (Bradford, 1976). The total protein content of each sample was measured after 10 mg of the sample was solubilized in 1 mL of 0.5 N NaOH. Finally, the hydrosolubility (%) was calculated via Equation 1.

$$\text{Hydrosolubility (\%)} = \frac{\text{Protein content in the supernatant}}{\text{Total protein content of each sample}} \text{x} 100 \quad (1)$$

Foaming properties of HIU-treated and untreated CUSP

The foaming capacity (FC) and foaming stability (FS) of HIU-treated and untreated CUSP were determined via the methodology of Calderón-Chiu *et al*. (2021) with modifications. Five percent (w/v) protein solutions in distilled water were prepared. Subsequently, aliquots of 10 mL of the sample were placed in graduated tubes. The dispersions were homogenized at 16,000 rpm for 1 min with an IKA T10 basic ultra-Turrax homogenizer (IKA, Staufen, DEU) at 25 °C to incorporate air. The total volume of the sample was measured immediately after homogenization (0 min) and after 30 min to calculate the FC (Equation 2) and FS (Equation 3), respectively.

$$\text{FC (\%)} = \frac{\text{B}-\text{A}}{\text{A}} \text{ x } 100 \quad (2)$$

$$\text{FS (\%)} = \frac{\text{C}-\text{A}}{\text{A}} \text{ x } 100 \quad (3)$$

where A is the initial volume (mL) before homogenization, B is the foam volume (mL) after homogenization (0 min), and C is the foam volume (mL) after 30 min of homogenization.

Emulsifying properties of HIU-treated and untreated CUSP

The emulsion activity index (EAI) and emulsion stability index (ESI) were determined (Yu *et al*., 2021). Therefore, an emulsion was prepared using 2 mL of olive oil and 6 mL of 0.5 % protein solution. Both phases were homogenized at 20,000 rpm for 1 min with an IKA T10 basic Ultra-Turrax (IKA, Staufen, DEU). After that, 50 µL of the emulsion was taken at 0 min and after 10 min and diluted in 5 mL of 0.1 % SDS solution. The diluted solutions were analyzed for

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absorbance at 500 nm via a spectrophotometer (Cary 50 Bio UV–Visible, Varian, Mulgrave, AUS). The absorbances of the diluted samples measured at 0 min (A_0) and 10 min (A_{10}) were used to calculate the EAI (Equation 4) and ESI (Equation 5).

$$\text{EAI}\ (m^2/g) = \frac{(2)(2.303)(\mathbf{A_0})(\mathbf{DF})}{(\text{g protein})(0.25)(1000)} \quad (4)$$

$$\text{ESI}\ (\text{min}) = \frac{\mathbf{A_{10}}}{\mathbf{A_0 - A_{10}}} * t \quad (5)$$

DF is a dilution factor (100), 0.25 is the fraction of olive oil used to formulate the emulsion, and *t* is the time.

Particle size distribution of emulsions

The emulsions acquired in the previous section were processed via a Digital Sonifier® Unit (Model S-150D, Branson Corporation, Danbury, CT, USA) operated at 24 kHz for 5 min. Subsequently, the particle size distribution was determined with a Mastersizer 3000 Hydro EV (Malvern, Worcestershire, UK). The assessment was performed at 25 °C, with the refractive index set at 1.46 for the dispersed phase (olive oil) and 1.33 for the dispersant (water). Initially, 400 mL of distilled water was introduced into the Hydro EV unit. The emulsion was carefully added dropwise until laser obscuration between 8 and 12 % was attained. The resulting emulsions were analyzed via a diffractometer five times, yielding a comprehensive mean size distribution curve. The particle size distribution parameters, including the PDI, $d_{[3,2],}$ and $d_{[4,3]}$, were derived via Mastersizer 3000 software (Worcestershire, UK).

