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High-power sonication of soy proteins: Hydroxyl radicals and their effects on protein structure

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Abstract

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Keywords

High power sonication, Soy protein, Spin trapping, Hydroxyl radicals, Protein oxidation, Protein structure

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High-power sonication of soy proteins: hydroxyl radicals and their effects on protein structure

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Introduction

The high-power sonication (HPS) involves lower frequencies of sound waves at 20 kHz to 100 kHz that generate higher power levels up to 16 kW [1]. Sound waves at lower frequencies produce greater acoustic energy and more cavitation; in contrast, very high frequency around 5 MHz does not produce cavitation [1,2]. In terms of size of sonoreactor or volume of the sample, low energy density ranges from 0.01 to 0.1 W/cm^3 whereas high density 1 to 10 W/cm^3 [3]. When HPS imparts energy to the aqueous medium in the form of sound waves, it creates alternative compression and rarefaction of bubbles [4–6]. The rarefaction results in the formation of microbubbles, which subsequently collapse violently creating strong hydrodynamic shear forces, immense pressure, and temperature gradient in the aqueous phase [1,6–8].

Ultrasound is one of the widely researched physical technologies that is valued in agri-food research, mostly, for its disruption of cell matrices at higher intensities and the ensuing effects on extraction of cell components [9–14]. The cavitation and shear forces generated during HPS disintegrate cellular matrices and decrease particle size by approximately 10-fold, thus, aiding in more extraction of biological constituents [10,15]. The conventional extraction and processing of soy proteins in aqueous system involves pH modulation, mostly to pH 8.5, which can solubilize approximately half of the proteins from defatted soybean meals [10]. Application of HPS was reported to increase soy protein extraction yield by 34% at a power density (PD) of 2.5 W/cm^3 for 2 min [16]. This technology is also widely studied to extract different components from plant sources besides protein, for example, sugar, oils, isoflavones, polyphenols, and saponins [3,10,17].

However, the HPS energy imparted in the aqueous phase can dissociate water molecules to form highly reactive hydroxyl radicals and hydrogen atoms ($H_2O \rightarrow {}^{\bullet}H + {}^{\bullet}OH$; $H_2O \rightarrow H_2 +$

•O). During rapid cooling, hydroxyl radicals and hydrogen atoms can react among themselves to produce hydrogen peroxide (•OH + •OH →H_2O_2) and molecular hydrogen (•H + H_2O → H_2 + •OH) [6,18]. The quantity of these radicals formed depends on the total energy imparted to the aqueous system in sonoreactor. In one study, the concentration of DMPO-OH was measured to be 4μM by EPR spin trapping during ultrasonication of 25cm^3 water for 60 min [7]. During sonication, the concentration of hydrogen radicals is one fourth of that of hydroxyl radicals and at higher concentrations, hydroxyl radicals recombine to form hydrogen peroxide (H_2O_2) [19]. These reactive oxygen species (ROS), i.e., free radicals (•OH) and nonradical species (H_2O_2), can be detrimental to protein quality and, possibly, nutrition by initiating oxidation reactions; however, most of the modifications in protein backbone are induced by ·OH radicals [9,12,20]. Although the mechanisms of free-radical induced protein oxidation in food systems are not completely understood, oxidation can change secondary and tertiary structures of proteins. These structural changes could modify ensuing physical and chemical properties of proteins, including functional characteristics. The oxidative modifications in protein take place mainly at the amino acids side chains, including thiol oxidation, aromatic hydroxylation, and formation of carbonyl groups [14,21]. In addition, the sulfur-containing amino acids, cysteine and methionine are the most susceptible to oxidative changes due to lesser electronegativity of sulfur than oxygen [22]. The oxidation of disulfide linkage in protein (R–S–S–R′) can form thiol bonds (R–SH and R′–SH, sulfhydryl), which may result in re-aggregation and re-polymerization, leading to the changes in surface hydrophobicity, solubility, water-holding capacity, and gelation properties [9,20,23]. Quantifying disulfide linkages in proteins, thus, can help understand free-radical induced protein oxidation.

Indeed, some published reports have linked the radicals generated during ultrasonication to oxidation of proteins [12–14,24]; however, there is no report to date quantifying in-situ the free radicals generated during ultrasonication of soybean proteins in aqueous solution and their impact on dominant protein fractions (7S or 11S). In addition, several studies have shown the effect of radical-induced oxidation on the structure and function of various food proteins [9,21,25–29]. The oxidation of 7S was shown to increase protein crosslinking and reduce potential allergenicity [21] and oxidation of egg white proteins were shown to increase surface hydrophobicity through cross-linking [9]. Also, oxidation of soy protein was shown to decrease the surface hydrophobicity and tryptophan residues, increase carbonyl group, form dityrosine and degrade free sulfhydryl groups [21,26,29]. Thus, it is important to measure the free radical(s) generated during sonication to control their possible effect on protein oxidation and resulting structure and function. One factor that could influence impact of free-radical generation in soy protein is the significant amounts of flavonoids with radical-quenching activity [30]. In addition, HPS was shown to impact isoflavones recovery during extraction, increase from 600 to 5813 mg/g in one instance [17]. Thus, the role of isoflavones in possibly reducing the free radicals during extraction needs evaluated.

