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Research Article

Enhancing Zinc Uptake through Dual-Modification of *Cicer arietinum* Protein

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Zinc is crucial for physiological processes; however, deficiency persists globally. Binding zinc to plant proteins enhances absorption, minimizing toxicity risks and offering a potential solution for deficiency. Mineral binding efficiency of the unmodified protein is limited; hence, dual modification (succinylation and ultrasonication) is potentially used to achieve higher binding efficiency. Enhancing zinc uptake is crucial for cellular health due to its vital roles in various biological processes including enzymatic activity, DNA repair, immune function, antioxidant defense, hormone regulation, brain function, signaling, growth, gene expression, and reproduction. Therefore, this research aimed to develop a chickpea protein-zinc complex and to evaluate the influence of dual modification on their physiochemical, bioavailability, and cellular mineral uptake attributes. Succinylation exhibited significant improvements in the water-holding capacity by 28.73%, oil-holding capacity by 34.09%, and solubility by 5.46% of the chickpea protein-zinc complex as compared to the native complex. Mineral bioavailability increased by 8.32%, and there were notable increases in cellular uptake of zinc by 2.10%, retention by 5.80%, and transport by 3.96%, respectively. Furthermore, the dual modification approach resulted in a notable decrease in the particle size of the chickpea protein-zinc complex, with a substantial reduction of 73.25% and an increased zeta potential value of −21 mV compared to the succinylated complex. As well, dual modification concurrently led to a substantial decline of 48.04% in the sulfhydryl (SH) content, coupled with a marked increase of 21.92% in the surface hydrophobicity. In addition, zinc bioavailability, cellular uptake, retention, and transport were further enhanced by 1.89, 3.34, and 4.8% through dual modification. Our findings highlight that the dual modification of the chickpea protein-zinc complex shows a promising strategy for enhancing the techno-functional characteristics, bioavailability, and cellular uptake of zinc, which could be a better platform for developing vegan foods.

1. Introduction

Zinc is a crucial trace element that plays an essential role in various physiological processes within the human body. It is involved in enzymatic reactions, immune function, DNA synthesis, and cell division, making it vital for overall health and well-being. However, zinc deficiency remains a significant global health concern, particularly prevalent in developing countries [1]. While zinc supplements are commonly used to address the deficiency, there are concerns

about the potential adverse effects of excessive zinc intake, including impaired copper absorption and immune system suppression [2]. Beyond the molecular level, zinc plays a pivotal role in boosting the body's immune system by supporting the development and function of immune cells, thereby enhancing its ability to fend off infections [3]. In addition, zinc contributes to the cell's antioxidant defense by being a component of superoxide dismutase, which neutralizes harmful reactive oxygen species and safeguards cellular structures from oxidative damage [4]. Zinc's influence extends to hormonal regulation, neurotransmitter function, and cell signaling, influencing critical processes ranging from hormone secretion to cognitive function. Moreover, zinc is indispensable for wound healing, tissue repair, proper growth and development, and gene expression regulation, impacting an array of physiological functions [5]. In essence, ensuring optimal zinc uptake is important for cellular health and for supporting the transposition of the essential biological processes [6]. Thus, exploring alternative approaches to enhance zinc bioavailability while minimizing potential toxicity is of great interest. Binding minerals like zinc to plant-based proteins has been shown to improve mineral bioavailability and absorption. Such mineral-protein complexes can exert influence over gene expression related to mineral transport, potentially leading to increased cellular uptake of zinc [7]. Moreover, these complexes may offer protective effects against zinc-induced toxicity by sequestering excess zinc ions, preventing their uncontrolled interactions with cellular components [8].

In recent years, there has been a growing demand for plant-derived protein sources, driven by the increasing popularity of vegetarian and vegan diets. Chickpeas, a widely consumed legume, have garnered attention as an attractive option due to their high protein content and nutritional profile [9]. Chickpea proteins, including globulins, albumins, and glutelins, possess a natural ability to bind zinc, making them a promising target for enhancing zinc bioavailability through protein-zinc complexes [10]. Beyond dietary choices, the demand for vegan protein sources has been influenced by concerns about the environmental impact of animal agriculture, making chickpeas a sustainable and environmentally friendly protein option [11]. Leveraging the nutritional potential of chickpea protein, especially its protein can bind and deliver essential minerals like zinc, which can lead to the development of functional ingredients to meet the increasing demand for vegan protein products [12].

Despite the importance of enhancing zinc bioavailability using chickpea proteins, there is a research gap in specifically investigating the zinc-bound chickpea protein complexes. To address this gap, it is crucial to explore different modification methods that optimize the interaction between chickpea proteins and zinc ions [13]. Among these, the most promising approaches are succinylation and ultrasonication [14], or their combined approach, which we are focusing on in this research. Succinylation chemically modifies proteins by attaching succinic acid moieties, which can enhance the zinc-binding capacity of proteins by introducing additional functional groups that facilitate zinc chelation [15]. This modification has been successful in improving the physicochemical properties, such as solubility and stability, of various proteins. However, the specific effects of succinylation on zinc-bound chickpea protein complexes and their subsequent bioavailability and cellular uptake of zinc require further exploration [16]. The combination of ultrasonication and succinylation involves using high-frequency sound waves to disrupt protein structures and promote interactions with zinc ions, potentially leading to enhanced zinc binding [17]. Subsequent succinylation further modifies the proteins, potentially resulting in synergistic effects on zinc binding and bioavailability. Challenges in cellular zinc uptake include the complexities of ZIP transporters, their regulation influenced by factors like zinc availability and signaling pathways, their ability to transport other cations, the use of receptor-mediated endocytosis in certain cells, methodological complexities in measuring zinc uptake, quantifying subcellular zinc distribution, and the need to investigate diverse regulatory mechanisms and signaling pathways. Moreover, cell type variability adds complexity to understanding zinc uptake across tissues and organs.

Thus, the primary aim of this study is to create a novel chickpea protein-zinc complex through dual modification of chickpea protein. This complex could serve as an effective zinc supplement in functional foods to reduce the risk of zinc deficiency in the human body. To achieve this, we assess the functional properties, characteristics, and bioavailability of the protein-zinc complex. We simulate gastrointestinal conditions in *in vitro* studies to evaluate zinc release. In addition, we conduct cellular uptake studies to uncover mechanisms of zinc transport and utilization.

2. Materials and Methods

2.1. Materials. Indian local variety of chickpeas was obtained from the Punjab Agriculture University, Ludhiana, India. The chemicals such as zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, hydrochloric acid (HCl), amphotericin, sodium carbonate (Na_2CO_3), nitric acid (HNO3), Folin–Ciocalteu (FC) reagent, bovine serum albumin (BSA), succinic anhydride (C_4H4O_3), hydrindatin ($C_{18}H_{10}O_6$), sodium hydroxide (NaOH), ninhydrin ($C_9H_6O_4$) and sodium potassium tartarate ($KNaC_4H_4O_6{\cdot}4H_2O$), streptomycin, and copper sulfate ($CuSO_4$) were purchased from CSIR-National Chemical Laboratories Pvt. Ltd., Pune, India. The study employed acid-washed glassware and analytical-grade chemicals to ensure an adequate level of purity and accuracy throughout the experimentation process.

2.2. Methods

2.2.1. Extraction of Protein from Chickpea. The chickpea protein extraction was conducted in two steps, following the procedure described by Gao et al. [18], with slight modifications. In the initial phase, 1 kg of chickpea kernels was immersed in deionized water at a ratio of 1 : 8 (w/v) for 16 h. A slurry of soaked chickpeas was prepared by grinding the kernels in a laboratory grinder (Precision Grind Pro, Grind

Master Technologies, Bangalore, India) with additional deionized water (1 : 8 w/v). The resulting mixture was then centrifuged at $6000 \times g$ for 30 min (NSAW-1609, NSAW Pvt. Ltd., Mumbai, Maharashtra, India) at 24°C. Following centrifugation, the supernatant was carefully collected, while the remaining material (pellets) was reconstituted in deionized water at a ratio of 1 : 8 (w/v). The reconstituted mixture was stirred using a magnetic stirrer (EIE Instruments Pvt. Ltd., Kathawada, Gujarat, India) at 900 rpm for 30 min. After reconstituting the remaining material in deionized water, the mixture underwent another round of centrifugation at $6000 \times g$ for 30 min. Subsequently, the supernatants from both samples were combined, and the pH was adjusted to 12 by adding 2M NaOH solution at 27°C. The blend was agitated at 900 rpm for 30 min using a magnetic stirrer. To induce protein precipitation, the pH of the supernatant was adjusted to 4.5 (the isoelectric point) with the addition of 2M HCL. The coagulated protein mass was then separated using centrifugation ($6000 \times g$ for 30 min) and collected. The protein mass underwent purification, with 4 rounds of washing using deionized water through centrifugation at $6000 \times g$ for 30 min each time. The protein mass was dissolved in deionized water, and the resulting suspension was adjusted to pH 7 by adding 2M NaOH while continuously stirring at 800 rpm using a magnetic stirrer. The protein suspension was stored at a refrigerated temperature of 4–7°C for subsequent spray drying. In the next step, the protein suspension underwent spray drying using a laboratory scale tall type drier (S. M. Scientech, Kolkata, India) equipped with a 0.7 mm diameter nozzle, employing a cocurrent flow mechanism. The protein suspension was fed into the drier through a positive displacement pump while maintaining the inlet temperature at 170°C and the outlet temperature at 65°C. The blower feed rate and speed were adjusted to 9 mL/min and 2400 rpm, respectively, with a pressure of 2.5 bars. Following the drying process, the chickpea protein powder was collected from the drier chamber and packaged in aluminum foil pouches for further analysis. The native chickpea protein powder was found to contain 77.52% protein content.

2.2.2. Modification of Protein. Succinylation is a chemical modification technique used in biochemistry and proteomics. It involves the selective addition of succinyl groups (-$COCH_2COOH$) onto specific amino acid residues in proteins, often lysine residues [19]. This modification is achieved through a controlled chemical reaction between the protein of interest and a succinylating reagent, such as N-hydroxysuccinimide (NHS) esters or succinic anhydride [20]. The reaction is carried out under controlled conditions, including temperature and pH, to ensure the precise modification of lysine residues. Once succinylated, the protein is often purified to remove excess reagents and impurities [20]. This technique is valuable for studying protein function, interactions, and post-translational modifications, particularly in proteomics studies, providing insights into complex biological processes [21]. Ultrasonication is a versatile technique used for particle dispersion and cell disruption. It employs high-frequency sound waves, typically above 20 kHz, to create alternating cycles of compression and rarefaction in a liquid medium, generating microscopic bubbles known as cavitation [22]. When these bubbles collapse, they release intense localized energy in the form of shockwaves and microjets, which can break down cell walls, disrupt molecular structures, and disperse particles in the liquid [23]. Ultrasonication can be performed using an ultrasonic bath or probe, with parameters like frequency, amplitude, duration, and temperature carefully controlled to optimize results. It finds applications in various fields, from sample preparation in laboratories to industrial processes like emulsification and cleaning [24]. Ultrasonication and succinylation were selected for dual modification of chickpea protein based on previous research findings. Ultrasonication enhances solubility and digestibility, improving versatility and nutrition while maintaining an eco-friendly, chemical-free approach. Succinylation is favored for refining functional properties like stability and emulsification, enhancing protein performance in food applications. It allows precise modifications, boosting protein quality for diverse food products. The method selection hinges on the targeted protein modification goals.

(1) Succinylation. The chickpea protein succinylation was performed based on the method described by Gavahian et al. [24] with slight modification. This modification included washing steps and centrifugation in the preparation of chickpea protein succinylation. The succinylation was performed at varying mole ratios of succinic anhydride to the number of free modifiable amino groups, particularly lysine content. A 100 mM blend of lysine (with a protein content of 7.86%) was prepared, and to improve the overall negative charge of the protein, the pH of the blend was modified to 8 using of 3M NaOH solution. In the lysine solution, a predetermined weight of dry succinic anhydride was introduced to achieve a concentration of 4 moles per mole of lysine in the chickpea protein. The blend was subjected to stirring using a magnetic stirrer (EIE Instruments Pvt. Ltd., Kathawada, Gujarat, India) at 800 rpm for 1 h at 25°C. Throughout the process, the pH of the solution was monitored continuously and upheld at pH 8 while maintaining constant stirring. The recovery of the chickpea protein involved precipitating the solution at pH 4.5 by adding 4M HCl, and subsequently centrifuging at 8000 ×g for 20 min (NSAW-1609, NSAW Pvt. Ltd., Mumbai, Maharashtra, India). The protein precipitate was retrieved and purified by washing it with 100 mL of triple deionized water while stirring for 1 h. The resulting blend was centrifuged and the resulting protein precipitate was further washed again with 100 mL of triple deionized water. The pH of the precipitate was determined; if the pH was found to be outside the range, it was again subsequently adjusted to 4.5 with the help of 4 M HCl. This washing process was iterated four more times to achieve extensive purification. Finally, the purified protein precipitates were solubilized again at pH 7 with the help of a 4 M NaOH solution, followed by spray drying. The spray

dryer (S. M. Scientech, Calcutta, India) was used at 180°C inlet and 85°C outlet temperatures, respectively, while maintaining the feed flow rate of 7 mL/min and 2.5 bar pressure.