ABTS⁺ radical scavenging activity

The ABTS⁺ radical scavenging activity was assessed as described by Re *et al*. (1999) with modifications. The ABTS⁺ solution was prepared to a final concentration of 7 mM ABTS⁺ in 2.45 mM potassium persulfate. The mixture was left in total darkness at room temperature for 16 h before use. The stock solution was diluted (distilled water) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Fifty microliters of HIU-treated and untreated CUSP solutions (4 mg/mL) were mixed with 950 µL of ABTS⁺ radical and vortexed for 10 s. The absorbance of the reaction mixture was read (734 nm) on a spectrophotometer (Cary 50 Bio UV–Visible Varian, Mulgrave, AUS) after 7 min for ABTS⁺ radical scavenging activity calculated (Equation 6).

$$ABTS^{+} radical\ scavenging\ activity\ (\%) = \frac{A_{control} - A_{Sample}}{A_{control}} * 100 \quad (6)$$

$A_{control}$ represents the absorbance value of the diluted ABTS⁺ solution, and A_{Sample} represents the absorbance of the ABTS⁺ reaction and the sample.

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Statistical analysis

The dataset underwent analysis through one-way analysis of variance (ANOVA). The LSD mean comparison test ($p < 0.05$) was subsequently conducted via Statistica v.12.0 (StatSoft, Inc., Tulsa, OK, USA).

Results and Discussion

Protein content and amino acid profile of CUSP

The protein content of the CUSP was 66.49 ± 2.10 g/100 g d. b., significantly different from the plum seed protein isolates at 19.4% (Xue *et al*., 2018) and the quinoa seed protein concentrate containing 57.3% (González-Muñoz *et al*., 2022). The variation in protein content can be attributed to the composition of the plant material and the method or conditions used for protein extraction. The protein content of CUSP exceeds 50%, surpassing that of protein concentrates from plum and quinoa seeds. This observation suggests that, compared to those sources, the CUSP may have superior potential for applications in the food industry.

In amino acid analysis, leucine, valine, isoleucine, and phenylalanine were identified in the HIU-treated and untreated CUSP (Table 1). However, the concentrations of these amino acids were lower in the CUSP treated with HIU compared to the untreated sample. A considerable increase in the levels of certain amino acids, such as glutamic acid, aspartic acid, proline, glycine, and serine, was evident in the HIU-treated CUSP. This behavior occurred because HIU treatment breaks down the protein matrix, primarily through van der Waals forces and hydrogen bonding at the molecular level. This process can alter the secondary and tertiary structures of proteins, enhancing the formation of more flexible structures that encourage internal rearrangements *(Jadhav et al*., 2024; Sun *et al.*, 2023). These changes have the potential to affect the functional properties.

The findings indicate that using low (600 W) and high (1080 W) levels of ultrasound for 15 min resulted in increased levels of hydrophobic amino acids (HAA), aromatic amino acids (AAA), and essential amino acids (EAA) compared to other treatments. However, the extension to 20 min corresponded to a decrease in these amino acids. This decrease in amino acids during prolonged processing may be due to the increased generation of free radicals by HIU, which can potentially trigger the oxidation of amino acid side chains within protein structures and impact their quality. Notably, the aromatic side chains found in phenylalanine, tyrosine, and tryptophan amino acid residues are especially vulnerable to this phenomenon (Rahman *et al*., 2020). This behavior could explain the observed low phenylalanine levels in samples treated for 20 min at 600 W and 1080 W. Unexpectedly, a medium level of ultrasonication (840 W) at 10–15 min resulted in the lowest amino acid recovery.

The untreated CUSP and the treatments obtained at 600 W and 1080 W (15 min) presented

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approximately 80.02% and 83.59% HAA. This HAA content exceeded that reported for quinoa seed protein (González-Muñoz *et al*., 2022) and *Stauntonia brachyanthera* (Yu *et al*., 2021). This high content of hydrophobic functional groups in proteins improves lipid hydrosolubility, increasing emulsifying activity and antioxidant capacity (Aderinola *et al*., 2018). The higher levels of HAA in the HIU treatments may stem from the turbulence created by ultrasonic cavitation and physical shearing. These forces cause the unfolding of protein molecules, revealing the hydrophobic amino acid residues within them, as observed in pumpkin seed protein isolates treated with HIU (Du *et al*., 2022). Cysteine, tryptophan, lysine, and tyrosine were not detected in HIU-treated and untreated CUSP. Meanwhile, glycine and serine were absent under specific HIU conditions, possibly due to the degradation of these amino acids during prolonged exposure to hot during amino acid analysis. HIU-treated and untreated CUSP contain valine, leucine, isoleucine, methionine, threonine, and phenylalanine. Importantly, methionine is the only essential amino acid that does not meet the requirements for adults set by the WHO/FAO (2007). All treatments are viable options for supplementing diets lacking essential amino acids.