There are several indirect methods to quantitate free radicals like iodometry or Fricke dosimetry, but electron paramagnetic resonance (EPR) spectroscopy is a widely used direct detection technique [31]. However, EPR is unable to detect highly reactive radical species like superoxide, hydroxyl radicals, sulfur centered radicals, and alkoxyl radicals due to their very short lifetime; for example, hydroxyl radicals have life span circa 10^{-9} s [31,32]. Spin trap compounds, which are diamagnetic often nitrone or nitroso compounds, can be made to react with free radicals and result in longer-lived radical adducts that can be spectroscopically detected

and analyzed afterwards using EPR [33]. The objectives of this study were to 1) identify and quantify major free radical species during HPS of soy proteins in aqueous solutions and 2) evaluate the effect(s) of radicals generate on soy protein structure. The novelty of this research is that it aims to identify and quantify the free radicals in-situ using direct method, which, in turn can help to explain the resulting modifications in soybean protein during high-power sonication.

2. Materials and Methods:

2.1 Materials. Soy protein isolate (SPI), obtained from Center for Crops Utilization Research (CCUR) at Iowa State University, was prepared according to Deak and Johnon [34]. Purified soy protein subunits namely β-conglycinin (7S) and glycinin (11S) were obtained from Dr. Mila P. Hojilla-Evangelista, USDA-ARS, Peoria, IL, and were prepared according to Thanh and Shibasaki [35]. The 90-PDI defatted soy flakes was gifted by Cargill Inc., (Cedar Rapids, IA).

The spin trap compound 5, 5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Enzo Life Sciences (Farmingdale, NY). The Pierce BCA protein assay kit, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), urea, ethylenediaminetetraacetic acid (EDTA), sodiumdodecyl sulphate (SDS), and β mercaptoethanol were purchased from Fisher Scientific (Waltham, MA). Dithiothreitol (DTT), L-cysteine hydrochloride monohydrate and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO). Milli-Q water (Millipore, Billerica, MA, USA) had a resistivity of 18.2 MΩ·cm at 25ºC. All other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA).

2.3 High-Power sonication (HPS) conditions

Ultrasonic treatments of sample solutions were carried with a sonicator (model VCX 750, Sonics & Materials, Inc., CT) at 20 kHz frequency with maximum power output of 750 W. A 3-mm

diameter and 171-mm length titanium alloy made tapered microtip produced ultra-high intensity at maximum amplitude of 40%. The ultrasonic power density (PD) was defined as the output power of the ultrasound per unit volume of the solution and regulated at ~2.5, ~5 and ~9 W/cm^3 by adjusting amplitude (20 - 40%), time (5-10 min) and volume of the sample solutions (2- 10 mL). Power density 2.5 W/cm^3 (LPD) and 5 W/cm^3 (HPD) corresponds to ~160 W/cm^2 and ~300 W/cm^2, respectively.

2.2 Sample preparations.

De-isoflavoned SPI (NO-ISO SPI) was prepared by removing isoflavones with a mixture of 59% aqueous acetonitrile, 11% of 0.1 mol/L HCl and 30 % water. SPI was mixed to the solvent at 1:10 and stirred for 2 h followed by centrifugation at 3000× *g* for 10 min. Solids were collected and dried in an oven dryer at 35°C for 48 h [17]. Regular SPI with isoflavone was termed 'SPI'. Solutions of 1% 11S subunit, 1% 7S subunit, 5%, SPI and 5% NO-ISO SPI (w/v) were prepared by adding ground samples to Milli-Q water and then gently stirred for 2 h, except for NO-ISO SPI sample that was stirred overnight at room temperature to get maximum solubility. All these samples were then centrifuged at 10000× g for 10 min.

Protein extraction from 10% soy flakes in water (w/v) was done by adjusting pH to 8.5 with stirring for 30 min at 60°C. Then centrifuged at 14000× g for 20 min to collect the supernatant and this supernatant used for further experiment named as 'soy flakes proteins'. Two sodium phosphate buffer solutions of pH 7.0 and pH 8.5 were also prepared. These supernatants and other protein samples were further sonicated as described in the following text.

2.4 Ultrasonic treatment of the samples

Two-mL samples that contained 100, 200, 300, 500 and 700 mM DMPO were placed in an ice bath kept within a blackout chamber along with the sonication unit that had a continuous purging of nitrogen gas to avoid the possible degradation of DMPO by light and oxygen. At the end of sonication at both PDs, average sample temperatures were measured to be below 55ºC. Samples were sonicated in centrifuge tubes wrapped fully in aluminum foil for saturation DMPO concentration during free radical quantitation. The HPS conditions were optimized based on exposure time and PD at which there was no loss of DMPO due to HPS itself and highest concentration of radical adducts were captured. All the samples were analyzed using EPR spectroscopy immediately after treatment. Separate sets of protein samples were prepared by sonicating 5 mL and 10 mL volumes at PD of 2.5 and 5 W/cm^3 followed by centrifugation at 12000× g for 10 min to evaluate protein molecular changes in electrophoretic patterns, secondary and tertiary structures, free sulfhydryl groups, and total disulfide bonds.