(I) Quantification of Succinylation (DS). The degree of succinylation (DS) of the sample was assessed using the ninhydrin method, following the procedure described by Pan et al. [25], with slight modifications, i.e., optimization of the standard level of protein and lysine in chickpea protein was carried out to enhance the accuracy of the estimation. In addition, we standardized the formula for determining the degree of succinylation of chickpea protein. A solution containing 15 mg of protein mixed in 15 mL of 0.1 M NaOH was prepared, and a 1 mL sample of this solution with 1 mL of triple distilled water was transferred to sealed-capped tubes and made into a 2 mL final volume. Subsequently, 2.0 mL of ninhydrin solution was introduced to each tube, including both the sample and the standard. Subsequently, tubes were heated in a hot water bath for 30 min, followed by immediate cooling in an ice bath. Following that, 7.0 mL of ethanol with a 50% concentration was included in each tube to achieve the final volume (10 mL), and the mixture was vortexed continuously for 15 sec. After that, centrifugation at 8000×g for 5 min was performed to eliminate insoluble particles and ninhydrin-negative material. The spectrophotometer (UV-visible spectrophotometer, SYSTRONICS, India) was utilized to determine the absorbance (570 nm) of the reaction mixture. The degree of succinylation (DS) of the sample was determined through an appropriate calculation method using the following equation:

$$\text{Degree of succinylation} = \left[\left(\frac{A-B}{A}\right) \times 100\right], \quad (1)$$

where A and B represent the absorbance value of the succinylated modified chickpea protein samples and the blank control, respectively.

(2) Ultrasonication. The ultrasonication treatment of the succinylated chickpea protein was performed following the methodology based on Kang et al. [26], with minor modifications. This modification includes ultrasonication treatment time. A suspension of chickpea protein (2% w/w) in 50 mL volume was prepared with the help of triple distilled water and treated with an ultrasonic device (SONOPLUS HD 4100, Berlin, Germany) equipped with a 13 mm diameter probe under ice bath conditions. The resulting chickpea protein dispersion was subjected to sonication using an ultrasonic device, the sonicator was set at a frequency of 20 kHz with a power output of 300 W, applying a sonication time of 4 sec followed by a resting time of 2 sec for varying durations of 25 min (USCP), respectively. The samples were then spray-dried. The spray dryer (S. M. SciTech, Calcutta, India) was used at 180°C inlet and 85°C outlet temperatures, respectively, while maintaining the feed flow rate at 6 mL/min and pressure at 2.5 bar.

2.2.3. Protein Content. The protein concentration of native and zinc-bound chickpea protein was assessed using the procedure outlined by Lees and Paxman [27]. In Lowry's method, a 500 μL diluted sample of both native and modified chickpea protein was mixed with 5 mL of alkaline copper reagent in a test tube using a vortex agitation machine. The combined sample was then incubated for 30 min. After incubation, 500 μL of Fc-reagent was added to the mixture, and the sample was mixed again on a vortex shaker (Tarson 3001 Spinix Vortex Shaker, Bengaluru, Karnataka, India) for 15 min. The mixture was incubated for an additional 30 min. Subsequently, the spectrophotometer was utilized to measure the absorbance of the sample at a wavelength of 660 nm. The protein content was determined by comparing the absorbance value with a standard curve.

2.2.4. Preparation of Protein-Zinc Complex and Estimation of Zinc-Binding Efficiency. The zinc-binding ability of native chickpea protein (NCP), succinylated chickpea protein (SCP), and succinylated chickpea protein treated with ultrasonication (USCP) was evaluated using ultrafiltration (UF) following the methodology outlined by Shilpashree et al. [28]. Individual solutions of NCP (native chickpea protein), SCP (succinylated chickpea protein), and USCP (ultrasonicated succinylated chickpea protein) were prepared at a concentration of 0.01 g/mL. To each protein solution, varying concentrations of zinc ranging from 1 to 12 mM/L were added gradually while constantly stirring using a magnetic stirrer. The pH of the protein-zinc dispersion was adjusted to 6.5, and the mixtures were incubated for 2 h at 24°C to facilitate optimal interaction between zinc and proteins. After incubation, the mixtures were subjected to centrifugation at 14000 × g for 30 min at 24°C to separate the supernatant containing soluble zinc and protein. The separated supernatant was then further processed using Amicon ultrafiltration (UF) membrane tubes (Millipore UFC900324, Amicon ultra, Tullagreen, Ireland) with a 10 kDa molecular weight cut-off. The collected permeate was analyzed to determine its zinc content using the Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) method (Toshvin Analytical Pvt. Ltd., Mumbai, Maharashtra, India). Following the analysis, a pilot-scale UF membrane system was utilized to create complexes of zinc and chickpea proteins. The diafiltration process was employed to verify the availability of free zinc in the collected permeate. Specifically, 2 mL of 4M NaOH was added to 20 mL of the collected permeate, and the mixture was then subjected to centrifugation to collect a solution devoid of any precipitated materials (pellet-free). Finally, the retentate was concentrated to a quarter of its

initial volume and subsequently subjected to spray drying. Zinc (Zn) retention and the overall yield of protein-zinc (Zn) complexes were assessed using the following equations, respectively.

$$\text{Zinc retention } (\%) = \left[\left(\frac{\text{The concentration of zinc in the complex}}{\text{Amount of zinc concentration added}}\right) \times 100\right], \tag{2}$$

$$\text{Yield } (\%) = \left[\left(\frac{\text{Weight of obtained complex } (g)}{\text{Weight of added protein source } (g)}\right) \times 100\right]. \tag{3}$$

The ultrafiltration (UF) process was conducted using a Sartorius Ultrafiltration unit equipped with a Masterflex easy load pump-7518. For the filtration, a membrane with a 10 kDa molecular weight cut-off and a filtration area of 0.1 m^2 was utilized. The membrane pressure was set to 15 psi, and the cross-flow rate was maintained at 300 mL/min. To monitor the filtration process, flow meters and pressure gauges were connected strategically to both the membrane's inlet and the outlet points of the permeate and retentate streams.

2.2.5. Estimation of Zinc Content. Zinc content in native chickpea protein (NCP), succinylated chickpea protein (SCP), and ultrasonication-treated succinylated chickpea protein (USCP) was evaluated using the method described by Hall et al. [29]. ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) (iCAP PRO ICP-OES, Thermo Fisher Scientific, Waltham, Massachusetts, US) was employed for zinc quantification. Laboratory dishes were immersed in 10% nitric acid (HNO3) for 24 h, then washed, dried, and used for analysis. The ICP-OES was calibrated using the ICAL method and a multistandard solution for zinc concentration. Calibration was based on radial observation at a 213.8 nm emission line without added flux or hydrochloric acid. Precipitation occurred beyond 10 mg/L, likely due to chloride. A 20 mg/L zinc solution served as an ICV control. ±10% deviation from expected values was set as the verification criterion, following recommendations by Spectro Analytical Instruments GmbH and the U.S. EPA. Control sample analysis ensured measurement accuracy.

2.2.6. Mineral Binding Efficiency of Chickpea Protein. The mineral binding efficiency of protein to zinc can be defined as the capacity of the protein to form a complex with zinc. The calculation of mineral binding efficiency is performed according to the following equation:

$$\text{Mineral binding efficiency } (\%) = \left[\left(\frac{\text{Total zinc in the sample}}{\text{Free zinc in the sample}}\right) \times 100\right]. \tag{4}$$

2.2.7. Functional and Physiochemical Properties

(1) Water-Holding Capacity (WHC) and Oil-Holding Capacity (OHC). The WHC and OHC of both native and modified zinc-bound proteins were evaluated using the methodology outlined by Li et al. [30]. To assess the water-holding capacity (WHC), 0.5 g of unaltered and modified chickpea protein powder with zinc binding was dispersed in 5 mL of deionized water within a beaker. Subsequently, the mixture was transferred to mini centrifuge tubes. For the oil-holding capacity (OHC) measurement, 0.5 g of the protein powder was evenly distributed in 5 mL of canola oil. This dispersion was performed in preweighed centrifuge tubes. The tubes were then subjected to incubation for 30 min, followed by centrifugation at $6000 \times g$ for 15 min using a Beckman Coulter Laboratory Centrifuge located in the United States, California. The resulting pellets were collected, and any remaining material (supernatant) was discarded. The centrifuged tubes containing the pellets were reweighed. WHC and OHC were quantified as the weight of water (g) or oil (g) retained per gram of the respective native and modified chickpea protein samples as given in the following equation:

$$\text{Water or oil holding capacity } (\text{g/g}) = \left[\left(\frac{\text{The dry weight of the sample } (\text{g}) - \text{Wet weight of sample } (\text{g})}{\text{weight of the sample } (\text{g})}\right) \times 100\right]. \tag{5}$$

(2) Foaming Capacity (FC) and Foaming Stability (FS). The FC and FS of native and modified zinc-bound chickpea protein samples were assessed using the procedure outlined by Wang et al. [31]. A 0.5 g of native and modified zinc-bound chickpea protein powder was mixed with 25 mL of deionized water and agitated for 30 min at the highest speed

using a laboratory grinder. After agitation, the dispersed sample was transferred to a 250 mL graduated glass measuring cylinder, and the initial and final volumes were measured carefully. The percentage change in volume was calculated to assess the foaming capacity or whipping ability of the sample. The foaming capacity and stability of the sample were determined according to (6) and (7).

$$\text{Foaming capacity } (\%) = \left[\left(\frac{\text{Whipped volume (mL)} - \text{Initial volume (mL)}}{\text{Initial volume (mL)}}\right) \times 100\right]. \tag{6}$$

The foaming stability was assessed by determining the retained foam volume after 30 min incubation at $25 \pm 2^{\circ}$C as a percentage of the initial foam volume.

$$\text{Foaming stability } (\%) = \left[\left(\frac{\text{Volume after resting (mL)} - \text{volume before resting (mL)}}{\text{Volume before resting (mL)}}\right) \times 100\right]. \tag{7}$$

(3) Emulsifying Capacity (EC) and Stability (ES). The ES and EC of chickpea protein were assessed using the procedure described by Opazo et al. [32]. An emulsion was prepared using a low-energy method. First, a 1% w/v solution of native and modified zinc-bound chickpea protein powder was created as the aqueous phase. To this solution, 1 mL of canola oil was added. The mixture was then subjected to magnetic stirring at 5000 rpm for 1 h until a creamy appearance indicated the formation of the emulsion.

To assess the emulsifying characteristics (EC) of the chickpea protein, the formulated emulsion was further subjected to centrifugation at $10000 \times g$ for 20 min. After centrifugation, the emulsified sample was carefully transferred to a graduated glass measuring cylinder, and the height of the emulsion in the cylinder was precisely measured. This measurement was carried out to evaluate the EC of the chickpea protein. The EC and EA of the sample are determined according to (8) and (9).

$$\text{Emulsion capacity } (\%) = \left[\left(\frac{\text{Emulsified layer height (cm)}}{\text{Total height (cm)}}\right) \times 100\right]. \tag{8}$$

The ES was then measured by subjecting the emulsion to heat treatment at 70°C for 20 min subsequently, followed by centrifugation at $14000 \times g$ for 15 min.

$$\text{Emulsion stability } (\%) = \left[\left(\frac{\text{Emulsified layer height (cm) after heating}}{\text{Emulsified layer height (cm) before heating}}\right) \times 100\right]. \tag{9}$$

(4) Protein Solubility (PS). The protein solubility (PS) of both native and modified zinc-bound chickpea protein powder was examined following the methodology described by Li et al. [33]. 0.5 g of native and modified zinc-bound chickpea protein was mixed with 5 mL of deionized water. The pH of the mixture was adjusted to 7 using a 2 M NaOH solution. The protein solutions were then stirred with a magnetic stirrer at an agitation rate of 850 rpm for 15 min afterward, centrifugation was carried out at $14000 \times g$ for 10 min at 24°C. The supernatant was collected, and the remaining material (pellets) was discarded. The protein content of the collected supernatant was determined using the method described by Lowry et al. [34]. To evaluate the solubility of the native and modified chickpea protein, triplicate

measurements of the powder samples were performed. The solubility of the sample was evaluated using the following equation:

$$\text{Protein solubility (\%)} = \left[\left(\frac{\text{Protein concentration in the supernatant}}{\text{Total protein concentration in the sample}}\right) \times 100\right]. \quad (10)$$

(5) Free Sulfhydryl Content (SH). The quantification of unbound sulfhydryl concentration was conducted employing the experimental approach outlined by Shui et al. [35]. The native and modified zinc-bound chickpea protein powder was thoroughly mixed with a phosphate buffer solution at a concentration of 2% (w/v). Ellman's reagent was prepared by dissolving 5,5-dithiobis-(2-nitrobenzoic acid) in Tris-glycine buffer at a pH of 8 under controlled low-light conditions, resulting in a final concentration of 8 mg/mL. A 1 mL portion of the diluted sample was combined with 5 mL of Tris-glycine buffer containing 8 M urea. In addition, 20 μL of Ellman's reagent was added to the mixture. The prepared blend was then incubated in a light-protected environment at 25°C for 20 min. After incubation, the mixture's absorbance was measured at 412 nm using a UV-visible spectrophotometer. Tris-glycine buffer served as the blank for comparison and calibration during the analysis. The SH content of the sample was measured according to the following equation:

$$\text{Free sulfhydryl content } (\mu\text{M/g}) = \left[73.53 \times A_{412} \times \left(\frac{\text{DF}}{C}\right)\right]. \quad (11)$$

In the described method, the absorbance (412 nm) of the sample is measured using a UV-vis spectrophotometer. The calculations incorporated the dilution factor (DF) and accounted for the quantity of the diluted sample to ensure accurate results.

