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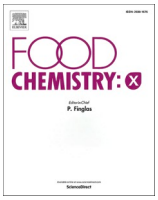

Effects of microwave on the structural and emulsifying properties and interfacial properties of oxidized soybean protein aggregates

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ABSTRACT

This research explored microwave treatment impact on the structuro-functional aspects of oxidized soy protein aggregates (OSPI). Data showed that oxidative treatment promoted the formation of high molecular weight aggregates through hydrophobic interactions, thereby disrupting the structure of natural soy protein isolates (SPI). Microwave treatment for an appropriate time (≤30 s) caused the molecular structure of OSPI to open up and reduction in molecular weight and disulfide bond content, while absolute zeta potential increased. These modifications increased emulsifying capacity of OSPI, as well as the interfacial adsorption of protein. Longer microwave treatment times (>30 s) caused OSPI to exhibit a tendency to aggregate in TEM and CLSM images. It indicated the appropriate microwave electromagnetic field effect and microwave heating effect could coordinatively regulate soy protein functional properties by modifying their aggregation behavior. The results provided new ideas for reducing resource waste, and further expanding soy protein application in the food industry.

Introduction

Soy protein isolate (SPI) is a food ingredient produced from defatted soybean meal with a protein content of over 90% (Puppo et al., 2005). Due to its excellent functional and physiological properties, it has been widely used in the food industry. The production and consumption of soybean protein have been continuously increasing, leading to significant challenges in storage and transportation. But during the production procedure of this protein, there is a residual presence of approximately 1% highly active lipoxygenase, which can generate an oxidative environment during conservation and conveying (Kumari et al., 2020). The catalysis of linoleic acid by lipoxygenase generates an important amount of ROS (reactive oxygen species), principally reacting with nutrients, especially proteins, causing oxidation of soybean protein and altering its physicochemical characteristics, leading to the formation of highly aggregated and emulsification-active impaired oxidized aggregates, thereby deteriorating its processing characteristics (Duque-Estrada, Kyriakopoulou, de Groot, van der Goot, & Berton-Carabin, 2020). Therefore, regulating protein aggregation is an important approach to enhance the functional activity of oxidized protein aggregates.

Currently, many research efforts were focused on using physical methods to change protein structure, and achieve regulation of protein aggregation degree. Yang, Liu, Zeng, and Chen (2018) found that high-pressure homogenization caused protein disaggregation and increased faba bean protein solubility. Cao et al. (2021) also suggested that ultrasonic treatment may regulate various properties of protein by regulating its degree of aggregation. However, due to their high power consumption and low production capacity, it is delicate to widely use ultrasonic homogenizer or a high pressure technology in food industry (Han et al., 2020). Microwave technology, as a green and clean energy source, has the advantages of short heating time, high energy utilization rate, and no pollution (Cao et al., 2018). Generally, microwave electromagnetic field effect and microwave heating effect could coordinatively enhance the functional aspects of proteins by modifying their molecular conformation. Research showed polar molecules in proteins with dipole moments underwent torque, generating a turning "variable pole" motion in the microwave field (Huang et al., 2022). This process could increase the interaction between the protein surface charges and

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lead to less water being implicated in the solvation layer of protein. As a result, protein unfold, exposing their hydrophobic groups, causing protein aggregates to undergo depolymerization, thereby altering their structural and functional characteristics. Additionally, special effects of microwave heating can also alter protein conformation. On the one hand, the fluctuation of charged groups on protein amino acid residues under alternating electric fields can cause the redistribution of the electric field and electrostatic interactions between these molecules. Moreover, the specific response of protein charged groups under microwave fields can also affect the depolymerization and aggregation of protein subunits. Existing works revealed that microwave application could possibly improve the functional aspect of protein aggregates. Li et al. (2020) found that microwave application helped to enhance hydrophobic interactions and stabilize weak protein-water hydrogen bonds, thereby regulating the aggregation and unfolding of protein molecules. Wang et al. (2020) indicated that microwave application exposed both polar and nonpolar fractions in proteins, increasing the flexibility of protein regions, and thereby improving foaming properties. Zhong et al. (2020) demonstrated that microwave treatment reduced the number of free sulfhydryl groups in rice protein, formed new disulfide bonds, caused cross-linking and aggregation of rice protein, and thereby altered its processing properties. However, there were currently few reports on how microwave alternating electromagnetic fields and microwave heating effects jointly regulate the structuro-functional aspects of protein aggregates. Particularly, information on microwaves effects on soy protein oxidation aggregates is scarce.