2.5 EPR Spectroscopy and free-radical quantitation

After sonication, the adduct concentration in samples were determined by EPR spectroscopy (ELEXYS E580 FT-EPR; Bruker, Germany) according to the method of Kubo et al. [5] with some modifications. Treated solutions were drawn to fill at least 40 mm of capillary tube height (1 mm inner dia) and placed in EPR spectroscopy unit. For each sample, the EPR spectra was measured at room temperature with microwave frequency of 9.8 GHz, modulation frequency of 100 kHz, modulation amplitude of 2 G, a field set of 3,520 G with a scan range of 200 G, and number of scan 10. The spin-adduct concentration was calculated from the EPR spectrum using Xepr acquisition software (Bruker BioSpin, Rheinstetten, Germany).

2.6 Electrophoresis

The sonicated and unsonicated protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) with some modifications [36]. Protein (1 mg/mL) solution was prepared in sample buffer (15.1 g/L tris, 300 g/L urea, 2 g/L SDS, 20 mL/L glycerol, and 0.1 g/L bromophenol blue) and incubated at 100°C for 10 mins. Cooled samples and marker (molecular weight 10 kDa to 250 kDa; Catalog# M4038, Sigma–Aldrich, St. Louis, MO) (10 µL) were loaded into 4% stacking gels, and subunits and/or peptides were separated on 12.5% resolution gels with electrophoresis at 180 V for 45 min. The gels were then stained for 40 min with staining solution (250 mL methanol, 110 mL of 1% Coomassie Blue solution, 50 mL acetic acid, and 90 mL Millipore water) and de-stained overnight with destining solution (500 mL methanol, 100 mL acetic acid, and 400 mL Millipore water).

2.7 Circular Dichroism (CD)

Changes in secondary structure of proteins due to HPS was quantitively estimated using the method developed by Provencher and Gloeckner (1981) with minor modifications [14]. All sonicated and unsonicated samples (0.1 mg protein/mL) were prepared using 0.01 M sodium phosphate buffer (pH 7.0) and centrifuged at 14000× g to remove any insoluble residues. CD spectrum were recorded at the far- UV range (260-180 nm) with a CD spectropolarimeter (Jasco 715, Jasco Corp) in a 0.1 cm quartz CD cuvette at 25 °C. The values of scan rate, response, and bandwidth were 50 nm/min, 0.25 s and 1.0 nm, respectively. Two scanning acquisitions were accumulated and averaged to obtain the final spectrum. The data were expressed in terms of mean residue ellipticity, $[\theta]$, in $deg.cm^2.dmol^{-1}$ and secondary structure was estimated using the DICHROWEB analysis software using the SELCON3 algorithm and reference data 6. Four

secondary structures of proteins were estimated namely, α-helix, β-sheet, β-turn, and unordered for comparison before and after sonication [37].

2.8 Intrinsic Fluorescence

The fluorescence spectra of the untreated and HPS untreated samples were obtained by a Synergy H1 Hybrid Multi-Mode Reader photometer (Hitachi, Tokyo, Japan) according to Wang et al. [13] with modifications. All of the protein samples (0.2 mg/mL) were prepared in 10 mM phosphate buffer (pH 7.0) and excited at 290 nm to get emission spectra from 300 to 460 nm at a constant slit of 5 nm for both excitation and emission.

2.9 Free sulfhydryl (SH) groups and total disulfide (SS)

The free sulfhydryl groups and disulfide bond of each unsonicated and sonicated soy protein samples were determined by Ellman's assay with some modifications [38]. Reaction buffer was prepared using 0.1 M sodium phosphate buffer, 6M guanidine HCl and 1.27 mM EDTA at pH 8.6. A 50 µL of Ellman's reagent was added to 2.5 mL of reaction buffer, followed by the addition of 250 µL of unsonicated and sonicated protein samples. Samples were then mixed and incubated at room temperature for 15 min to measure the absorbance at 412 nm using UV–visible spectrophotometer (Shimadzu UV 160). The free SH bonds were calculated using a molar extinction coefficient 14150 M^{-1} cm^{-1} and presented as µmol/g protein.

Soluble disulfide bonds were reduced to free sulfhydryl groups using reducing agent to measure the disulfide bonds in protein samples. Reduction was done by adding 0.75 mL 300 mM DTT (prepared in reaction buffer) to 1.5 mL of filtered protein solution followed by incubated for 2 h at room temperature within a nitrogenous condition. Then DTT washed away by adding 1 mL 39% trichloroacetic acid and vortexed at low speed followed by centrifuged at 12,000 × g for

20 min. After decanting the supernatant, pellets were washed again by adding 0.444 mL 39% TCA followed by vortex and centrifuge. The supernatant was decanted after final wash and pellets were solubilized by adding 1 mL of reaction buffer and stored overnight at 4°C stored in an environment of depleted oxygen. Then 250 µL supernatant was taken to follow the procedure of free SH bonds, as above, to determine the SS bonds.