Techno-functional properties

Soluble protein content and hydrosolubility

The soluble protein content significantly increased ($p < 0.05$) following ultrasound application (Table 2). CUSP treated at 600 W exhibited lower protein content than other HIU treatments, regardless of processing time. The samples treated at 840 and 1080 W for 20 and 15 min showed the highest protein contents, respectively. Extending the treatment time to 20 min for samples treated at 1080 W did not significantly impact the protein content. The hydrosolubility (loss of hydrophobicity) also significantly improved after HIU treatment (Table 2). However, increasing the HIU level from 600 W to 1080 W or processing time did not significantly increase the hydrosolubility. The hydrosolubility values ranged from 79.90% at the lowest HIU level and time to 87.53% at 1080 W and 15 min. A similar pattern was observed for millet protein concentrate after 12.5 min of HIU, since extending the processing time to 20 min did not further improve this parameter (Nazari *et al*., 2018). Notably, the soluble protein content and water solubility of the samples exposed to HIU were twice as high as those of the control sample. This highlights the potential of ultrasound to modify plant proteins and improve their functional properties.

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Table 1. Amino acid profile (g of amino acids/100 g dry material) of HIU-treated and untreated CUSP.

Amino acid/ HIU treatment	0 W (Control)	600 W			840 W			1080 W			WHO/ FAO*
	CUSP	10 min	15 min	20 min	10 min	15 min	20 min	10 min	15 min	20 min	
Alanine	3.06±0.32^{A}	8.57 ± 0.26^{B}	9.71 ± 1.04^{B}	7.22 ± 0.66^{BC}	10.38 ± 1.02^{B}	9.93 ± 0.17^{B}	8.49 ± 3.35^{B}	10.17 ± 0.97^{B}	7.65 ± 0.97^{BC}	9.28 ± 2.23^{BC}	
Glycine	nd^{A}	1.74 ± 0.08^{B}	3.97 ± 0.57^{C}	3.83 ± 0.66^{C}	4.46 ± 0.95^{C}	4.94 ± 0.32^{C}	4.11 ± 2.73^{BC}	5.62 ± 2.21^{C}	nd	4.78 ± 2.9^{C}	
Valine*	20.26 ±0.59^{A}	14.33 ± 0.67^{B}	18.86 ± 4.12^{AB}	14.67 ± 1.52^{B}	9.83 ± 0.56^{C}	14.6 ± 1.13^{B}	13.88 ± 1.56^{B}	14.29 ± 1.01^{B}	20.06 ± 0.38^{A}	12 ± 1.1^{D}	3.9
Leucine*	23.71±0.37^{A}	20.82 ± 0.1^{B}	21.27 ± 3.2^{B}	17.43 ± 1.22^{B}	10.96 ± 0.94^{C}	16.27 ± 0.92^{B}	18.21 ± 0.^{B}	16.96 ± 0.41^{B}	24 ± 0.21^{AB}	15.75 ± 1.89^{A}	5.9
Isoleucine*	13.31±0.09^{A}	12.7 ± 0.01^{B}	12.84 ± 2.36^{AB}	10.55 ± 0.84^{C}	7.11 ± 0.3^{D}	9.58 ± 0.43^{C}	10.63 ± 1.14^{BC}	9.88 ± 0.58^{C}	15.42 ± 0.31^{AB}	8.48 ± 1.32^{C}	3.0
Proline	4.87±0.27^{A}	4.27 ± 0.08^{B}	5.57 ± 0.87^{A}	6.2 ± 0.07^{A}	5.64 ± 0.04^{A}	8.55 ± 0.21^{C}	5.35 ± 1.68^{AB}	4.46 ± 1.11^{AB}	4.05 ± 0.01^{B}	5.64 ± 1.92^{AB}	
Methionine*	1.55±0.06^{A}	0.98 ± 0.16^{B}	1.27 ± 0.02^{C}	1.27 ± 0.35^{ABC}	1.21 ± 0.06^{C}	nd	1.9 ± 0.08^{D}	1.51 ± 0.08^{A}	1.7 ± 0.06^{E}	1.54 ± 0.32^{AC}	2.2***
Serine	Nd^{A},	3.79 ± 0.28^{B}	2.56 ± 0.33^{C}	2.35 ± 0.44^{C}	5.01 ± 0.09^{D}	1.67 ± 0.15^{E}	3.61 ± 1.73^{B}	4.22 ± 0.59^{F}	1.9 ± 0.02^{C}	4.65 ± 0.76^{D}	
Threonine*	1.82±0.50^{A}	6.14 ± 0.36^{B}	4.19 ± 1.09^{C}	3.86 ± 1.62^{AC}	7.32 ± 0.46^{D}	3.32 ± 0.04^{C}	4.04 ± 1.29^{C}	5.17 ± 0.18^{C}	3.05 ± 0.01^{C}	5.51 ± 1.16^{BC}	2.3
Phenylalanine*	19.19±0.09^{A}	10.05 ± 0.92^{B}	12.1 ± 1.99^{B}	10.23 ± 1^{B}	7.28 ± 0.7^{C}	7.29 ± 0.21^{C}	10.78 ± 0.84^{B}	8.76 ± 0.63^{D}	14.76 ± 0.38^{E}	8.43 ± 1.25^{CD}	3.8**
Aspartic acid	8.87±0.15^{A}	10.22 ± 1.4^{A}	6.21 ± 1.8^{B}	17.17 ± 0.58^{C}	18.23 ± 0.68^{C}	11.46 ± 0.97^{A}	11.33 ± 3.51^{A}	12.37 ± 1.8^{A}	4.72 ± 0.02^{B}	14.2 ± 2.99^{A}	
Glutamic acid	3.36±0.32^{A}	6.39 ± 1.3^{B}	1.45 ± 0.25^{C}	5.21 ± 3.41^{AB}	12.57 ± 1.12^{D}	12.38 ± 1.04^{D}	7.68 ± 2.7^{B}	6.58 ± 0.2^{B}	2.68 ± 0.65^{A}	9.73 ± 2.77^{BD}	
HAA	81.08	69.19	80.02	65.20	51.23	62.61	68.00	67.19	83.59	60.26	
AAA	19.19	10.05	12.10	10.23	7.28	7.29	10.78	8.76	14.76	8.43	
EAA*	79.84	65.02	70.53	58.01	43.71	51.06	59.44	56.57	78.99	51.71	