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) is a free radical initiator with good controllability, stability, repeatability, and applicability. Research shows that by changing the concentration of AAPH, the generation of peroxide radicals that cause protein oxidation can be regulated to preserve protein during long-term storage and transportation, and obtain an oxidized protein model similar to actual storage and transportation (Chen, Zhao, & Sun, 2013). Therefore, in this work, an AAPH-peroxide radical soy protein oxidation system was constructed. Protein oxidation aggregates were treated with microwave modification for different durations to investigate microwave consequence on structuro-functional aspects of protein oxidation aggregates, through a strategy combining the microwave electromagnetic field effect with microwave heating effect in order to provide novel strategies for ameliorating the functional aspects of soybean protein.

Materials and methods

Materials

Soybean protein isolate (92.4% protein) comes from Shandong Yuwang Co., Ltd. All other chemicals are analytical grade which from Thermo Fisher.

Oxidized soybean protein aggregates preparation

The oxidized aggregates of soybean protein (OSPI) were obtained according to a previous study by Wu et al (Wu, Hua, Lin, & Xiao, 2011). Soybean protein was softened in a phosphate buffer mixture (0.01 mol/L) at pH 7.2 (reached using 0.5 mg/mL NaN3) to prepare a 10 mg/mL soybean protein solution. AAPH was added to a final concentration of 0.5 mmol/L AAPH. After applying the oxidation treatment for 12 h at 37 °C in the dark, dialysis was carried out at 4 °C for 72 h with a 14000 kDa dialysis bag and H2O was altered every 6 h. Finally, an oxidized soybean protein aggregate solution with 12 h of oxidation time was obtained. After freeze-drying, the samples were obtained and named OSPI.

Microwave treatment

The sample after oxidation treatment was dissolved in PBS (photosphate buffer solution, pH 7.0) and exposed to a fixed power of 350 W microwave for 0, 10, 20, 30, 40, 50, 60, and 70 s. Centrifuge and freeze dry the microwave treated solution. A total of 8 samples were obtained, named SPI, OSPI, WOSPI-a, WOSPI-b, WOSPI-c, WOSPI-d, WOSPI-e, WOSPI-f, and WOSPI-g.

Molecular weight circulation

Following Ma et al. (2019), molecular weight of the tested proteins was calculated at 280 nm using a Waters 2175 UV finder.

Thioflavin T (Th T) fluorescence

ThT stock solution was prepared and filtered, then diluted in PBS and each sample (50 μL) was mixed with Th T working solution. Fluorescence spectra was read in the wavelength range of 460–560 nm using spectrophotometer (F-7000, HITACHI, Tokyo, Japan).

Inherent fluorescence

For this test, the approach of Jiang et al. (2014) was followed. Briefly, soy samples were diluted in 0.01 mol/L PBS to obtain a protein concentration of 0.4 mg/mL and spectroscopy were determined from 300 to 400 nm using fluorescence spectroscopy (Hitachi, Japan).

Surface hydrophobicity (H_0)

Jiang et al. (2014) method was followed for this test. Briefly, 4 mL aliquots of the treated sample were dissolved in PBS (10 mM, pH 7.0), then mixed with ANS (25 μL, 20 min). The comparative fluorescence intensity (FI) was measured fallowing an excitation wavelengths rate of 374 nm and emission rate of 485 nm.

The free sulfhydryl group (-SH) and disulfide bonds (S—S) contents

For this approach, the protocol of Wang et al. (2022) was followed in which, Ellman's assay and DTNB, which possess the capacity to react with free sulfhydryl groups were used to determine SH group levels in the mixture.

Transmission Electron Microscopy (TEM)

Sample were dispensed in 30 μL droplets (After dH_2O dilution), then applied on a carbon net and the net was dyed using 2% uranyl acetate solution. Noting that a transmission electron microscope (model TEM-JEM-1230 80 kV) was used for this purpose (Keppler et al., 2019).