2.10 Statistical analyses

The experimental design was randomized complete block design (RCBD) with two replications. Statistical analyses were performed using the SAS statistical methods (Version 9.3, SAS Institute; Cary, NC). Ultrasonic power densities were independent variables, whereas, quantifying free radicals and relevant changes in protein structures were dependent variables. Two levels of independent variables were HPD and LPD, calculated based on ultrasonic output (J), time of exposure (s) and volume of the solution (cm^3). Analysis of variance (ANOVA) was performed to assess the effect of treatments on total radical concentration and structural characteristic as well as treatment means were separated by the least significant difference (LSD) test at $P < 0.05$.

3. Results and discussion:

3.1 High power sonication and generation of free radicals

3.1.1 Ultrasonic power density and excess DMPO concentration

Generation of free radicals depends on several conditions, such as power density (PD), configuration of ultrasonic system, reaction temperature, concentration of dissolve gas and others [39]. Therefore, generation of radicals was optimized for this study based on time of sonication, PD applied into the sonoreactor and saturation DMPO concentration needed to capture most or

all of the radicals (Fig. 1). The highest concentration of radical adduct was obtained at PD 5 W/cm^3 irradiation for 10 min at 500 mM of DMPO (Fig.1). Between PDs 2.5 and 5 W/cm^3, there was a sharp increase in the adduct concentration; however, it fell sharply at PD 9 W/cm^3 (Fig.1b). Generally, it is reported that higher ultrasound power intensity induces a larger number of localize high temperature and pressure regions, resulting in higher OH radical generation [7] and this was seen in our results for up to 5 W/cm^3. However, it is also reported that too high local temperature at very high PD could negatively affect the rate of adduct generation [7] and similar result in adduct generation was observed at PD 9 W/cm^3 (Fig 1b). At localized too high temperatures, water vapor pressure and content of water vapor inside the bubble increase; when the bubbles collapse, the heat capacity of vapor suppress further rise in reaction temperature as well as radical formation [7]. Additionally, during the formation of radical adducts, the degradation of the adducts occurs simultaneously, therefore, the rate of adduct formation changes over time and reaches a plateau at a specific time [7]. In the present experiment, there was a sharp and significant ($p<0.05$) increase in the adduct concentration between 5- and 10-min exposure, whereas, the difference in adduct concentrations between 10 and 20 min was not significant (Fig. 1c). At a specific PD and treatment time, the stability of DMPO was one of the important parameters to capture highest adduct concentration. The radical adduct concentration significantly ($p<0.05$) increased and reached the highest with 500mM of DMPO; however, there was no significance ($p<0.05$) difference in adduct concentrations captured between 500mM and 800mM (Fig.1a). This indicated that 500mM of DMPO was an excess concentration for the given conditions that ensured all of radicals were captured; further sonication of protein solutions for measuring radical concentration were carried out at this DMPO concentration. PD 2.5 W/cm^3

and 5 W/cm^3 were used to sonicate rest of the samples and PD 2.5 W/cm^3 was denoted as lower PD, whereas, 5 W/cm^3 was denoted as higher PD in subsequent discussion.

3.1.2 Radicals identification

During HPS, a 4-line EPR signal (Fig. 2) with a calculated g value of 2.0054, intensity ratio of 1:2:2:1, and calculated hyperfine constants of 1.5 (a_N) and 1.5 mT (a_H) was observed. This specific signal is attributable to DMPO-OH adduct [32], verifying that hydroxyl radicals were generated during the sonication of aqueous protein solution; however, no hydrogen radicals were detected. Same spectral pattern was found for all sonicated protein samples in this experiment. During ultrasonic treatment, dissociation of water into hydroxyl and hydrogen radicals had been reported in many studies, however, many studies had not report hydrogen radicals [7,40], as was the case with this study. One reason could be that the hydrogen and hydroperoxy radicals can disappear faster before reacting with DMPO [7].

3.1.3 Concentration of DMPO-OH adducts in various substrates

Two power densities, 2.5 and 5 W/cm^3, were applied for 10 min to MQ water, tap water, two different buffer solutions, soy flakes protein, 5% SPI, 5% NO-ISO SPI, and 1% 7S and 1% 11S subunit solutions. Higher PD 5 (W/cm^3) induced more cavitation; therefore, relatively higher concentration of radicals was seen for MQ water, tap water, 7S, NO-ISO SPI, and soy flake protein solution (Fig. 3). There was no significant difference ($p<0.05$) in radical adducts concentration between NO-ISO SPI and SPI at lower PD. However, NO-ISO SPI solution displayed significantly ($p<0.05$) higher radical concentrations of 2.14 μM at higher PD than SPI (1.14 μM). Both SPI and NO-ISO SPI showed significantly lower radical adducts than both types of water at each PD. Flavonoids are well-known for their free radical quenching activity

and soy proteins contain approximately 0.2–1.6 mg flavonoids/g of dry weight [30], consequently, more radicals were expected in NO-ISO SPI solution. Moreover, HPS breaks down cell matrix and reduced particle size approximately 10-fold as well as increased the recovery of isoflavones [10,17]. Therefore, higher PD could extract more isoflavones from substrate, which in turn, resulted in fewer radicals at higher PD in SPI solution. Among the protein solutions, the lowest radical adducts were captured in soy flakes. Defatted soy flakes contain carbohydrates, fiber, ash, isoflavones (combinedly approximately 50% of its total weight), besides proteins of approximately 50% [41]. When HPS was applied to soy flakes protein solution, the energy in the form of cavitation ruptures the cell wall, which in turn could increase protein extraction yield [16] as well as recovery of isoflavones [17]. Thus, it's possible that most of the HPS energy was consumed in rupturing cell walls than dissociating water molecules resulting in fewer radicals being generated in soy flakes protein solutions.