(6) Surface Hydrophobicity (H_0). The assessment of H_0 was carried out utilizing the methodology established by Ji et al. [36]. The native and modified chickpea protein powder samples were diluted to create a concentration range of 0.1 to 1 mg/mL. This dilution was achieved by adding 10 mM phosphate-buffered solution at pH 7 while maintaining an inclined position. After preparing the mixture, it was left undisturbed in a dark room for 30 min. Next, 20 μL of a solution containing 1-anilino-8-naphthalene-sulfonate (ANSA) was added to each 4 mL of the sample solution at a lower concentration. The ANSA solution was prepared by dissolving it in a phosphate-buffered solution with a concentration of 10 mM and a pH of 7. The fluorescence intensity of the sample was then measured using a fluorescence spectrophotometer (Hitachi Hi-tech, Mumbai, India) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm. To assess the SH reactivity of the protein, the slope of the initial fluorescence intensity for the concentration of protein was calculated.

2.2.8. Characterization of Chickpea Protein-Zinc Complex

(1) Scanning Electron Microscope (SEM). The Field Emission Scanning Electron Microscope (FE-SEM: JEOL JSM-7610F Plus, Tokyo, Japan), equipped with an Energy Dispersive Detector (EDS: MRB EDS, SDD 150 LN2 free, UK) and a Gold Sputter Coater (ACG World Smart Coater), was utilized to examine the morphological characteristics of powdered chickpea protein. For the analysis, 5 mg of both native and modified zinc-bound chickpea protein powder was weighed and mounted on carbon-coated copper tape. The sample then underwent a sputtering process at 30 mA for 2 min to enhance its conductivity by applying a thin coating of gold. Micrographs of the sample were captured at different magnifications: 250x, 5000x, and 600x, and using accelerating voltages of 15 kV and 20.0 kV. The working distance during imaging was maintained within the range of 8.0–7.9 mm for each set of conditions.

(2) Fourier-Transform Infrared Spectroscopy (FTIR). Fourier-transform infrared spectroscopy (FTIR) was utilized to analyze the functional groups present in the native and modified zinc-bound chickpea protein powder. Attenuated Total Reflectance (ATR) and Pellet accessories (PERKIN ELMER X400) were used to collect the spectral data. The sample preparation involved mixing 10 mg of chickpea protein powder with 100 mg of KBr to form a mixture. This mixture was then carefully applied to the clean surface of the machine mirror. Subsequently, the lens was positioned on the sample to ensure proper containment. The measurements were conducted in the wavenumber range of 4000 to 400 cm^{-1}, with air used as the background. The obtained data were collected in terms of transmittance using dedicated Spectrum 10 software. This allowed for the observation and analysis of the functional groups present in the chickpea protein powder through FTIR spectroscopy.

(3) Particle Size and Zeta Potential. The zeta potential and size of the native and modified zinc-bound chickpea protein were measured at a temperature of 24°C using a particle size and zeta potential analyzer (Microtac MRB NANOTRAC Wave II, Osaka, Japan). To prepare the sample, a 1% solution of the native and modified zinc-bound chickpea protein was created. This solution was then subjected to ultrasonication treatment in an ultrasonic water bath for 10 min to ensure proper dispersion. Each sample was measured in triplicate to ensure accurate and reliable results for the zeta potential and size analysis of the chickpea protein samples.

(4) Thermogravimetric Analysis (TGA). The thermal stability and mass loss of the native and modified zinc-bound chickpea protein powder were evaluated using thermogravimetric analysis (TGA 340 Em Prepash, Scinco Co. Ltd., Korea). A 15 mg sample was placed in an aluminum pan and subjected to a temperature range from 10 to 950°C. The sample was heated at a constant rate of 10°C per minute under a nitrogen atmosphere with a flow rate of 50 mL/min. During the analysis, the changes in mass were recorded to assess the thermal stability and decomposition behavior of the chickpea protein samples.

(5) Differential Scanning Calorimeter (DSC). The thermal properties of the native and modified zinc-bound chickpea protein powder were analyzed using the DSC technique (TA instrument, New Castle, Delaware). DSC analysis was performed under a controlled atmosphere of 99.999% pure nitrogen gas. The temperature ramp rate ranged from 0.01°C/min to 100°C/min. Calibration of the instrument was performed using zinc and indium as reference materials to adjust temperature and heat flow. A 5 mg sample of the powdered substance was placed in an aluminum pan cell (PerkinElmer, 0219-0071), and measurements were recorded at a scan rate of 10°C/min. The temperature range covered in the experiment was from 10 to 450°C, with an empty pan serving as the reference point. The results were detected by thermocouple-based temperature sensors, and corresponding graphs were generated.

2.2.9. Mineral Bioavailability

(1) In vitro Mineral Bioavailability. To determine the *in vitro* bioavailability of zinc in chickpea protein samples, the method described by Soliman et al. [37] was employed. The study focused on evaluating how effectively the zinc present in chickpea protein could be absorbed and utilized by the body. The experiment involved simulating the gastrointestinal digestion process and utilizing Caco-2 cell lines in a Transwell assay to assess cellular absorption. Caco-2 cell lines are commonly employed in Transwell assays to study how substances are absorbed across the intestinal barrier. These human colorectal cells closely mimic the small intestine's characteristics, making them a valuable model. In this assay, cells are grown on a porous membrane that separates two compartments, representing the intestinal lumen and the basolateral side. The test substance is introduced into the upper compartment, mimicking the intestinal conditions. After incubation, samples from both compartments are collected to assess absorption by measuring substance concentration. By employing these techniques, the researchers aimed to gain insights into the bioavailability of zinc from chickpea protein and its potential impact on human nutrition.

(2) Stimulated Gastrointestinal Digestion. To evaluate zinc bioavailability in the chickpea protein sample, a simulated gastrointestinal digestion process was conducted. Firstly, 5 mL of the protein sample was mixed with 1.90 mL of saliva solution and incubated in an orbital shaker at 35°C and 95 rpm for 10 min to simulate the conditions of the gastrointestinal tract. Next, 3.1 mL of gastric juice was added, and the pH was adjusted to 2 using hydrochloric acid (HCl). The mixture was then incubated in a dark room at 35°C for 2 h to simulate the gastric digestion phase. Following the gastric phase, a combination of 1.97 mL of bile juice and 5.30 mL of duodenal juice was introduced to the sample to represent the intestinal digestion phase. This mixture was incubated for 3 h. After completing the digestion process, the solution was centrifuged using an ultrafiltration centrifuge tube with a 10 kDa molecular weight cut-off. This step separated the soluble fraction from the insoluble components of the sample. The percentage bioavailability of zinc was calculated based on the concentration of zinc in the soluble fraction according to the following equation:

$$\text{Zinc bioavailability (\%)} = \left[\left(\frac{\text{Zinc concentration of dialysate (permeate)}}{\text{Zinc concentration of the sample}}\right) \times 100\right]. \quad (12)$$

(3) Cellular Absorption Study (Transwell Assay). Caco-2 cells were cultured based on the methodology specified in the study conducted by Isik et al. [38]. The growth medium contained 30 μg/mL streptomycin, 25 μg/mL amphotericin, 100 μg/mL penicillin, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% DMEM. Caco-2 cells were cultured at 35°C in a CO_2 incubator with 96% humidity and 4% CO_2 levels. The medium was changed every other day, and cell passaging was performed when cells reached approximately 90% confluence, which usually took 7-8 days. Cell subculturing was performed using 0.05% EDTA and 0.5% trypsin. Caco-2 cells from the 39th passage were seeded at a density of 50,000 cells per well in six-well plates with 24 mm sterile polyester membranes having a 0.4 μm pore size. This setup was used for the mineral absorption assay and facilitated zinc transfer within the cells. The cells were allowed to differentiate further in the CO_2 incubator. The culture medium was refreshed every other day, and the cells were washed with PBS five times. After an initial 4-5 days, an additional 10-day period of differentiation was allowed before conducting the transepithelial cellular absorption assay.

(4) Valuation of Cell Monolayer Integrity. After ensuring 95–100% confluence, the integrity of the Caco-2 monolayer on the Transwell was tested with the help of a phenol red dye test, by using a modified method developed by Vij et al. [39]. The procedure began with washing both the apical and basal

chambers of six-well Transwell plate inserts. Subsequently, 2 mL of DMEM was introduced into the apical chamber, while an equivalent volume of PBS devoid of phenol red was added to the basal chamber. The culture was then subjected to an incubation period at 37°C within a CO_2 incubator for a duration of 1 h. Afterward, 100 μl of solutions from both the apical and basal chambers, along with PBS and DMEM serving as controls, were gathered in a 96-well microtitre plate. The absorbance of these samples was measured spectrophotometrically at 558 nm, and their potential for phenol red leakage through intercellular spaces was assessed. This integrity assessment procedure was carried out both before and after the completion of the main experimental protocol.

Following the integrity assessment, the apical and basal chambers of the wells underwent three washes with 2 mL of HBSS. Subsequently, preincubation of the apical and basal chambers of each well was performed with 2 mL of HBSS for a period of 30 min. Except for the control well, a portion of HBSS was replaced with the respective peptide solution, resulting in final concentrations of 100, 200, 300, 400, and 500 μg/mL (equivalent to 128.20–690.01 μM) in the respective wells. The plate was once again incubated for 60 min. At 0 min, 500 μl of supernatant was collected from both the apical and basal chambers, while after the experiment, 1.5 mL of supernatant was collected from both chambers. The integrity of the cells was reevaluated using the phenol red test. The procedure employed for estimating the saturation time was the same as described for saturation concentration estimation as discussed above, with the exception that the final concentration of the solution in the apical chamber was 400 μg/mL (512.82 μM) in each well. The plate was incubated for varying time intervals, specifically 0, 5, 10, 15, 30, 60, and 90 min, at 37°C in a 5% CO_2 environment.

(5) Transport Studies of Mineral. To investigate the transepithelial transport of minerals, previously washed Caco-2 cells were exposed to 2 mL of DMEM medium containing mineral dialysate obtained from the simulated gastrointestinal treatment. The mineral concentration in the medium was set to 50 μM, and this prepared medium was added to the wells positioned on the upper surface of the cells. In parallel, 2 mL of regular DMEM was added to the bottom compartment. After a 3 h incubation at 37°C, the DMEM medium was removed to assess the transepithelial absorption through the cell line. Subsequently, the cells were cultured for an additional 22 h to induce ferritin synthesis. To analyze the mineral transfer across the cellular barrier from both chambers, the ICP-OES method was employed. This analytical technique allowed for the quantification of minerals present in the samples obtained from the upper and lower compartments of the cell culture.

2.3. Statistical Analysis. All tests were performed in triplicate, and the data were reported as the mean ± standard deviation of three independent measurements. Statistical analysis involved one-way analysis of variance (ANOVA) and *t*-tests, with a significance level set at $p < 0.05$. The critical difference (CD) was calculated using Microsoft® Excel®, version 2021 (Microsoft Corporation, Redmond, Washington, USA).

3. Results and Discussion

3.1. Zinc-Binding Efficiency of Chickpea Protein. The mineral-binding efficiency of proteins is crucial for the effective absorption and utilization of essential minerals like zinc. As shown in Figure 1, the zinc-binding efficiency of the native chickpea protein (NCP) increases significantly ($p < 0.05$) from 93.64 ± 0.20 to 98.26 ± 0.42% up to the addition of 1–3 mM concentration of zinc sulfate in the protein, and reaching its maximum at 3 mM concentration of zinc (98.26 ± 0.42%). This increase in mineral binding efficiency is attributed to the formation of stable chelates between the chickpea protein and zinc ions. The specific amino acids present in the protein, such as histidine, cysteine, and aspartic acid, contain functional groups like imidazole and thiol, which facilitate strong coordination bonds with zinc ions [40]. As a result, stable complexes (chelates) are formed, with multiple amino acids surrounding and binding to individual zinc ions. The strategic arrangement and abundance of these amino acids in the chickpea protein enhance its affinity for zinc, allowing it to efficiently capture and bind a larger number of available zinc ions, leading to the observed significant increase in mineral binding efficiency [41]. Our study aligns with the study of Fasae et al. [41], in which the mineral binding efficiency of succinylated sodium caseinate is maximum at a 2.4 mM concentration of zinc sulfate. As the zinc sulfate concentration increases in the range of 4–12 mM, the zinc-binding efficiency of chickpea protein increases nonsignificantly ($p > 0.05$) (98.39 ± 1.04 to 99.04 ± 0.17%). This may be attributed to the limited availability of binding sites. Zinc ions may already occupy these sites, leading to saturation and reduced binding efficiency. Factors like steric hindrance, electrostatic interactions, and protein conformation changes further contribute to this phenomenon [42]. Steric hindrance occurs when nearby zinc ions hinder the accessibility of other binding sites, affecting overall efficiency [43]. Electrostatic interactions between charged amino acids and zinc ions influence the stability of the binding interactions. Changes in protein conformation at higher zinc concentrations can impact the availability of binding sites, influencing the overall efficiency [44]. Our research is consistent with the study conducted by Nguyen et al. [44], where the mineral binding efficiency of succinylated sodium caseinate shows a nonsignificant increase beyond a zinc sulfate concentration of 3 mM.