*Essential amino acids specified by the WHO/FAO (2007) for adults: **Tyrosine + phenylalanine. ** *Methionine + cysteine.** nd: not detected. Different uppercase letters in the row indicate significant differences ($p < 0.05$) between control and HIU treatments for an amino acid. Hydrophobic amino acids (HAA), aromatic amino acids (AAA), and essential amino acids (EAA).

Source: Prepared by the authors based on results.

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Table 2. Soluble protein content and hydrosolubility of HIU-treated and untreated CUSP.

HIU Treatment (W)	Soluble protein content (mg/mL)			Hydrosolubility (%)		
	10 min	15 min	20 min	10 min	15 min	20 min
0 (Control)	1.1057 ±0.029^{a}	1.1057 ±0.029^{a}	1.1057 ±0.029^{a}	44.23 ±1.15^{a}	44.23 ±1.15^{a}	44.23 ±1.15^{a}
600	1.998 ±0.070^{b,A}	2.118 ±0.050^{b,A}	2.105 ±0.065^{b,A}	79.90 ±4.55^{b,A}	84.73 ±5.60^{b,A}	84.18 ±2.10^{b,A}
840	2.044 ±0.060^{b,A}	2.088 ±0.080^{b,A}	2.158 ±0.092^{b,B}	81.75 ±3.98^{b,A}	83.51 ±5.08^{b,A}	86.33 ±4.70^{b,A}
1080	2.068 ±0.060^{b,A}	2.188 ±0.020^{c,B}	2.138 ±0.072^{b,AB}	82.71 ±3.21^{b,A}	87.53 ±6.81^{b,A}	85.53 ±4.55^{b,A}

Different lowercase letters in a column indicate significant differences ($p < 0.05$) between treatments at a specific time. Different uppercase letters in a row signify significant differences ($p < 0.05$) between treatment times at a specific HIU level.