There was no significant ($p<0.05$) difference in radical adduct concentrations among MQ water and tap water at higher PD and 11S subunit solution at both PDs. However, 7S subunit solution at both PD showed significantly ($p<0.05$) lower adduct concentration than 11S subunit. The difference in the adduct concentration between 7S subunit and 11S subunit attributed to their structural difference as seen in following discussion. The 11S subunit is a hexamer of six subunits of acidic A polypeptides (37–42 kDa) and basic B polypeptides (17–20 kDa) dimers linked by disulfide bond, on the other hand, 7S subunit is a trimer contains three subunits of 71 kDa, 67 kDa and 50 kDa linked through non-covalent interaction, predominantly hydrophobic interaction [30]. HPS of protein was reported to rearrange the molecules by exposing the hydrophobic interaction sites, thus, improving hydrophobicity of amino acid side chains [13]. As hydrophobic bonds are weaker than covalent bonds, applied ultrasonic energy could have

resulted in breakdown of more hydrophobic bonds in 7S subunit than covalent bonds in water, thus less free radicals. On the other hand, as 11S subunit is highly convoluted and larger in structure due to presence of more disulfide bonds, applied ultrasonic energy more likely dissociated water into its radicals than breaking disulfide bonds. In addition, 7S subunit is glycosylated and HPS can dissociate the carbohydrate moiety that scavenges the radicals, possibly reducing the free radicals in 7S subunit than 11S subunit solutions [42].

Conventional protein extraction is basically a pH modulation protocol, so, two buffer solutions at pH 7 and pH 8.5 were also subjected to similar HPS treatment to assess their relative contribution in radical generation. Figure 3 shows that higher PD resulted in significantly ($p<0.05$) lower radicals for pH 7 buffer than tap water, whereas, there was no significant difference in radical adduct concentrations at higher PD for pH 8.5 buffer, MQ water and tap water. pH 7 buffer is a neutral solution contains equal concentration of hydrogen and hydroxyl ion whereas pH 8.5 buffer contains more hydroxyl ion and less hydrogen ion than that of pH 7 buffer. Therefore, there was no significant ($p<0.05$) difference in DMPO-OH concentration at both PDs for pH 7 buffer, and being slightly more acidic, tap water dissociated into more radicals than MQ water. HPS generated significantly ($p<0.05$) higher DMPO-OH at lower PD for pH 7 buffer than pH 8.5 buffer, because radical generation decreases with increasing pH after pH 3.2 of buffer solution and radical concentration rapidly decrease in alkaline region [43].

3.2 Effect of HPS on protein structure

3.2.1 Gel electrophoresis of sonicated and unsonicated protein samples

SDS-PAGE was carried out to visualize the effect of HPS on soy proteins and/or their subunits. Fig. 5 shows sonicated and unsonicated SDS-PAGE profiles of the protein in solutions indicating

no changes in the bands of protein subunits, SPIs and soy flakes protein due to HPS. These results agree with literature, reports [14,16,26], suggesting that either the energy levels during current HPS treatments were not high enough to modify the primary structure of protein, or radical-induced re-aggregation occured during or right after the sonication. NO-ISO SPI showed lower intensity for high-molecular weight bands compared to SPI because the acidic isoflavonoids removal procedure lost some protein that reduced the high-molecular weight bands and intensified low molecular weight band in NO-ISO SPI [17,44].

3.2.2 Effect on secondary structure of protein samples:

The proportions of α-helix, β-sheet, β-turn, and unordered structure in protein molecules of untreated and treated soy protein samples, as estimated using circular dichroism, are listed in Table 1. All samples except SPI showed significant ($p<0.05$) changes in β-sheet. Higher PD significantly ($p<0.05$) increased the β-sheet % as well as significantly decreased the unordered % than unsonicated 11S subunit. However, there were no significant ($p<0.05$) differences in the estimated β-turn % of all the samples, and also no differences in unordered % of all the samples except 11S subunit due to HPS treatments. These findings are similar to some previous studies [13,14,24]. Hu et al. [14] reported that ultrasonic cavitation might disrupt the tertiary structure but not secondary. In another report, they also estimated secondary structure using FT-IR beside CD and concluded that high intensity sonication of 7S subunit and 11S subunit cannot significantly ($p<0.05$) breakdown the hydrogen bonds between C=O and H=N groups on the polypeptide backbone [24]. However, Wang el al. [13] found that ultrasonic treatment increased the proportion of α-helix, and β-turn, but decreased β-sheet and unordered in both 7S subunit and 11S subunit. They also concluded that increase in ultrasonic power, time, and temperature could not decrease β-sheet because these conditions are responsible to increase molecular motion as

well as molecular collision in protein so that protein molecule get rearranged [13]. Moreover, oxidation leads to unfolding, dissociation, and rearrangement of protein molecular structure [21]. Secondary structure is local sequence of amino acids and the interactions between different parts of the protein molecule [14], so oxidation by hydroxyl radicals detected in sonoreactor could take part in changing the secondary structure. These studies used power density (approx. 0.35 W/cm^3) at much lower intensity than present study (2.5 and 5 W/cm^3), which eventually leads to lower radical generation and less oxidation. It can thus be inferred that very high-power density or more cavitation could have rearranged molecular structure of both 7S subunit and 11S subunit; therefore, small changes in secondary structure were observed in these subunit molecules.