Preparation of oil–water emulsions

Emulsions were prepared according to the method of Kharat, Skrzynski, Decker, and McClements (2020), but with slight modifications (Kharat et al., 2020). The samples powder were dispersed in PBS solution (5 mM at pH 7.0) to form a 0.1% (w/v) solution. The samples were then hydrated at 4 °C overnight. Emulsions were prepared by blending 10 wt% 5 mL of corn oil and 15 mL of 0.1% (w/v) protein solution for 2 min at 9000r/min using a high-speed blender.

Particle size distribution and ζ-potential

For this test, dynamic light scattering approach were applied according to Nunes et al. (2020). Briefly, all samples were diluted in PBS (pH 7.0) and refractive indices of the particles and dispersant were respectively 1.450 and 1.330.

Confocal laser scanning microscope

The emulsion microstructure was evaluated using a Leica TCS SP2 CLSM. Briefly, dye containing 0.02% Nile red (40 μL) and 0.1% Nile blue (45 μL) was mixed with 1 mL of the suspension. Tinted emulsion was placed in the slide center. Noting that, laser confocal microscope was used for viewing droplets (Cheng, et al., 2019).

Percentage of adsorbed proteins (AP%)

The proportion of adsorbed proteins was determined based on Ma et al. (2019) and calculated with Equation:

$$AP(\%) = \frac{C_s - C_f}{C_0} \times 100$$

where C_0 is the original protein level of the protein mixes and C_f is the unabsorbed protein level of the protein mixes.

Interfacial tension

This parameter was evaluated using a tensiometer K100 (Krüss, Germany). Briefly, the Wilhelmy plate was submerged in 20 g of aqueous solution and the measurement of the surface tension was performed with ring tensiometer. Noting that oil–water interfacial tension was realized by pipetting oil stage (50 g) throughout aqueous stage.

Apparent viscosity

For this test, according to Wang et al. (2020), AR 1500 regulated stress rheometer was used to characterize the viscosity of samples. Briefly, emulsions were fractionated into 2.0 mL aliquots and measured on stage at 25 ± 0.1 °C. Noting that viscosity ranged from 0 to 200 s^{-1}.

Viscoelastic Properties

According to Sun and Arntfield (2012) approach, an RST-CPS rheometer was used to measure the rheological characteristics. Briefly, samples were sandwiched between two parallel plates (1 mm space, 40 °C) and a strain reference was set at 1 Hz and principally applied to determine sample linear viscoelastic area.

Statistical analysis

Samples were independently prepared in triplicate for analysis. Statistical analyses were performed using SPSS 20.0. The results were assessed using Duncan's manifold series and ANOVA tests and are presented as the mean ± SD of triplicate analyses. A p-value ≤ 0.05 was considered significant.

Results and discussion

Molecular weight distribution

Size exclusion chromatography (*SEC*) can characterize the molecular weight distribution and aggregation degree of soluble components in microwave treated soybean protein oxidized aggregates. As shown in Fig. 1A, In comparison to untreated SPI, the molecular weight distribution of oxidized SPI (OSPI) undergoes significant changes, with the area of the first molecular weight peak showing little change, while the areas of the remaining peaks decrease significantly, as evidenced by Fig. 1A. This observation could be attributed to the oxidation-induced aggregation of low molecular weight proteins into higher molecular weight oxidized aggregates. Oxidized application may possibly elevate molecular weight of protein aggregates by modifying intermolecular forces (Cheng et al., 2021). Upon microwave treatment for <30 s, the peak areas decrease, and a multi-peak distribution is observed within the retention time range of 13 to 25 min. This could be attributed to non-thermal effects, like magnetic fields engendered internal group exposure of protein but also molecular collision of polar molecules, which had a tearing effect on OSPI. Consequently, rupture of chemical bonds separated protein into small molecules. With microwave application time exceeding 30 s, peak areas within the retention time range of 0 to 14 min