Source: Prepared by the authors based on results.

The soluble protein content and hydrosolubility exhibited similar patterns, consistently yielding the most favorable outcomes at the same power and duration levels of HIU (1080 W for 15 min). Ultrasound exposure revealed hydrophilic amino acid regions and charged groups (NH_4^+, COO^-) on the protein surface, enhancing electrostatic forces. As a result, the electrostatic forces enhance the interactions between the protein and water (hydrogen bonds), ultimately increasing the hydrosolubility and soluble protein content (Corzo-Martínez *et al*., 2017). The high hydrosolubility and protein content values correlate with the high levels of HAA, AAA, and EAA resulting from the treatment at 1080 W for 15 min. A greater amino acid content may suggest enhanced interaction between the hydrophilic regions of the amino acids and water. The high hydrosolubility of proteins is essential for their use in food formulations and a necessary property for their role as emulsifiers (Adal, 2024).

Foaming properties

The implementation of HIU positively affected the foaming capacity (FC) and foam stability (FS) of CUSP (Table 3). In comparison to the other treatments, the CUSP treated at 600 W for 10 min exhibited superior FC and FS values. Still, an increase in the duration or intensity of the ultrasound did not enhance these properties. Meanwhile, increasing the intensity from 600 to 840 W for 15 and 20 min significantly improved FC but decreased FS ($p > 0.05$). The enhanced foaming properties can be attributed to the reduction in protein size because of HIU, since smaller protein molecules allow more air into the solution than larger molecules, increasing protein adsorption at the air–water interface (Rahman & Lamsal, 2021; Xue *et al*., 2018). This would explain the lack of foaming properties in untreated CUSP despite their high HAA content (Table 1), as the formation of the air/water interface is influenced not only by the surface's hydrophobicity but also by the orientation, size, and structural flexibility of the protein (Rahman & Lamsal, 2021).

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The results indicated that high ultrasonic energy levels (higher than 600 W) and long processing times negatively impacted the foaming properties. Under these conditions, the hydrophobic groups of amino acids are exposed on the surface, leading to more significant protein aggregation (Justino *et al*., 2024). This phenomenon would explain why the high-HAA samples presented reduced foaming properties. The application of HIU has also improved the foaming of seed protein isolates from pumpkin (Du *et al*., 2022) and plum (Xue *et al*., 2018). However, the foaming properties of these isolates were not considerably affected by increases in ultrasound power. Alternatively, Ramondo *et al*. (2024) reported superior foaming properties in pumpkin seed protein treated with HIU for 5 min at 150 W, and that prolonging the processing time did not enhance these properties. The results of this study showed differences from those reported for other plant proteins, which are attributed to the ultrasound conditions (ultrasound power <600 W), processing time, and the type of protein.

Emulsifying properties

Regarding emulsifying properties, HIU-treated CUSP showed higher EAI and ESI values than the control (Table 3). The EAI increased with higher ultrasonic power levels and processing time from 10 to 15 min, but longer processing times diminished this property. Regarding the ESI, raising the HIU from 600 to 840 W for the 10- and 20-min samples enhanced the ESI. However, increasing the HIU to 1080 W for these samples notably decreased this parameter. Thus, the results showed that exposing the sample to 600 and 1080 W power levels for 15 min yielded the highest EAI and ESI values, while extending the time decreased these indices. This finding highlights the direct correlation between emulsification properties, hydrosolubility, and HAA detected in these samples. The improvement in emulsification properties was attributed to the pressure generated by ultrasonication. These forces significantly alter the tertiary and quaternary structures of proteins, resulting in a more disordered configuration (Xue *et al*., 2018), which favors greater mobility and rapid absorption at the oil-water interface of the HIU-treated CUSP than the control, which probably had a more compact structure.