Proteins in SPI solutions, however, showed a significant ($p<0.05$) increase in α-helix structure between untreated and both treated samples. Higher PD showed a significant ($p<0.05$) decrease in β-sheet proportion and increase in α-helix proportions in NO-ISO SPI than unsonicated one. Soy flakes protein showed a significant ($p<0.05$) increase in α-helix and decrease in β-sheet at lower PD than unsonicated and higher PD. However, Hu et al. [14] reported that α-helix and unordered structure of SPI decreased at lower PD and increased at higher PD . In our experiments, proteins in NO-ISO SPI were highly, and soy flakes were least susceptible to ultrasonic cavitation depending on the sample purity. More cavitation effect on secondary structure was exhibited in NO-ISO SPI for being purer protein. At the same time, proteins in both SPI and NO-ISO SPI were more susceptible to the rearrangement of molecular structure than proteins in soy flakes because of possible exposure to varying levels of energy within cells. Presence of other cellular components like cell walls may shield proteins in soy flakes so they are exposed to least of the applied energy than other purer matrices. Thus, it can be

inferred that HPS can change the secondary structure of the protein, at the same time, help in the rearrangement of protein molecular structure depending on the radical induced oxidation and amount of energy applied. Therefore, highly purified 7S and 11S subunits could have been rearranged and exhibit few discernible effects. Having more complex substrate matrix than 7S and 11S subunits, both SPIs and soy flakes also showed some changes in secondary structure due to cavitation effect although still susceptible to molecular rearrangement.

3.2.3 Changes in the tertiary structure of the protein samples

The changes in the fluorescence intensity during HPS is shown in a representative figure (Fig. 6) and tabulated in Table 2. The fluorescence spectrum are indicator of oxidation of Trp residues that change conformation based on local environment; the Trp local environment is nonpolar when the λ_{max} is <330 nm and polar when λ_{max} is >330 nm [13,29]. In this experiment, all the samples showed λ_{max} to be ≥330 nm, thus polar, with no changed in the λ_{max} value for each sample after HPS. However, relative fluorescence intensity changed after treatments as shown in the Table 2. Decrease in the fluorescence intensity could be attributed to the loss of the Trp residues, whereas, increase in the fluorescence intensity attributed to protein unfolding during oxidation that also expose buried Trp residues [21]. Both PDs significantly ($p<0.05$) increased the fluorescence intensity for 11S subunit and NO-ISO SPI, whereas, lower PD decreased fluorescence intensity for 7S subunit and SPI. The changes in the fluorescence intensity can be correlated with the radical generation, as higher PD generates more radicals leading to oxidation. 11S subunit showed no significant ($p<0.05$) changes in radical concentration as well as in fluorescence intensity. In addition, soy flakes protein showed minimal radicals and no significant ($p<0.05$) changes in the fluorescence intensity at both PDs. NO-ISO SPI showed highest positive changes or increase in fluorescence intensity because of having no isoflavoids and for more

399 oxidation. Our findings are similar to Xu et al. [21], who report that polarity of 7S proteins
400 changes at very high hydrogen peroxide concentration of 5 mM, whereas, fluorescence intensity
401 can even change at 0.1 mM. However, Huang et al. [45] found that ultrasonication did not
402 change the fluorescence intensity of SPI when done at 600W and 20 kHz for 5 min. It can thus
403 be inferred that although the polarity changes were not detected, the protein tertiary structure did
404 change due to HPS exposure, as indicated by relative fluorescence intensity.

405 *3.2.4 Free sulfhydryl groups and disulfide bonds*

406 Ultrasound treatments can reorganize protein molecules due to cavitional high pressure and shear
407 forces, therefore, it is likely that buried sulfhydryl groups would be exposed [13] and susceptible
408 to oxidation. The results of quantitation of free sulfhydryl groups and disulfide bonds as
409 impacted by sonication of proteins is presented in Fig. 4. Unsonicated 7S and 11S subunits had
410 3.66 and 14.01 µmol free SH/ g of protein, respectively, and 131.73 and 170.15 µmol SS/gm
411 protein respectively. This findings falls in the range of free sulfhydryl group and disulfide bond
412 content reported in the literature; for example, Hu et al. [24] and several other studies reported
413 free sulfhydryl groups in the range of 2.3-9.5 µmol/g of protein in unsonicated 7S subunit, and 0-
414 32.2 µmol/gm of protein in unsonicated 11S subunit. They also found that sonication reduced the
415 amount of free sulfhydryl groups in 7S and 11S subunits when exposed to power density of
416 0.33–0.35 W/cm^3 for 5 min [24]. However, Gulseren et al. [12] reported free SH in BSA
417 decreased with increasing sonication time at 20 W/cm^2. However, these studies did not employ
418 very high sonication power density like in this experiment. In this experiment, HPS treatments
419 showed significantly ($p<0.05$) lower free sulfhydryl groups of 7S subunit than control; however,
420 there was no significant ($p<0.05$) difference in free sulfhydryl groups of 7S subunit between the
421 HPS treatments. (Fig. 4a). In case of proteins from soy flakes, there was no significant ($p<0.05$)