Furthermore, the zinc-binding efficiency of succinylated chickpea protein (SCP) ($p < 0.05$) significantly increases (95.96 ± 1.14 to 98.69 ± 0.36%) in the range of 1–3 mM zinc sulfate concentration. The observed increase is due to the succinylation process, where the addition of negatively charged succinyl groups to specific amino acid residues, particularly lysine, using succinic anhydride [45]. These succinyl groups enhance electrostatic interactions with

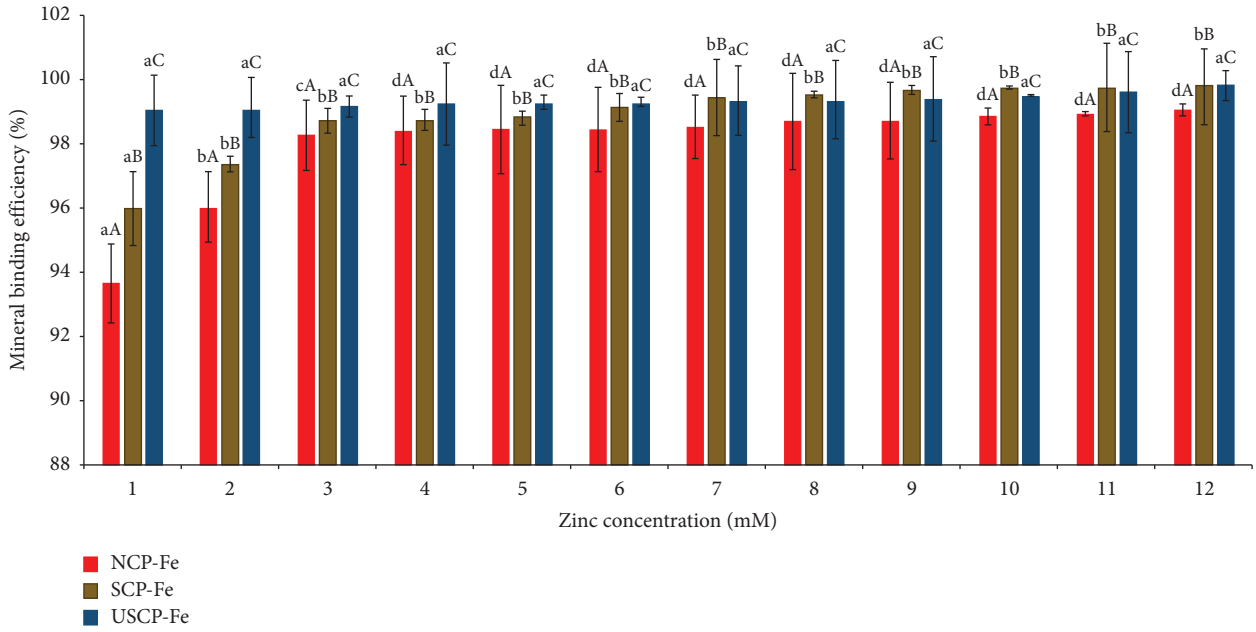


Figure 1: Mineral binding efficiency of native chickpea protein (NCP); succinylated chickpea protein (SCP); ultrasonication-treated succinylated chickpea protein (USCP). The results were expressed as the mean ± standard deviation of ≥3 independent replicates, and error bars represent the standard deviation from the mean values, while different lowercase and uppercase letters above each bar represent significantly different values within zinc concentration and protein sample, respectively, based on analysis of variance (ANOVA) and post hoc tests.

positively charged zinc ions, acting as additional binding sites for zinc. This increases the protein's mineral binding capacity and efficiency, enabling more effective and stable zinc-protein interactions, resulting in higher mineral binding efficiency compared to the native protein [46]. Our research is in line with the findings of Muduli et al. [46], wherein the maximum mineral binding efficiency of succinylated milk protein occurs at a zinc sulfate concentration of 7 mM. Moreover, as the zinc concentration increases in the range of 4–12 mM, the zinc-binding efficiency of chickpea protein increases nonsignificantly ($p > 0.05$) (98.73 ± 0.33 to 99.77 ± 1.17%). This is due to the at higher zinc concentrations, the binding sites on the SCP become saturated, and already-bound zinc ions hinder nearby binding sites' accessibility, causing steric hindrance. This reduces the efficiency of additional zinc binding, leading to a nonsignificant increase in mineral binding efficiency [47]. Our study is in agreement with Proctor et al. [47], where the mineral binding efficiency of succinylated milk protein demonstrates a nonsignificant increase beyond a zinc sulfate concentration of 7 mM.

Moreover, the zinc-binding efficiency of dual modified (ultrasonication + succinylation) ultrasonicated-treated succinylated chickpea protein (USCP) significantly ($p < 0.05$) increased by 3.10% (95.96 ± 1.14 to 99.03 ± 1.10%) by adding the concentration of zinc sulfate at 1 mM as compared to SCP. Ultrasonication treatment of succinylated chickpea protein significantly increases zinc-binding efficiency through multiple mechanisms. Firstly, ultrasonication subjects the protein to high-frequency sound waves, inducing conformational changes that expose more binding sites for zinc ions. This altered protein structure is more conducive to forming stable complexes with zinc. Secondly, the treatment improves protein solubility by disrupting aggregates, ensuring a higher effective concentration of available binding sites for zinc [48]. Furthermore, the process fragments the protein, increasing its surface area and providing more opportunities for zinc ions to interact with the protein. Ultrasonication also enhances mass transfer, allowing zinc ions to readily diffuse and react with the modified protein, thereby increasing binding efficiency. Ultrasonication prevents aggregation and precipitation, ensuring that zinc ions remain accessible for binding. Collectively, these effects make succinylated chickpea protein more efficient in binding zinc ions, facilitating their incorporation into the protein matrix [49]. In addition, ultrasonication also accelerates chemical reactions, facilitating faster zinc-protein interactions. Moreover, ultrasonication-induced conformational changes may expose additional binding sites and alter surface charge distribution, further enhancing zinc affinity [50]. Enhancing zinc-binding efficiency improves mineral bioavailability by converting zinc into more easily absorbed forms. These forms are efficiently transported into the bloodstream, increasing the amount available for distribution to tissues [51]. Improved binding efficiency also aids cellular mineral uptake, facilitating zinc's crucial role as a cofactor for various enzymes and proteins, ultimately benefiting overall cellular health and function [52]. These combined effects contribute to the observed significant increase in zinc-binding efficiency. Moreover, as the zinc concentration increases at 1 to 12 mM, shows a nonsignificantly ($p > 0.05$) increase in mineral binding efficiency up to 99.80 ± 0.48. This could be due to binding site saturation, competitive binding, steric

hindrance effects, and solute-induced conformational changes, all of which limit the further enhancement of zinc-protein interactions [53].

Protein samples with the highest zinc-binding capacities, including native chickpea protein bound with zinc at 3 mM (NCP-Zn), succinylated chickpea protein bound with zinc at 3 mM (SCP-Zn), and ultrasonicated treated succinylated chickpea protein bound with zinc at 1 mM (USCP-Zn), were selected for further investigation. A comprehensive analysis will assess their functional, physicochemical properties, and characteristic features. *In vitro* mineral bioavailability studies will determine the availability of bound zinc for human body absorption, and cellular mineral uptake of zinc will be examined to evaluate the proteins' efficiency in delivering zinc to cells.

3.2. Impact of Succinylation on the Protein Content of Zinc-Protein Complex. Succinic anhydride can modify certain amino acids, including histidine, lysine, serine, and threonine, through succinylation. Among these, lysine exhibits the highest reactivity and is commonly used for succinylation. The measurement of lysine content in chickpea protein serves as an indicator to assess the degree of succinylation. Figure 2 shows a nonsignificant ($p > 0.05$) difference in the protein content of SCP-Zn ($81.58 \pm 1.95\%$) compared to SCP ($80.15 \pm 0.57\%$). This is because the succinylation process and zinc binding do not significantly alter the overall protein amount. The specific amino acid residues targeted during succinylation and the occupancy of zinc-binding sites are already present in the nonbound protein, resulting in minimal changes to the total protein content [54]. In addition, the stability of the protein structure is not significantly affected by succinylation and zinc binding, leading to a nonsignificant difference in protein content [54]. Our study is consistent with Shiplashree's study [41]," where the protein content of zinc-bound succinylated whey protein exhibits a non-significant increase when compared to nonbound whey protein.

3.3. Impact of Dual Modification (Ultrasonication and Succinylation) on the Protein Content of Zinc-Protein Complex. Ultrasonication, a high-frequency sound wave-based process used in the food industry, can modify proteins to improve solubility and control particle size. However, in Figure 2, there was no significant ($p > 0.05$) difference in protein content between ultrasonicated-treated succinylated chickpea protein bound with zinc (USCP-Zn) (84.26 ± 0.62) and ultrasonicated-treated succinylated chickpea protein without zinc binding (USCP) (84.33 ± 0.79). This is because zinc binding does not significantly alter the protein content, and the ultrasonication process primarily influences protein modifications such as solubility and particle size control [55].

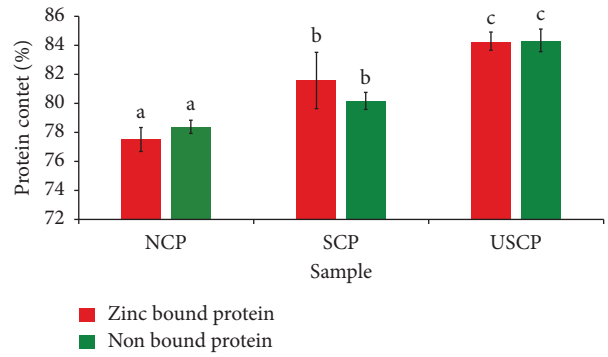


Figure 2: Protein content in zinc-bound and nonbound native chickpea protein (NCP), succinylated chickpea protein (SCP), and ultrasonicated-treated succinylated chickpea protein (USCP). The results were expressed as the mean ± standard deviation of ≥3 independent replicates, and error bars represent the standard deviation from the mean values, while different lowercase letters above each bar represent significantly different values based on analysis of variance (ANOVA) and *t*-tests.

3.4. Impact of Dual Modification Methods on Functional and Physiochemical Properties of Zinc-Protein Complex

3.4.1. Solubility. Chickpea protein exhibits minimal solubility around its isoelectric point (pH 4-5) but demonstrates the highest solubility at pH levels ranging from 1 to 3 and 7 to 10. When comparing the solubility of SCP-Zn and NCP-Zn at pH 7 (Figure 3(a)), it is observed that the solubility of SCP-Zn significantly increased from $81.42 \pm 0.89\%$ to $86.13 \pm 1.05\%$, representing a 5.46% improvement over NCP-Zn (with $p < 0.05$ significance). This is due to the succinylation process, which introduces more negative charges and enhances the protein's hydrophilicity. This modification leads to a more water-compatible protein structure, making it highly soluble in aqueous environments [55]. In addition, the presence of zinc ions further stabilizes the succinylated protein, contributing to its improved solubility compared to its native counterpart. Our study is in agreement with Shilpashree et al. [56], wherein the solubility of zinc-bound succinylated whey protein demonstrates a significant increase of 77.77% at a zinc sulfate concentration of 8 mM. Accordingly, USCP-Zn shows a significant ($p < 0.05$) increase of 86.13 ± 1.05 to $91.61 \pm 0.56\%$ as compared to SCP-Zn. The significant increase in solubility can be attributed to the synergistic effects of ultrasonication and succinylation with zinc binding. Ultrasonication creates cavitation and microstreaming, breaking down aggregates and reducing particle size, thus exposing more hydrophilic regions and promoting dispersion in the solvent. The resulting structural modifications make the protein more amenable to water interactions, leading to higher solubility [56]. In addition, the ultrasonication process enhances the binding of zinc ions to the succinylated chickpea protein, improving protein stability and preventing the reformation of aggregates during zinc binding [57].