However, with longer sonication times, larger protein aggregates are formed due to depolymerization from noncovalent interactions or oxidation, resulting in decreased surface hydrophobicity compared to samples treated for a shorter duration (Rahman & Lamsal, 2021). Moreover, excessive mechanical force over extended periods can lead to protein denaturation, diminishing their functionality. Nazari *et al*. (2018) and Zhao *et al*. (2022) reported a significant relationship between the exposure of hydrophobic groups by HIU and the enhancement of emulsifying properties in both millet protein concentrate and potato protein isolates.

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Table 3. Foaming and emulsifying properties of HIU-treated and untreated CUSP.

HIU Treatment (W)	Foaming capacity (%)			Foaming stability (%)		
	10 min	15 min	20 min	**10 min**	**15 min**	**20 min**
0 (Control)	0± 0^{a}	0± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0a	0 ± 0^{a}
600	41.67±3.64^{b,A}	19.44± 4.81^{b,B}	5± 0.4^{b,C}	25.56 ± 4.71^{b,A}	13.89 ± 4.81b^{b,B}	13.39± 2.81^{b,B}
840	10.00± 1.89^{c,A}	23.99± 6.3^{b,B}	23.89± 5.31^{c,B}	16.78± 3.48^{c,A}	12.78 ± 9.48^{b,AB}	9.72 ± 2.41^{b,B}
1080	2.78± 0.21^{d,A}	5.56± 1.2^{c,B}	15.00± 3.01^{d,C}	4.44 ± 4.19^{d,A}	4.44 ± 4.19^{c,A}	8.89 ± 7.7^{b,A}
HIU Treatment (W)	**Emulsifying activity index (m²/g)**			Emulsion stability index (min)		
	10 min	15 min	20 min	10 min	**15 min**	**20 min**
0 (Control)	11.97 ± 3.56^{a}	11.97 ± 3.56^{a}	11.97 ± 3.56^{a}	47.98 ± 1.88^{a}	47.98 ± 1.88^{a}	47.98 ± 1.88^{a}
600	20.08 ± 4.77^{b,A}	42.06 ± 0.29^{b,B}	13.72 ± 1.18^{a,C}	131.47 ± 10.48^{b,A}	287.93 ± 12.15^{b,B}	194.92 ± 27.96^{b,C}
840	14.49 ± 0.39^{a,A}	39.27 ± 1.13^{c,B}	21.3 ± 0.29^{b,C}	261.63 ± 22.4^{c,A}	51.91 ± 14.08^{c,B}	219.31 ± 15.05^{b,C}
1080	23.61 ± 8.28^{ab,A}	43.09 ± 0.02^{d,B}	28.02 ± 0.39^{c,A}	138.13 ± 8.02^{b,A}	284.35 ± 0.82^{b,B}	110.06 ± 12.19^{c,C}

Different lowercase letters in columns show significant differences ($p < 0.05$) between treatments at a specific time. Different uppercase letters in the rows indicate significant differences ($p < 0.05$) between treatment times at a specific HIU level.

Source: Prepared by the authors based on results.

Droplet size distribution of emulsions

The particle size of an emulsion is fundamentally essential for understanding its structural characteristics. The stability of emulsions is closely related to the distribution of dispersed particles, with smaller particles contributing to greater system stability. The emulsions stabilized by HIU-treated CUSP exhibited bimodal particle size distributions (Figure 1). However, the data indicated that increasing the power of HIU decreased the diameter and PDI of the 10- and 15-min samples, while extending treatment to 20 min adversely affected the droplet size distribution. These results align with the emulsifying properties mentioned above, in which samples treated with HIU for 15 min had higher EAI and ESI values than the other treatments. Notably, the PDI and droplet size of emulsions stabilized with HIU-treated CUSP were lower than those reported for emulsions stabilized with jackfruit leaf protein hydrolysates (Calderón-Chiu *et al*., 2022). These findings indicate that employing HIU to enhance plant protein functionality may be an alternative to enzymatic methods, frequently necessitating process condition optimization, such as enzyme type, concentration, and hydrolysis duration.