difference in free sulfhydryl groups between unsonicated and lower PD samples; however, higher PD sonication showed significantly ($p<0.05$) lesser free sulfhydryl groups than both untreated and lower PD samples. Other than 7S subunit and soy flakes proteins, there was no significant ($p<0.05$) differences in free sulfhydryl groups in 11S subunit, SPI and NO-ISO SPI, although, each of these samples generated significant amount of hydroxyl radicals upon HPS treatment. The hydroxyl radicals can react among themselves to produce hydrogen peroxide. These reactive oxygen species (ROS) generated during HPS may oxidize susceptible free sulfhydryl groups to form disulfide bonds, and/ or to its reversible form, sulfenic acid or to irreversible form, sulfinic acid [12,13,47]. Among amino acids, sulfur-containing cysteine and methionine are the most susceptible to oxidative changes due to lower electronegativity of sulfur [22]. The changes in free sulfhydryl groups and disulfide bonds could be attributable to oxidation of cysteine residue, whereas, methionine can be oxidized to sulfoxide and sulfone. Oxidation can modify protein structure either by changing the redox state of cysteine or equilibrium constant of the thiol-disulfide interchange reaction, which in turn, change the content and distribution of free sulfhydryl groups and disulfide bonds [21].

Some researchers have reported decrease in free sulfhydryl groups in protein with increasing sonication time [12,24], which implies that when a constant energy is applied for longer periods of time , more free sulfhydryl groups and free radicals become available for oxidative reactions and form disulfide bonds. The 7S protein structure predominantly contains non-covalent hydrophobic interactions, which are comparatively weaker bonds, therefore, higher PD could expose more free sulfhydryl groups than lower PD sonication. At the same time, we observed that higher PD sonication also generated significantly ($p<0.05$) more hydroxyls radicals than lower PD (Fig. 3). Therefore, free sulfhydryl groups have more available hydroxyls radicals

to react and oxidize to disulfide bonds at higher PD, which in turn, lowers the number of free sulfhydryl groups. This explanation can be bolstered by the contents of disulfide bonds observed in 7S subunit (Fig. 4b), as there was no significant ($p<0.05$) difference in the disulfide bonds among the treated and untreated samples. On the other hand, there was no significant ($p<0.05$) difference in the number of free sulfhydryl groups and disulfide bonds among treatments in 11S subunit, although lower PD resulted in lesser mean value of both free sulfhydryl groups and disulfide bonds than higher PD and unsonicated. Because of the stronger covalent bonds in 11S subunit, lower PD resulted in lesser free sulfhydryl groups (Fig. 4); at the same time, hydroxyl radical generation was not significantly ($p<0.05$) different between the PDs (Fig.3).

In case of SPI and NO-ISO SPI, there was no significant ($p<0.05$) difference in free sulfhydryl among treatments; however, disulfide bonds at higher PD was significantly lower than in untreated samples. NO-ISO SPI showed higher number of disulfide bonds than SPI, because NO-ISO SPI solution generated more radicals (Fig. 3) to oxidize free sulfhydryl groups to disulfide bonds. SPI at higher PD generated significantly ($p<0.05$) lower free radicals than lower PD that react with free sulfhydryl groups to lower down the number of disulfide bonds at higher PD. In soy flakes, free sulfhydryl groups at higher PD were significantly ($p<0.05$) lower than at lower PD; however, there was no significant difference in disulfide bonds between these treatments. Higher PD generated significantly ($p<0.05$) higher number of radicals than lower PD, therefore, at higher PD, it is possible that more free sulfhydryl groups are being oxidized to disulfide bonds than at lower PD. These results suggest that HPS used in this experiment generated significant ($p<0.05$) amount of hydroxyl radicals as well as quick oxidation of free sulfhydryl groups to disulfide bonds in all the protein samples. Therefore, differences in free

sulfhydryl groups as well as in disulfide bonds among HPS treatments were not detectable in most cases.