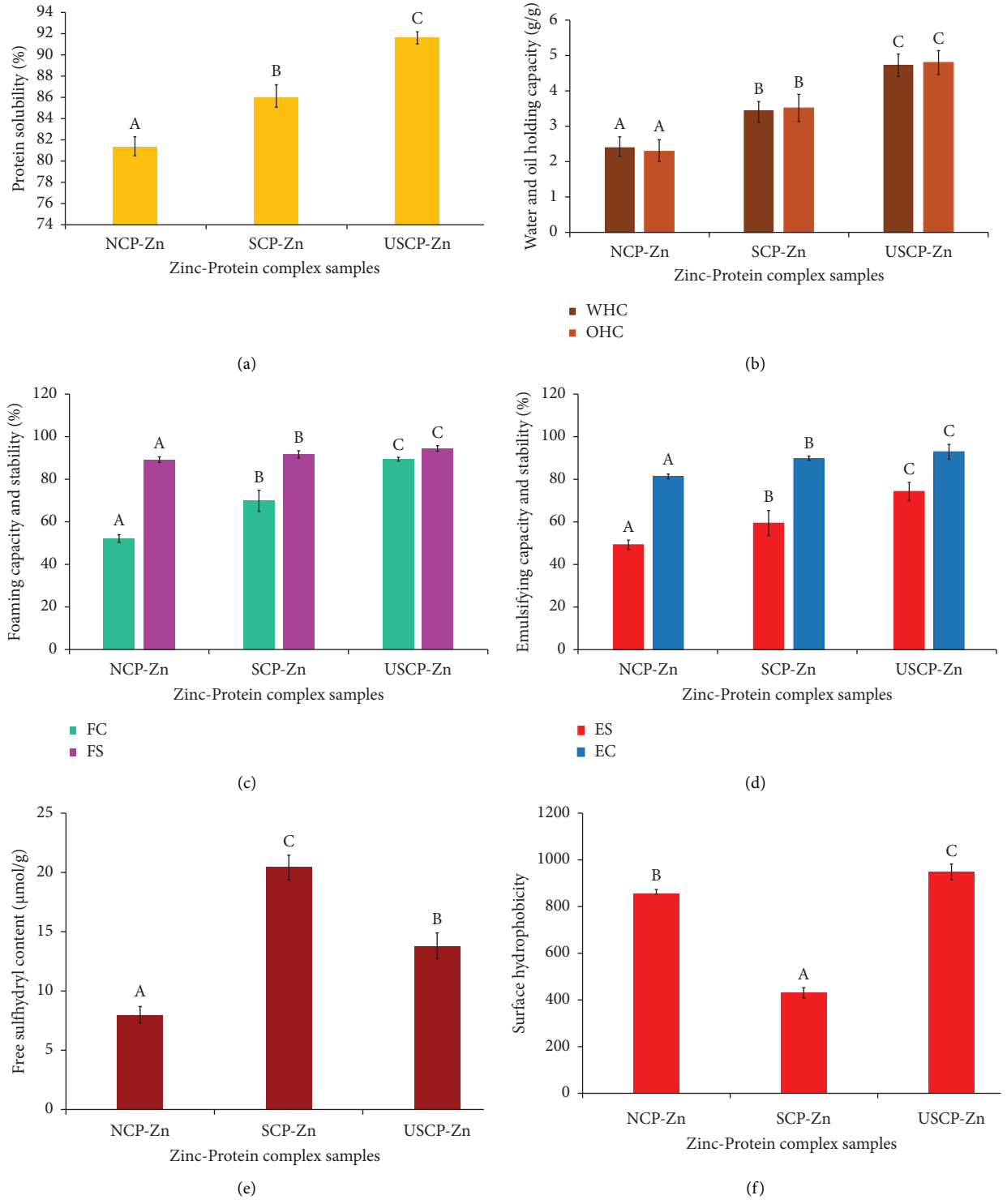


FIGURE 3: Protein solubility (a), water- and oil-holding capacity (b), foaming capacity and stability (c), emulsifying capacity and stability (d), free sulfhydryl content (e), and surface hydrophobicity (f) of the zinc-bound native chickpea protein (NCP-Zn), zinc-bound succinylated chickpea protein (SCP-Zn), and zinc-bound ultrasonication-treated succinylated chickpea protein (USCP-Zn). WHC, water-holding capacity; OHC, oil-holding capacity; FC, foaming capacity; FS, foaming stability; EC, emulsifying capacity; ES, emulsifying stability. The results were expressed as the mean ± standard deviation of ≥3 independent replicates, and error bars represent the standard deviation from the mean values, while different uppercase letters above each bar represent significantly different values based on analysis of variance (ANOVA) and *t*-tests.

3.4.2. Water and Oil-Holding Capacity (WHC and OHC). WHC (water-holding capacity) refers to a substance's ability to retain water, which is essential for maintaining moisture in food products. On the other hand, OHC (oil-holding capacity) represents a substance's capacity to absorb and retain oil, affecting the texture and mouthfeel of food formulations. As shown in Figure 3(b), the WHC of SCP-Zn was significantly enhanced ($p < 0.05$) from 2.43 ± 0.27 to 3.41 ± 0.29, indicating a 28.73% increase compared to NCP-Zn. This increase is because succinylated chickpea protein demonstrates enhanced solubility, increased hydrophilicity, and potential structural changes, resulting in a more open and accessible conformation with additional binding sites for water molecules [58]. Moreover, the coordination complexes formed between zinc ions and the succinylated protein further stabilizes its structure, contributing to its improved ability to hold and retain water [59]. Furthermore, WHC of USCP-Zn significantly increased ($p < 0.05$) from 3.41 ± 0.29 to 4.73 ± 0.32 as compared to SCP-Zn, this can be attributed to the fact that ultrasonication induces mechanical energy that disrupts protein aggregates and unfolds the protein, while succinylation introduces hydrophilic groups and prevents aggregation [60]. These combined modifications optimize the protein's structure and surface properties, making it more accessible to water molecules and allowing it to form stronger interactions with water [61].

Figure 3(b) shows that the OHC of SCP-Zn significantly ($p < 0.05$) increased by 34.09% as compared to the NCP-Zn. This can be attributed to several factors related to the modifications induced by succinylation. Succinylation introduces additional negative charges on the protein surface, facilitating stronger interactions with oil droplets. It also induces conformational changes that create more binding sites for oil molecules while reducing hydrophobic patches and enhancing protein dispersibility [62]. These modifications lead to improved emulsifying properties, stabilizing the oil-in-water emulsion, and promoting a more efficient oil binding process, ultimately resulting in higher oil-holding capacity [63]. Following the ultrasonication treatment for 20 min, the oil-holding capacity of USCP-Zn significantly increased from 3.52 ± 0.39 to 4.81 ± 0.33, which is 1.2 times greater than that of SCP-Zn**.** This increases due to synergistic effects. Ultrasonication disrupts aggregates, increases surface area for oil binding, and combined with succinylation-charged groups, enhances oil-protein interaction while reducing aggregation, resulting in improved oil binding efficiency [64].

3.4.3. Foaming Capacity and Stability (FC and FS). Foaming capacity (FC) and foaming stability (FS) are critical in food and beverage applications as they determine a protein's ability to create and maintain stable foam structures. These attributes depend on factors like protein structure, concentration, pH, and interactions with other components, all influencing the foam formation and longevity. In Figure 3(c), SCP-Zn shows a significant ($p < 0.05$) increase in foaming capacity by 24.59% and foaming stability by 2.76% compared to NCP-Zn. This is due to the enhanced solubility, altered surface properties, zinc-induced cross-linking, conformational changes, and potential pH effects. Succinylation improves protein solubility, allowing better dispersion in the aqueous phase, while altered surface properties enable stable air-water interfaces during foaming [65]. Zinc binding acts as a cross-linking agent, creating a cohesive protein network. Conformational changes and potential pH effects further contribute to the enhanced foaming properties of the succinylated protein [66]. The ultrasonication treatment was given to the succinylated chickpea protein for 20 min (USCP-Zn) increased the FC and FS significantly ($p < 0.05$) in the range of 68.70 ± 4.8 to 88.24 ± 0.85 and 90.54 ± 1.75 to 93.38 ± 1.37 as compared to SCP-Zn. This increases significantly due to the ultrasonication treatment's ability to enhance protein unfolding, increase surface area, and improve interfacial interactions [67]. This treatment also leads to improved protein solubility, allowing for a higher amount of protein to be available at the air-water interface during foam formation. In addition, the synergistic effect with zinc binding further strengthens the protein network at the interface, resulting in more stable foams [68].

3.4.4. Emulsifying Capacity and Stability (EC and ES). Emulsifying capacity (EC) and stability (ES) significantly increased after succinylation modification compared to NCP-Zn, as shown in Figure 3(d). EAI rose from 49.52 ± 2.92 to 59.41 ± 5.89, and ESI increased from 80.15 ± 0.95 to $90.08 \pm 0.83\%$ (both with $p < 0.05$ significance). This increase is due to several factors resulting from the succinylation process. Succinylation involves the introduction of charged functional groups, which enhances the protein's solubility and dispersibility in water or oil [59]. This improved solubility allows the succinylated protein to form a more uniform and stable emulsion. In addition, the modification alters the protein's surface properties, leading to increased adsorption at the oil-water interface and better stabilization of the emulsion [60]. The succinylation process also promotes stronger protein-protein interactions through cross-linking, further contributing to the emulsion's stability [69]. The USCP-Zn shows a significant increase ($p < 0.05$) in EC and ES within the range of 59.41 ± 5.89 to 74.39 ± 4.12 and 90.08 ± 0.83 to $93.11 \pm 3.37\%$ as compared to SCP-Zn. This enhancement can be attributed to the effects of ultrasonication, which lead to enhanced protein dispersion, surface modification, reduced interfacial tension, particle size reduction, and potential structural changes [70]. These combined alterations result in a larger interfacial area between the oil and water phases, a stable protein layer at the interface, and increased interaction with both phases in the emulsion system [71].

3.4.5. Free Sulfhydryl Content. Free sulfhydryl content refers to the concentration of unbound sulfhydryl (-SH) groups in a sample, indicating the presence of reactive thiol groups with important roles in chemical reactions and protein functionalities. Figure 3(e) shows that SCP-Zn exhibited a significant ($p < 0.05$) increase in SH content, rising from

$7.98 \pm 0.70\,\mu$mol/g to $20.46 \pm 1.02\,\mu$mol/g, which is 60.99% greater than NCP-Zn. This can be attributed to the succinylation process. Succinylation introduces succinyl groups to the protein structure, creating new binding sites for zinc ions. This results in the formation of more stable zinc-protein complexes, leading to higher zinc incorporation. With more zinc ions bound to the succinylated protein, there are increased interactions with sulfhydryl groups, elevating the free sulfhydryl content [72]. Furthermore, in USCP-Zn, the SH content significantly decreased ($p < 0.05$) from $20.46 \pm 1.02\,\mu$mol/g to $13.82 \pm 0.98\,\mu$mol/g as compared to SCP-Zn**.** The reduction in sulfhydryl content can be ascribed to the impact of ultrasonication on the zinc-protein binding process, resulting in modified interactions between zinc ions and sulfhydryl groups [73]. Ultrasonication generates mechanical stress and induces conformational changes in the protein structure, leading to the exposure or alteration of zinc-binding sites. These modifications may lead to the masking or chemical modification of sulfhydryl residues, impeding their ability to interact effectively with zinc ions. As a consequence, the quantified sulfhydryl content decreases when zinc binds to the protein under the influence of ultrasonication [74].