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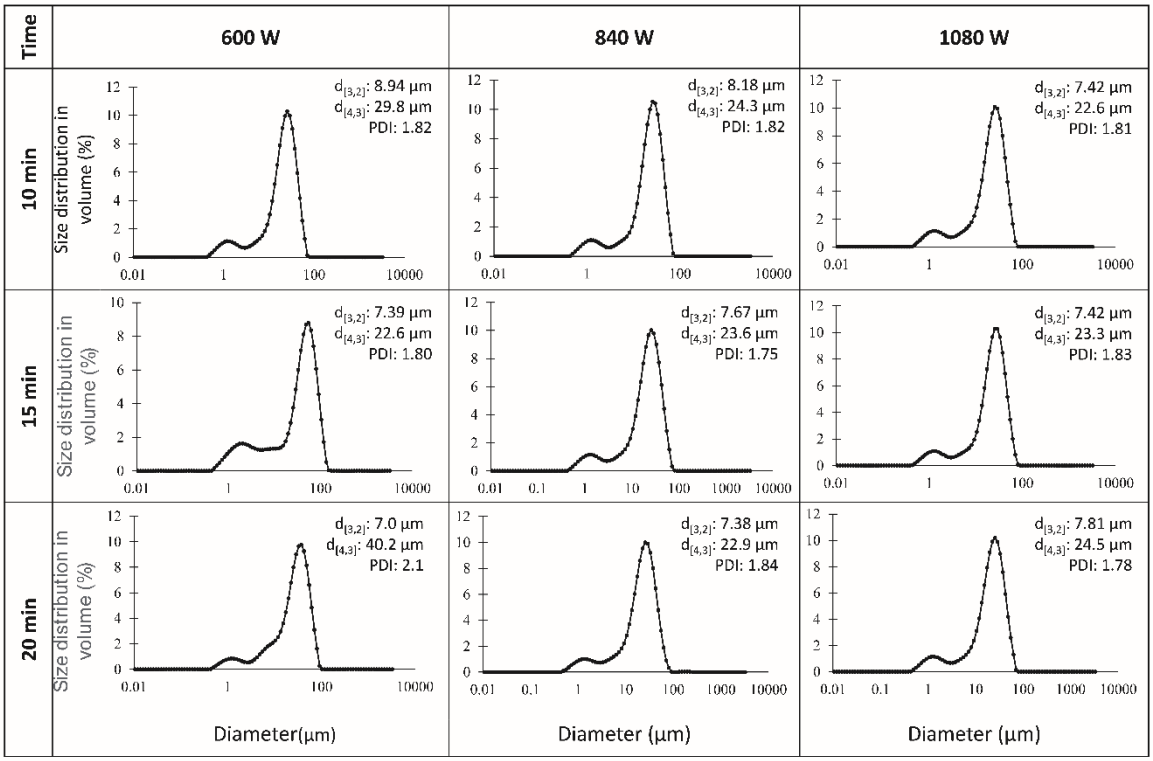

Figure 1. Droplet size distribution of emulsions stabilized by HIU-treated and untreated CUSP.

Source: Prepared by the authors based on results.

ABTS⁺ radical scavenging activity

The ABTS⁺ radical scavenging activity of the HIU-treated CUCP was significantly ($p < 0.05$) more remarkable than that of the control at all power levels and durations tested (Figure 2). The antioxidant capacity significantly increased in HIU-treated CUSP at 600 W ($p < 0.05$), with no notable changes due to processing time. Raising the power level to 840 W for 10 and 15 min significantly enhanced the antioxidant capacity of the sample. Meanwhile, at 1080 W, the antioxidant capacity decayed with increasing ultrasonication time. The highest antioxidant capacity was observed at 840 W and 15 min (97.21 ± 0.23 %), which was approximately double the radical scavenging activity of the control (51.07 %). These results suggest that using HIU in CUSP potentially enhances antioxidant activity by releasing amino acid groups from the protein structure, facilitating effective reactions with oxidants (Hussain *et al*., 2024).

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The amino acids released or exposed during HIU treatment play a vital role in the antioxidant capacity of proteins. Interestingly, the treatments with high HAA exhibited slightly lower $ABTS^{+}$ radical scavenging activity compared to those with the highest antioxidant capacity (840 W and 15 min), suggesting that antioxidant capacity is not solely determined by exposure to HAA. The treatment with the highest antioxidant capacity presented increased glutamic acid and prolamin levels. The antioxidant properties of proline and glutamic acid arise from the surplus electrons that interact with free radicals. This behavior was also noted in sunflower proteins treated with HIU (Rawat & Saini, 2023).