4. Conclusions:

In this experiment, highest concentration of radical adduct was captured with 500 mM DMPO at 5 W/cm^3 ultrasonic power density when exposed to an aqueous system for 10 min. Only hydroxyl radicals were detected in each of the protein solution at PD of 2.5 and 5 W/cm^3. Between these two PDs, higher PD generated significantly ($p<0.05$) higher hydroxyl radicals for most of the protein samples, except for 11S subunit and SPI. SPI without isoflavonoids generated more radicals than SPI with them because of the radical quenching activity of isoflavonoids. Soy flakes protein was the least pure among samples because of the presence of cell matrix that generated lowest radicals among the protein samples. The concentrations of hydroxyl radicals in the samples were high enough for proteins to refold and oxidize free sulfhydryl groups to disulfide bonds. Therefore, no changes in the protein electrophoretic patterns were detected. For the same reasons, some changes were detected in estimated secondary structures and tertiary structure as well as in free sulfhydryl group and disulfide bond contents depending on the purity of samples and applied sonication energy. Overall, this research showed that the hydroxyl radicals generated during HPS led to oxidation-induced partial changes in protein molecular structures due to highly convoluted nature of soy proteins. These findings provide explanations on the possible protein modifications, thus resulting potential functionality changes, due to HPS when employed during protein extraction or modification.

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Highlights:

- ˙OH was the dominant species during high power sonication (HPS) of soy protein
- High power (5 W/cm^3) sonication induced more radicals than lower power
- ˙OH led to protein oxidation but did not change protein electrophoretic patterns
- Provide evidence to future research on oxidative protein modifications during HPS

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Table 1: Changes in secondary structure of control and sonicated soy protein samples (Means with same letter on each column for each sample are not significantly different at $p<0.05$. Each sample was compared for difference from respective control.

Sample	Treatment	α- Helix (%)	β- Sheet (%)	β- Turns (%)	Unordered (%)
1% 7S	Control	16.2 ± 3.5^{a}	32.7 ± 3.9^{ac}	18.2 ± 0.3^{a}	32.9 ± 0.8^{a}
	LPD	19.1 ± 0.8^{a}	30.1 ± 0.4^{ab}	20.1 ± 1.7^{a}	30.7 ± 2.9^{a}
	HPD	19.1 ± 2.1^{a}	25.8 ± 0.8^{b}	21.1 ± 0.9^{a}	33.9 ± 2.3^{a}
1% 11S	Control	16.9 ± 1.1^{a}	18.1 ± 4.2^{a}	11.4 ± 3.5^{a}	53.6 ± 1.8^{a}
	LPD	9.2 ± 6.2^{a}	30.8 ± 8.9^{ac}	19.3 ± 0.8^{a}	40.8 ± 3.3^{b}
	HPD	7.3 ± 2.5^{a}	39.5 ± 3.4^{bc}	15.6 ± 5.7^{a}	37.6 ± 4.8^{b}
5% SPI	Control	10.2 ± 0.8^{a}	44.5 ± 4.2^{a}	15.6 ± 6.6^{a}	29.7 ± 3.2^{a}
	LPD	17.1 ± 1.1^{b}	42.1 ± 1.0^{a}	12.5 ± 1.2^{a}	28.4 ± 1.2^{a}
	HPD	15.6 ± 1.7^{b}	36.4 ± 2.9^{a}	19.2 ± 0.2^{a}	28.9 ± 4.8^{a}
5% NO-ISO SPI	Control	9.7 ± 1.3^{a}	24.4 ± 3.1^{a}	14.4 ± 4.4^{a}	51.4 ± 2.5^{a}
	LPD	11.2 ± 0.9^{a}	23.7 ± 2.1^{a}	11.7 ± 0.5^{a}	53.4 ± 3.6^{a}
	HPD	16.8 ± 0.6^{b}	16.1 ± 0.6^{b}	15.6 ± 0.9^{a}	50.9 ± 2.0^{a}
Soy flakes protein	Control	14.3 ± 2.1^{a}	38.8 ± 2.3^{a}	19.8 ± 0.4^{a}	27.2 ± 0.5^{a}
	LPD	25.1 ± 2.2^{b}	22.4 ± 0.7^{b}	21.9 ± 0.5^{a}	30.4 ± 2.2^{a}
	HPD	17.4 ± 2.9^{a}	36.9 ± 4.7^{a}	19.7 ± 3.5^{a}	25.9 ± 4.1^{a}

659 LSD= Least significance difference at p= 0.05, LPD= Low power density (2.5 W/cm^3), HPD=
660 High power density (5.0 W/cm^3)

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Table 2: Relative fluorescence intensity (AU) for protein samples and their respective controls. Means with the same superscript letter on each row are not significantly different at $p<0.05$. Each sample was compared for difference from respective control.

Sample	λ_{max} (nm)	Relative fluorescence intensity (AU)x1000		
		Control	PD: 2.5 W/cm^3	PD: 5 W/cm^3
1% 7S	335	60.6 ± 1.6^{a}	56.4 ± 0.8^{b}	64.4 ± 4.3^{a}
1% 11S	330	88.4 ± 1.6^{a}	92.2 ± 1.7^{b}	91.7 ± 2.0^{b}
5% SPI	335	57.4 ± 2.0^{a}	54.0 ± 2.7^{b}	58.6 ± 2.3^{a}
5% NO-ISO SPI	340	53.0 ± 0.5^{a}	61.1 ± 0.8^{b}	58.4 ± 1.0^{c}
Soy flakes protein	335	84.4 ± 5.5^{a}	78.5 ± 6.6^{a}	81.8 ± 5.4^{a}

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