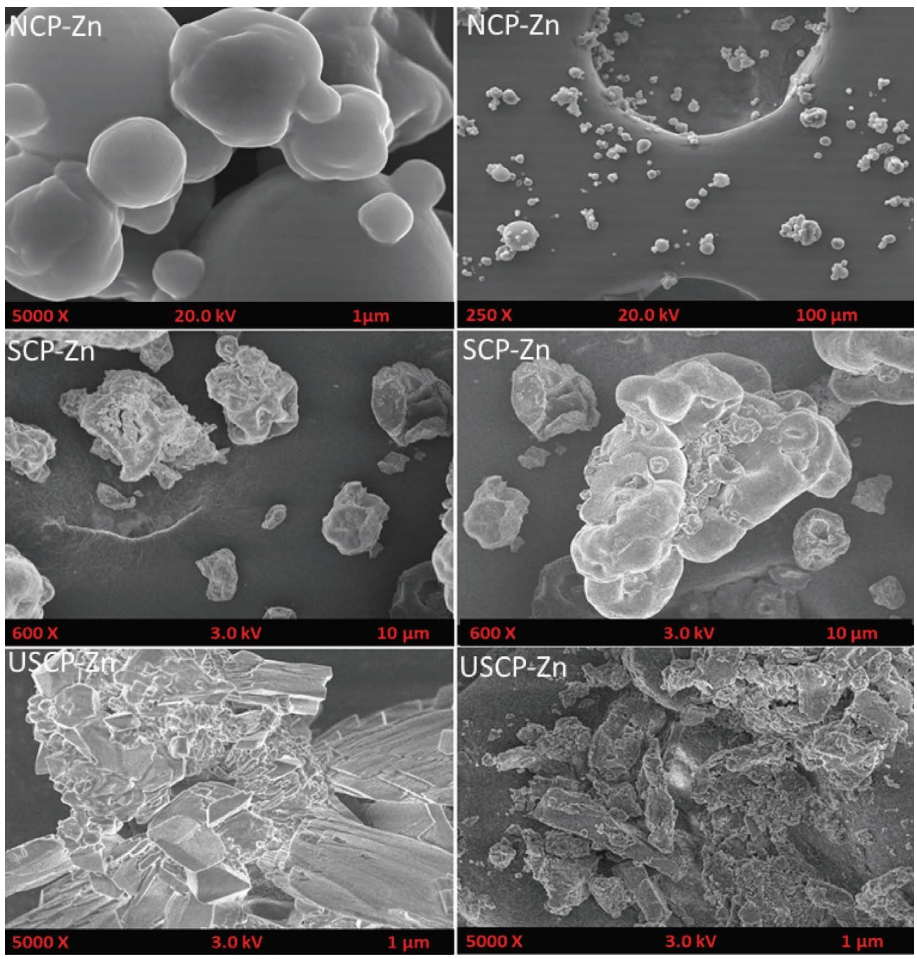
3.4.6. Surface Hydrophobicity. Surface hydrophobicity, which affects protein adsorption and surface wettability, refers to a surface's ability to repel water and is influenced by its chemical composition and characteristics. In Figure 3(f), zinc-bound succinylated chickpea protein (SCP-Zn) exhibited a substantial ($p < 0.05$) decrease ($p < 0.05$) in H_o value from 861.23 ± 9.71 to 429.58 ± 17.84, representing a 1.75-fold reduction compared to NCP-Zn. This reduces due to the succinylation-induced conformational changes, potential masking of hydrophobic patches, alterations in electrostatic effects, zinc's influence on surface properties, and the interplay between zinc and succinylation [48]. These factors collectively contributed to the reduction in the protein's hydrophobicity, impacting its solubility, stability, and interactions with other molecules [56]. Furthermore, the application of a 20 min ultrasonication treatment (USCP-Zn) to succinylated chickpea protein significantly increased ($p < 0.05$) surface hydrophobicity, ranging from 429.58 ± 17.84 to 946.68 ± 32.04 as compared to SCP-Zn. This can be attributed to the effects of ultrasonication. The mechanical forces generated during ultrasonication induce conformational changes, uncovering buried hydrophobic regions on the protein's surface [75]. In addition, the disruption of succinyl groups further promotes hydrophobic interactions. The process also leads to protein aggregation and surface roughness, both of which contribute to the overall enhancement of the protein's hydrophobicity [76].

3.5. Characteristics of Zinc-Chickpea Protein Complex

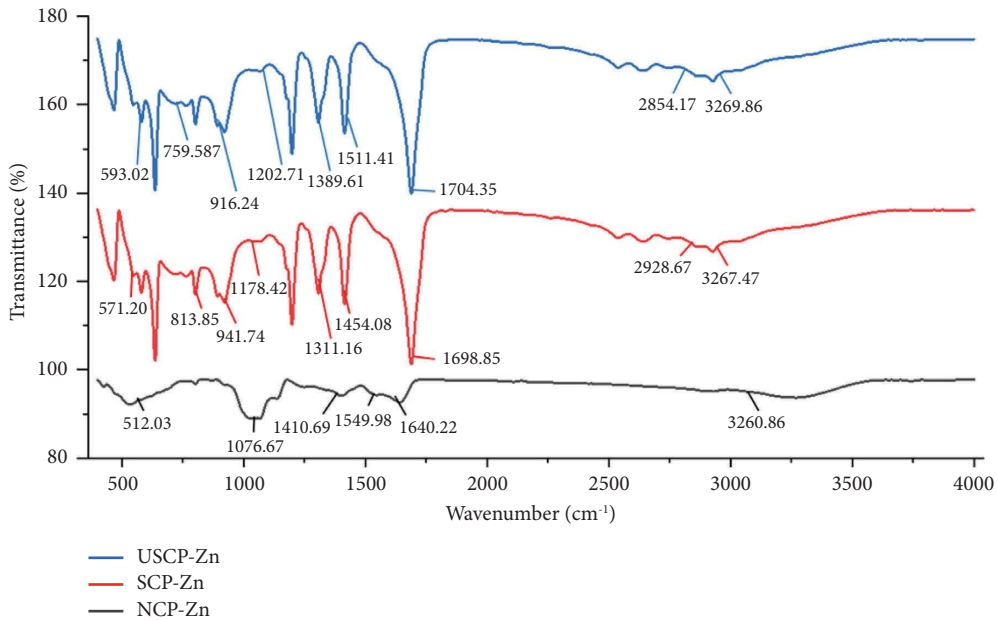
3.5.1. Scanning Electron Microscope (SEM). The surface morphology of NCP-Zn, SCP-Zn, and USCP-Zn samples was investigated using a scanning electron microscope (SEM) at various magnifications. In Figure 4(a), the SEM images revealed distinct microstructures for each sample. NCP-Zn displayed a smooth spherical structure at 5000x and 250x magnification. In contrast, SCP-Zn exhibited irregular flakes with a rough surface and minor cracks at 600x and 600x magnification, attributed to the spray drying process during succinylation. Similarly, USCP-Zn showed surface irregularities resulting from ultrasonication treatment, as observed at 5000x and 5000x magnification. High-intensity ultrasonication induced cavitation and mechanical effects, leading to the formation of microfractures and fragmentation of the protein structure. Previous studies have shown that combining chemical methods like alkalization and acidification with ultrasonication can lead to protein fragmentation and the presence of microfractures [77]. The structural analysis indicates that the incorporation of negatively charged succinyl groups in SCP-Zn increased electrostatic repulsion and steric hindrance, enhancing the protein's structural flexibility [67]. The mechanical and cavitation effects of ultrasonication further contributed to modifications in the secondary and tertiary protein structures, resulting in increased disorder [78]. The combination of ultrasonication and succinylation caused significant alterations in protein structure, directly impacting their functional properties. Our study is consistent with Shokri et al. [75], as it demonstrates similar structural changes in succinylated milk protein.

3.5.2. Fourier-Transform Infrared Spectroscopy (FTIR). The FTIR frequencies reported for the zinc-bound native chickpea protein (NCP-Zn) provide valuable insights into its molecular composition and structural characteristics. As shown in Figure 4(b), the presence of amide groups, which are common in proteins, is evident from the peak observed at $3260.86\,\text{cm}^{-1}$, which corresponds to the N-H stretching vibrations. This signifies the involvement of peptide bonds in the protein's primary structure. In addition, the frequency at $1640.22\,\text{cm}^{-1}$ indicates the C=O stretching vibration in the amide group, further confirming the presence of peptide bonds. The amide II band is represented by the peak at $1549.98\,\text{cm}^{-1}$, which involves N-H bending coupled with C-N stretching. This band provides information about the secondary structure of the protein. Furthermore, the frequency at $1410.69\,\text{cm}^{-1}$ is likely associated with C-H bending in the aliphatic side chains of the amino acids, indicating the presence of hydrocarbon groups within the protein. The FTIR spectrum also reveals a peak at $1076.67\,\text{cm}^{-1}$, which could be attributed to the C-O stretching in the peptide backbone, contributing to the overall conformation of the protein. Importantly, the presence of zinc coordination with oxygen atoms in the protein is suggested by the peak at $512.03\,\text{cm}^{-1}$, indicative of Zn-O stretching.

Similar to NCP-Zn, the presence of N-H stretching vibrations is evident from the peak at $3267.47\,\text{cm}^{-1}$, indicating the involvement of amide groups and peptide bonds in the protein's primary structure. The peak observed at $2928.67\,\text{cm}^{-1}$ is associated with C-H stretching vibrations, suggesting the presence of aliphatic groups in the succinylated protein. The frequency at $1698.85\,\text{cm}^{-1}$ corresponds



(a)



(b)

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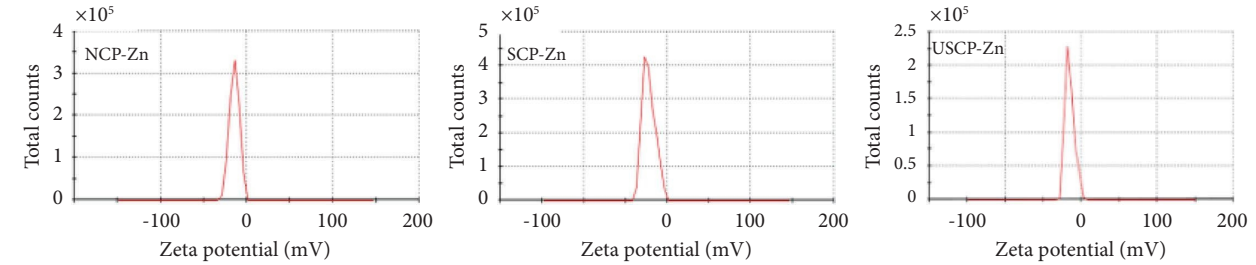
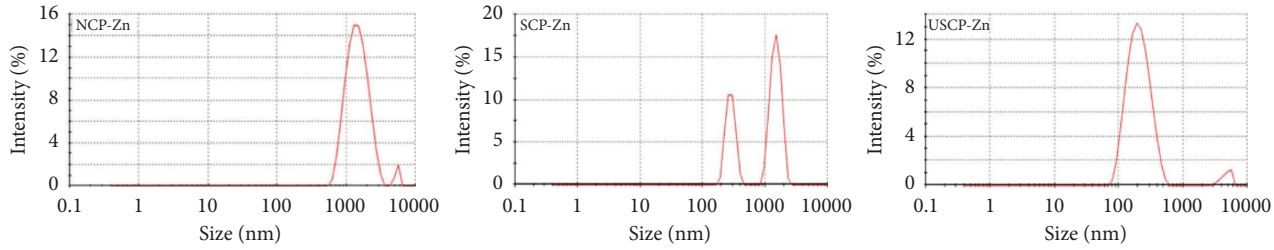
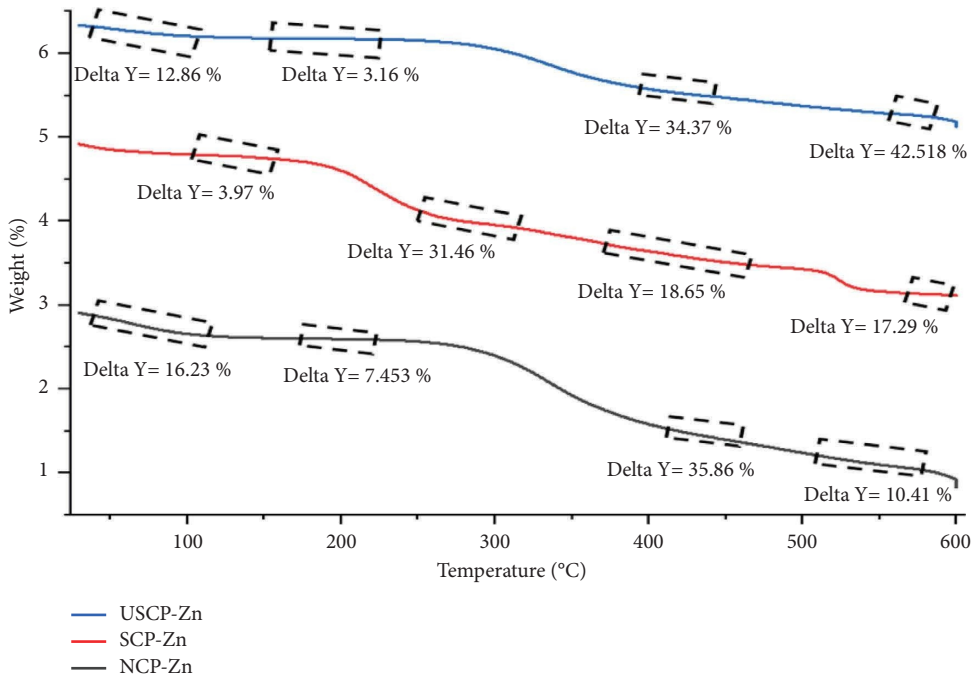


(c)



(d)



(e)

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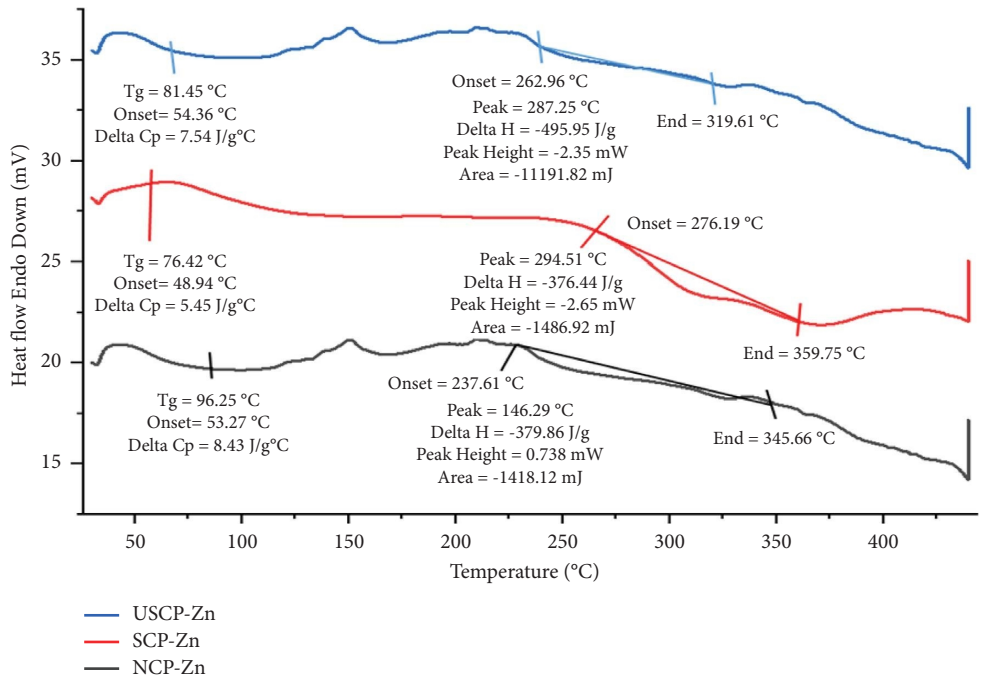