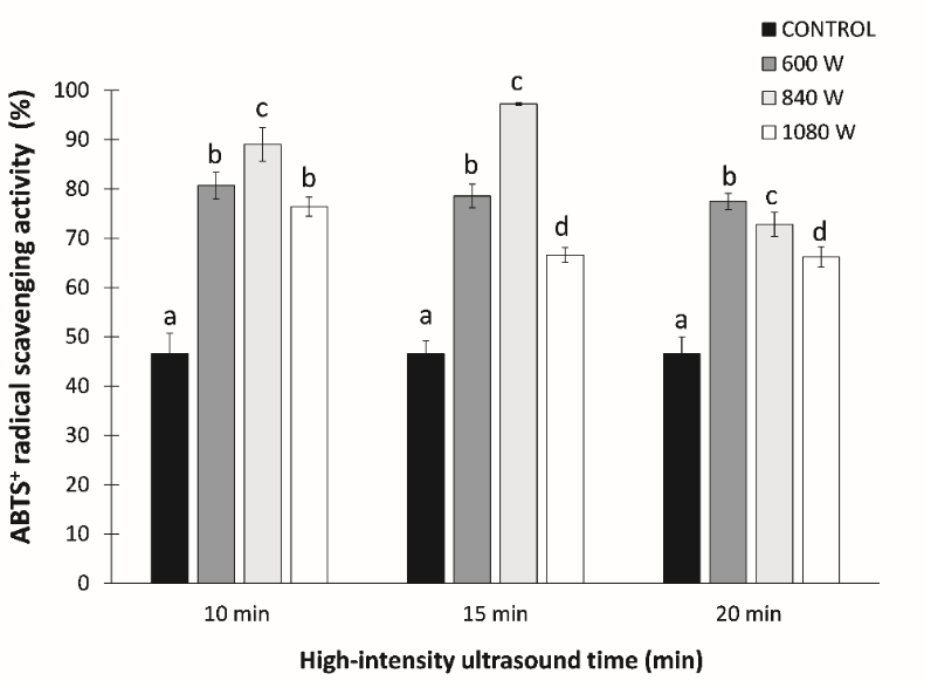

Figure 2. Radical scavenging activity of HIU-treated and untreated CUSP. Different letters show significant differences ($p < 0.05$) between treatments with HIU at specific times.

Source: Prepared by the authors based on results.

Conclusions

Applying high-intensity ultrasound (HIU) to *Coccoloba uvifera* seed protein (CUSP) altered the amino acid profile, significantly affecting its functionality. The CUSP exposed to HIU exhibited

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enhanced amino acid profiles, hydrosolubility, foaming and emulsifying abilities, and antioxidant properties compared to untreated samples. Notably, this enhancement depended on both the duration and the level of HIU applied. Exposing CUSP to HIU at 600 and 1080 W for 15 min resulted in a more significant recovery of hydrophobic amino acids, directly leading to improved emulsifying properties. Applying HIU at 600 W for 10 min to CUSP enhanced the foaming properties.

All HIU treatments demonstrated higher antioxidant capacity compared to the untreated sample. However, treatment at 840 W for 15 min had superior antioxidant properties due to a high release of proline and glutamic acid. These findings suggest that applying suitable HIU conditions in CUSP can achieve specific nutritional, functional, or antioxidant properties. Thus, the application of HIU in CUSP enhanced functionality, indicating its potential use as a new plant protein for the food industry when cultivated expansively. Nevertheless, further optimization studies are required to maximize the properties identified in this study.

Author Contributions

Conceptualization: FZRC, MCS, and JARS. Methodology, Software management, Experimental validation, Results analysis: FZRC, CCC, MCS, DEHM, KEMR, and JARS. Data management, Writing and manuscript preparation, Writing, Reviewing, and editing: FZRC, CCC, MCS, and JARS. Project manager and Funding acquisition: JARS**.**

“All authors have read and accepted the published version of the manuscript.”

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Ethical declarations

Not applicable.

Declaration of informed consent

Not applicable.

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Conflict of interest

The authors encourage that they do not have any conflicts of interest.

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