(f)

Figure 4: Scanning electron microscope (SEM) (a), Fourier-transformation infrared spectroscopy (FTIR) (b), zeta potential (c), particle size (d), thermogravimetric analysis (TGA) (e), and differential scanning calorimetry (DSC) (f) of the zinc-bound native chickpea protein (NCP-Zn), zinc-bound succinylated chickpea protein (SCP-Zn), and zinc-bound ultrasonication-treated succinylated chickpea protein (USCP-Zn).

to the C=O stretching vibration in the succinyl group, further confirming the presence of this functional group. The asymmetric stretching of carboxylate (COO-) groups in the succinylated protein is suggested by the peak at 1451.08 cm^{-1}, providing insights into the succinylation modification. The frequency at 1311.16 cm^{-1} might indicate the presence of amide III bands, involving a combination of N-H bending and C-N stretching, which provides information about the secondary structure of the protein. Furthermore, the peaks at 1178.42 cm^{-1} and 941.74 cm^{-1} are likely associated with C-O stretching and C-H bending vibrations, respectively, in the succinyl group. The presence of Zn-O stretching vibrations is indicated by the peak at 813.85 cm^{-1}, while the peak at 571.20 cm^{-1} suggests the coordination of zinc with nitrogen atoms (Zn-N stretching) in the succinylated protein. The appearance of new peaks in the FTIR spectrum of SCP-Zn compared to NCP-Zn can be attributed to the combined effects of succinylation and zinc binding. Succinylation introduces new functional groups, altering the protein's composition and potentially its conformation [76]. Zinc binding further induces structural changes, resulting in distinctive spectral features. These modifications arise from altered hydrogen bonding, secondary structure, and specific interactions between zinc ions and the succinylated protein [79].

The FTIR spectrum of USCP-Zn exhibits similar peaks to SCP-Zn, with noticeable frequency shifts. These shifts indicate significant structural and conformational changes induced by ultrasonication. The mechanical impact of ultrasonication, involving cavitation and shear forces, perturbs the secondary structures of the protein, resulting in partial unfolding. Moreover, the process promotes enhanced interactions with solvent molecules [80]. Concurrently, ultrasonication may lead to alterations in the coordination environment of zinc ions with neighboring residues.

3.5.3. Particle Size and Zeta Potential. The zeta potential and mean particle size of NCP-Zn subjected to SCP-Zn and USCP-Zn were investigated, as depicted in Figure 4(c) (NCP-Zn and SCP-Zn). SCP-Zn exhibited a significant ($p < 0.05$) increase in zeta potential value, from -12.4 ± 0.29 to -17.8 ± 0.61 mV, as compared to NCP-Zn. The increase in zeta potential observed in SCP-Zn can be attributed to the combined effects of zinc binding and succinylation. Succinylation introduces additional negative charges to the protein by attaching succinic acid groups, contributing to the overall negative zeta potential value observed in SCP-Zn [81]. In addition, when zinc ions bind to the protein surface, they may interact with the succinyl groups, further increasing the negative charges introduced by succinylation. As a result, there is a slight increase in the zeta potential value due to zinc binding [82]. However, it is important to note that the increase caused by zinc binding is not substantial. The primary influence of succinylation-induced negative charges prevails, and the presence of zinc ions serves to augment the negative surface charge [83]. Our study is consistent with Shilpashree et al. [56], where the

negative charge of zinc-bound succinylated whey protein increased by 33.89% in comparison to nonbound whey protein. Following the USCP-Zn shows a significant increase ($p < 0.05$) in zeta potential values of SCP-Zn, from -17.8 ± 0.61 to -23 ± 0.79 mV. The observed increase in zeta potential in USCP-Zn can be attributed to the exposure of a greater number of negatively charged groups during ultrasonication. The application of high-frequency sound waves induces mechanical disruptions in the protein solution, leading to physical changes in the protein molecules [84]. These disruptions expose previously concealed negatively charged groups on the protein surface. Consequently, some of the negative charges introduced by succinylation may be neutralized, and the presence of zinc ions due to zinc binding can further contribute to this effect by forming electrostatic interactions with the exposed negative charged groups, resulting in a more pronounced increase in the zeta potential [85].

Furthermore, as shown in Figure 4(d), the SCP-Zn exhibited a significant ($p < 0.05$) reduction of 30.39% in particle size compared to the NCP-Zn (1243 ± 0.8 to 1160 ± 0.48 nm). The reduction in particle size can be attributed to several factors. Firstly, the succinylation process, which involves attaching succinic acid groups to the protein molecules, alters the protein's surface charge and hydrophilicity. This modification increases protein solubility and dispersion, leading to smaller particle sizes [86]. Moreover, the binding of zinc ions to the succinylated protein may further contribute to particle size reduction. The presence of zinc ions can interact with the protein molecules, inducing conformational changes and promoting protein aggregation, resulting in the formation of smaller and more compact particles [87]. Our study is in line with Shilpashree et al. [56], where the particle size of zinc-bound succinylated whey protein decreased by 25.65% compared to nonbound whey protein. Moreover, zinc-bound ultrasonication-treated succinylated chickpea protein resulted in particle size reductions of 83.19% compared to SCP-Zn. The decrease in particle size can be attributed to the combined effect of ultrasonication and zinc binding. Ultrasonication generates intense shear forces and microstreaming within the zinc-protein solution, breaking apart larger protein aggregates into smaller particles [88]. The formation and implosion of cavitation bubbles during ultrasonication create localized high-energy zones, contributing to the breakage of protein aggregates [80]. In addition, zinc binding to the protein can alter its conformation and stability, influencing the aggregation behavior and overall particle size distribution. The synergy between ultrasonication and zinc binding enhances the dispersion of the protein, preventing the formation of larger aggregates and promoting a more uniform distribution of protein particles [89].

3.5.4. Thermogravimetric Analysis (TGA). The thermogravimetric analysis was conducted on the NCP-Zn, SCP-Zn, and USCP-Zn as shown in Figure 4(e). The specimens underwent thermal treatment with a heating rate of 10°C/min, starting from 25°C and reaching 600°C, within a nitrogen environment. The TGA curve of the NCP-Zn sample exhibited four weight-loss phases. The first phase was reported at 30 to 100°C. At this phase, 16.23%, the protein undergoes a moderate weight loss within a specific temperature range, typically associated with the desorption or removal of adsorbed or weakly bound water molecules from its structure [90]. The second phase which was reported for 7.453% of weight loss of native chickpea protein occurred within 150 to 200°C, the observed temperature range indicates the denaturation of the protein, where its native conformation is unfolded due to the disruption of weak intermolecular forces such as hydrogen bonds and hydrophobic interactions. This leads to progressive degradation of the protein's structure and further decomposition [91]. The third weight loss of 35.86% was reported at 400 to 450°C. At this stage, a substantial loss is observed in the protein. The temperature range indicates the occurrence of thermal degradation, during which the protein backbone begins to undergo fragmentation into smaller fragments. This process likely leads to the decomposition of the protein's amino acid residues, resulting in the release of volatile compounds, including gases and small organic molecules [92]. The fourth weight loss of 10.41% was reported at 500 to 574°C. At this stage, the protein undergoes further decomposition. The elevated temperatures in this range expedite the cleavage of the protein's structure into smaller peptide fragments. The remaining amino acids are susceptible to additional degradation, leading to the generation of more volatile compounds and a consequent reduction in protein mass [93]. Moreover, SCP-Zn exhibited a first weight loss of 3.97% between 100 and 150°C. The second weight loss of 31.46% was observed at 250 to 300°C. The third weight loss of 18.65% occurred between 350 and 450°C. The fourth weight loss was found at 550 to 570°C of 17.29%. Similarly, in USCP-Zn, the first weight loss of 12.86% occurred at 30 to 100°C. The second weight loss of 3.16% was exhibited at 150 to 200°C. The third weight loss of 34.37% occurred between 400 and 450°C. The third weight loss was found at 550 to 575°C of 42.518%.

3.5.5. Differential Scanning Calorimeter (DSC). The differential scanning calorimeter (DSC) provides valuable insights into the thermal properties of zinc-bound chickpea proteins, including NCP-Zn, SCP-Zn, and USCP-Zn as shown in Figure 4(f). The glass transition temperature (T_g) of NCP-Zn is measured at 96.25°C. This suggests a critical transition point in the protein's behavior where it changes physical properties, transitioning from a solid to a rubbery state. This transformation could be attributed to structural reorganization or enhanced molecular mobility within the protein [94]. The onset temperature of NCP-Zn is recorded at 53.27°C which signifies the initiation of thermal degradation or decomposition in the protein. The unfolding of the protein's native structure is induced by the application of heat, leading to disruptions that affect its solubility and functional properties [95]. In addition, the peak temperature of decomposition is observed at 294.51°C. This suggests the occurrence of the peak rate of degradation, characterized by

the rapid breakdown of weak interactions or bonds within the protein, such as hydrogen bonds or hydrophobic interaction [96]. Significantly, the negative values for peak height (−2.65 mW) and area (−1486.92 mJ) suggest exothermic reactions occurring during the decomposition of NCP-Zn. This indicates that exothermic chemical reactions are taking place within the protein structure, resulting in the release of heat. The presence of zinc ions in the protein complex may have a potential influence on these reactions, either by catalyzing specific reactions or by inducing changes in the protein's conformation and stability [97]. Moreover, the negative value of delta H (−376.44 J/g), representing the enthalpy change associated with the decomposition process, indicates that heat is being released during the reaction.

The SCP-Zn exhibits distinct thermal properties compared to NCP-Zn. SCP-Zn displays a lower glass transition temperature (T_g) of 76.42°C, indicating alterations in its physical properties and structural behavior. The onset temperature of decomposition for SCP-Zn is recorded as 48.94°C, while the peak temperature is observed at 146.29°C. The lower peak height of 0.738 mW and area of −1418.12 mJ suggest a relatively lower heat release during the decomposition process compared to NCP-Zn. These observations indicate that SCP-Zn undergoes a less intense decomposition reaction compared to NCP-Zn. The negative value of delta H (−379.86 J/g) indicates an exothermic reaction during the decomposition of SCP-Zn, suggesting the release of energy. The observed discrepancies in thermal properties between SCP-Zn and NCP-Zn can be ascribed to the combined effects of succinylation and zinc binding. Succinylation involves the covalent attachment of succinic acid groups to the protein molecules, inducing structural modifications that impact the protein's flexibility and overall behavior [97]. These alterations subsequently affect the thermal stability and decomposition characteristics of the protein [98]. Moreover, the binding of zinc ions to the chickpea protein further influences its structural properties and thermal behavior. The presence of zinc ions can lead to interactions with the protein, thereby altering its conformation and stability. Consequently, these modifications and interactions play a crucial role in shaping the protein's response to heat and its decomposition behavior [99].

The USCP-Zn exhibits distinct thermal properties compared to SCP-Zn. USCP-Zn shows a lower glass transition temperature (T_g) of 81.45°C, indicating further modifications in the protein's physical properties and structural behavior. The onset temperature of decomposition for USCP-Zn is recorded at 54.36°C, while the peak temperature of decomposition remains the same as NCP-Zn, at 294.51°C. However, the negative peak height of −2.35 mW and negative area of −1191.82 mJ suggest a more pronounced exothermic reaction during decomposition compared to SCP-Zn. The negative value of delta H (−495.95 J/g) further supports the occurrence of an exothermic reaction during the decomposition of USCP-Zn. The improved thermal behavior observed in USCP-Zn can be attributed to the combined effects of succinylation and ultrasonication treatment. Ultrasonication, accomplished using high-frequency sound waves, introduces physical disruptions in the protein solution, resulting in enhanced solubility and dispersion [100]. Moreover, it influences molecular interactions, leading to the rearrangement of protein domains and increased flexibility. The acoustic energy generated during ultrasonication contributes to localized heating, further enhancing the thermal behavior of USCP-Zn [101]. These alterations, coupled with the structural modifications induced by succinylation, synergistically enhance the protein's thermal stability and decomposition characteristics.

3.6. Zinc Content in a Zinc-Protein Complex. As shown in Figure 5(a), the zinc content of the SCP-Zn significantly ($p < 0.05$) increased by 9.90% as compared to the zinc content in NCP-Zn. This is due to the structural changes caused by succinylation, which create new binding sites for zinc ions or expose existing ones. The process of succinylation leads to alterations in the three-dimensional structure of the protein, enhancing its zinc-binding affinity [87]. Furthermore, succinylation results in a reduction of lysine content, an amino acid that plays a role in zinc binding. This reduction prompts other available amino acids to take on the role of zinc-binding sites, ultimately contributing to the overall increase in zinc-binding capacity of the succinylated chickpea protein [102]. Our study aligns with Shilpashree et al. [56], as both studies demonstrate similar zinc content in zinc-bound succinylated proteins. Moreover, the zinc content in the USCP-Zn significantly increases ($p < 0.05$) by 12.48% as compared to SCP-Zn. The increase can be attributed due to several important factors. Firstly, ultrasonication induces structural changes in the chickpea protein, such as unfolding, denaturation, and fragmentation, which expose more zinc-binding sites and enhance zinc-protein interactions [103]. Secondly, succinylation reduces the lysine content in the protein, decreasing competition for zinc binding and allowing more zinc ions to bind to available sites [104]. Thirdly, the ultrasonication process improves protein solubility and accessibility, further promoting zinc-protein interactions [104].

3.7. Mineral Bioavailability. Mineral bioavailability pertains to the degree to which minerals can be absorbed and effectively utilized by the human body for diverse physiological functions and processes. As depicted in Figure 5(b), the bioavailability of SCP-Zn demonstrates a statistically significant increase ($p < 0.05$), rising from $77.26 \pm 0.73\%$ to $84.28 \pm 0.71\%$. This increase of 8.32% is notably higher in comparison to NCP-Zn. This increase is attributed to the fact that the succinylation of chickpea protein leads to increased resistance to digestion by proteolytic enzymes, making more of the zinc bound to the protein remain intact and available for absorption in the small intestine [58]. The modified structure of the protein ensures that it is better protected in the acidic environment of the stomach and can resist complete breakdown until it reaches the absorptive surface of the small intestine [105]. This enhanced stability allows for a higher concentration of zinc-bound protein to interact with specific transporters in

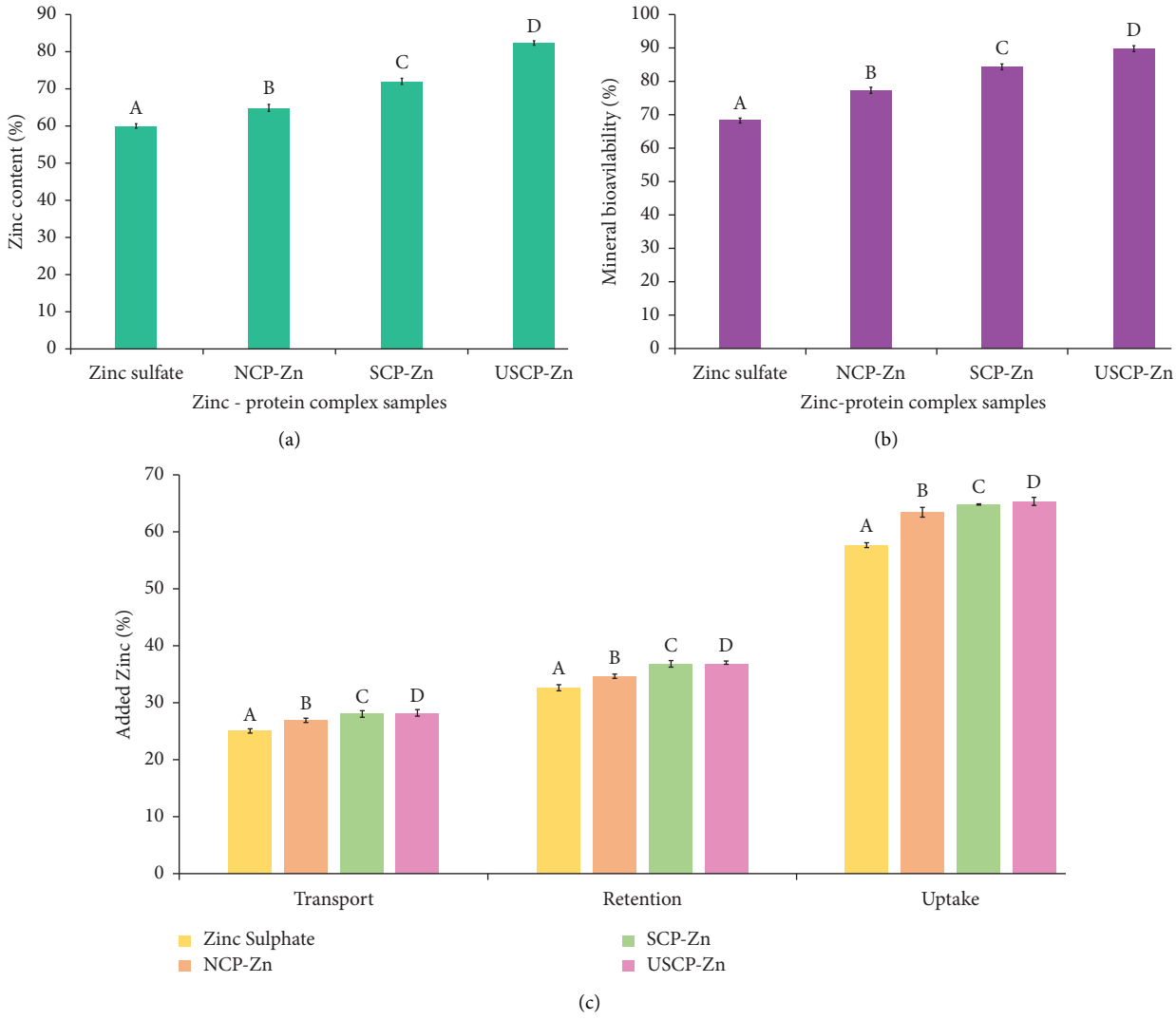

Figure 5: Zinc content (a), mineral bioavailability (b), and cellular transport, retention, and uptake (c) of the zinc-bound native chickpea protein (NCP-Zn), zinc-bound succinylated chickpea protein (SCP-Zn), and zinc-bound ultrasonication-treated succinylated chickpea protein (USCP-Zn). The results were expressed as the mean ± standard deviation of ≥3 independent replicates, and error bars represent the standard deviation from the mean values, while different uppercase letters above each bar represent significantly different values based on analysis of variance (ANOVA) and *t*-tests.

the intestinal lining, facilitating greater zinc uptake into the bloodstream [106]. As a result, the mineral bioavailability of SCP-Zn significantly improves compared to NCP-Zn. In addition, as shown in Figure 5(b) the USCP-Zn has significantly increased ($p < 0.05$) in the range of 84.28 ± 0.71–$89.66 \pm 0.61\%$ which is 6% greater than the SCP-Zn. This improvement can be attributed to enhanced digestion and mineral uptake mechanisms. The ultrasonication treatment likely breaks down protein structures into smaller peptides, allowing for better access to mineral binding sites and facilitating the formation of soluble zinc-chickpea protein complexes [107]. Consequently, more zinc ions are released from the bound complexes during digestion, making them available for absorption in the digestive tract. In addition, the treatment may reduce antinutritional factors, further promoting mineral uptake [108].

3.7.1. Zinc Uptake by Caco-2 Cells. Digested samples obtained from simulated gastrointestinal digestion were utilized in a Transwell assay to assess the cellular absorption of minerals. Caco-2 cells were positioned in the apical chamber and exposed to a 50 μM concentration of zinc. The results are shown in Figure5(c), indicating the cellular uptake, retention, and transport of zinc in NCP-Zn are $62.82 \pm 0.34\%$, $33.55 \pm 0.39\%$, and $25.58 \pm 0.84\%$. In addition, cellular uptake, retention, and transport of zinc in SCP-Zn have significant ($p < 0.05$) increases by 2.54, 6.44, and 4.15% as compared to NCP-Zn. The cellular uptake, retention, and transport of zinc significantly increase in SCP-Zn compared to NCP-Zn because succinylation introduces negatively charged succinyl groups, enhancing zinc-binding capacity and making more zinc ions available for absorption. The modified protein exhibits improved solubility and digestibility, leading to the release of zinc ions during

digestion, which becomes more accessible for uptake by intestinal cells [59]. Once inside the cells, the succinylated chickpea protein's structural changes facilitate interactions with specific cellular receptors or transporters, promoting efficient uptake and retention of zinc [109]. Furthermore, the USCP-Zn has significant increases ($p < 0.05$) in the cellular uptake, retention, and transport of zinc is 1.89%, 3.34%, and 4.8% as compared to SCP-Zn. This is attributed due to the combined effect of ultrasonication and succinylation, where the ultrasonication-induced structural changes further expose the succinylated sites on the protein surface. This increased surface area, altered charge distribution, and modified. The enhanced accessibility of binding sites on the protein surface, along with altered charge interactions, makes it easier for the zinc-bound chickpea protein to interact with specific cellular receptors responsible for mineral uptake [110]. The structural changes and surface modifications may promote favorable interactions with cellular transporters, facilitating the movement of zinc-bound chickpea protein across the cell membrane and into the cell interior [111].

The results of the study on the dual-modified chickpea proteins zinc complex and their enhanced zinc uptake hold significant promise for the broader field of zinc uptake research. These findings offer a valuable outline for improving zinc bioavailability from plant-based sources, focusing on effective modification strategies like succinylation and ultrasonication. Researchers can use this knowledge to explore the biofortification of staple crops, develop functional foods or supplements, and design nutritional strategies to combat zinc deficiency, especially in populations dependent on vegetarian or vegan diets. Moreover, the study provides insights into the molecular mechanisms underpinning enhanced zinc uptake, opening doors for future research to delve deeper into the complex biology of zinc transport and absorption. Ultimately, these findings not only address the practical challenge of zinc deficiency but also have broader implications for improving overall health and well-being by ensuring the optimal functioning of essential biological processes that depend on zinc.

4. Unexpected Findings

The study revealed an unexpected synergistic effect when succinylation and ultrasonication (a dual modification) were combined, resulting in a more substantial increase in zinc uptake compared to a single modification. This surprising outcome underscores the complex nature of protein modifications and their profound influence on mineral absorption. Furthermore, dual modification not only enhanced zinc-binding capacity but also remarkably enhanced chickpea protein solubility and digestibility. This unexpected improvement in protein properties highlights the impact of modification strategies.

5. Limitations

Limitations in the experiment include variations in pH and temperature, variability in zinc species, cellular heterogeneity, transporter regulation changes, measurement technique inconsistencies, sample contaminants, interference from other ions, cellular adaptation, sample handling errors, and mistakes in data analysis. Mitigating these limitations requires rigorous experimental design, quality control, calibration standards, and validation methods, as well as attention to detail and methodological consistency.

6. Future Direction

Future research in the field of cellular zinc uptake is composed to yield valuable insights and practical applications across multiple domains. Advancements in measurement techniques and subcellular imaging will enhance our understanding of zinc's intracellular distribution and dynamics. Investigating the complicated molecular regulation of ZIP transporters and their interaction with cellular factors could lead to targeted therapies for diseases associated with zinc dysregulation. Moreover, the application of this knowledge extends to the development of functional foods that improve zinc absorption, personalized nutritional strategies, and environmentally informed policies. In addition, the utilization of zinc in drug delivery systems and its role in biotechnology and agriculture holds the promise of addressing health and food security challenges. Finally, exploring the potential of zinc nanoparticles for various applications underscores the interdisciplinary nature and far-reaching implications of future research in cellular zinc uptake.

7. Conclusion

This research highlights the significant impacts of ultrasonication and succinylation on chickpea protein-zinc complexes. Succinylation notably enhances water-holding and oil-holding capacities, improving functionality and solubility. It also enhances zinc bioavailability and cellular uptake of zinc as compared to the native protein-zinc complex which makes it more accessible. However, thermal stability decreases while the zeta potential charge increases as compared to the native protein-zinc complex. Moreover, the dual modification approach (ultrasonication and succinylation) has yielded significant improvements in the chickpea protein-zinc complex as compared to single modified (succinylation) protein-zinc complex. Notably, this approach has resulted in a substantial reduction in particle size and an increased zeta potential value compared to the succinylated chickpea protein-zinc complex, indicating improved stability and dispersion properties. The dual modification approach has also led to a considerable decrease in the sulfhydryl (SH) content and an increase in surface hydrophobicity, suggesting alterations in the protein's structural characteristics that may contribute to its enhanced functionality. Importantly, these modifications have further augmented zinc bioavailability, cellular uptake, retention, and transport, emphasizing the potential of this complex as a valuable nutritional resource. The research findings indicate that modified chickpea protein-zinc complexes have potential applications in the food

industry, improving product functionality and nutritional value. In addition, the enhanced zinc bioavailability and cellular uptake are significant for human health, especially for those with dietary zinc concerns, contributing to better overall well-being. The study faces challenges in optimizing succinylation and ultrasonication conditions, ensuring consistency in results across chickpea protein batches, addressing safety and environmental concerns associated with ultrasonication, and assessing the bioavailability of zinc bound to the modified chickpea protein in the digestive system.

Data Availability

All data generated or analyzed during this study are included in the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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