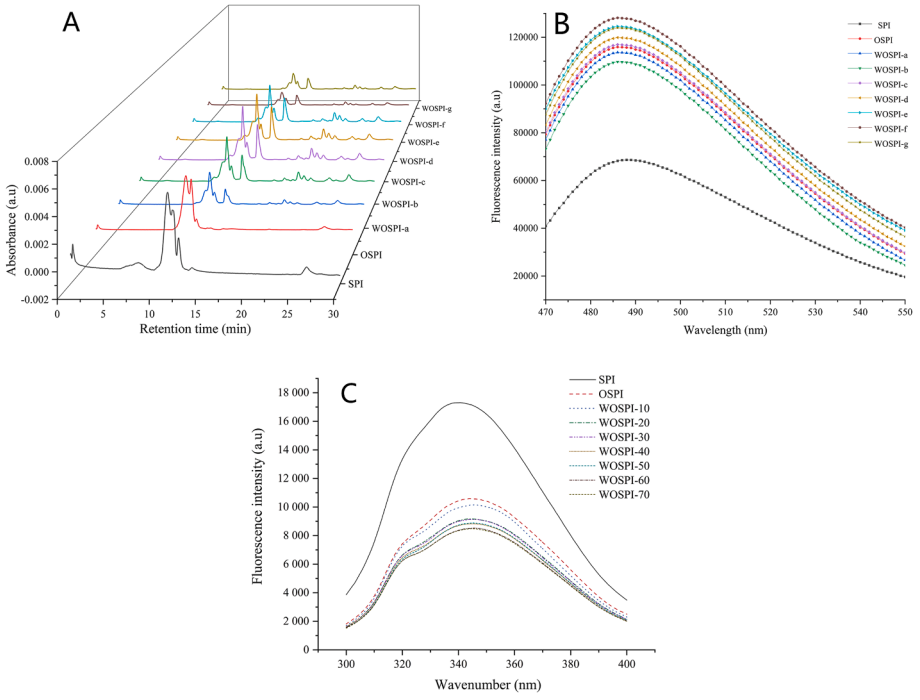

Fig.1. (A) Molecular weight distribution, (B) Fluorescence intensity of Th T, and (C) Inherent fluorescence of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). SPI: soy protein isolate; OSPI: oxidized soy protein aggregates; WOSPI: oxidized soy protein aggregates treated with microwave.

show an increasing trend, while peak areas within the retention time range of 14 to 28 min show a decreasing trend. This could be attributed to the increasing microwave treated thermal effects with longer treatment times, causing covalent and non-covalent crosslinking of SPI. In addition, the flocculent structure of protein clusters progressively stretched under the effect of electromagnetic field, generating by the same way a significant increase in hydrodynamic radius in protein (Zheng et al., 2020). The coupling of microwave thermal and non-thermal effects promoted the growth of intermolecular forces. The intertwining of protein molecular chains caused the aggregation between protein molecules, which resulted in the increase of molecular weight (Perez & Pilosof, 2004).

Thioflavin T (Th T) fluorescence

Thioflavin T (Th T) can specifically be inserted into the internal β_1 structure (intermolecular antiparallel β-fold) of soy protein aggregates and bind with it (Klunk et al., 2001). Therefore, the fluorescence intensity of Th T is proportional to β_1 structural content and can be used to characterize changes in the degree of oxidized soy protein after microwave treatment. Fig. 1B showed that Th T fluorescence intensity of OSPI was significantly increased compared to SPI, indicating that oxidation remarkably enhanced β_1 structural content in SPI. This may because the oxidative treatment caused protein molecules to gradually aggregate into small, amorphous aggregates through hydrophobic and ionic bonds, followed by the induction of covalent cross-linking through disulfide other covalent bonds, generating large, oxidized aggregates through β_1 structures. Studies by Cui et al. (2012) have shown that oxidative treatment promotes disulfide bonds formation, resulting in the emergence of large, oxidized aggregates. Another reason may have been that insoluble oxidized aggregates were destroyed in the microwave physical field, exposing the β-sheet structure in the aggregate core, increasing fluorescence intensity. After microwave treatment, the Th T fluorescence intensity of WOSPI trended to decrease first, then to increase with the processing time, reaching a minimum at 20 s. This was because the non-thermal effects such as the electric field generated by microwave treatment caused structural fragmentation within the soy protein isolate oxidized aggregates, resulting in some hydrogen bonds the breaking and a decrease in β_1 structure content. As the microwave time exceeded 20 s, the thermal effect gradually increased, leading to denaturation of small soy protein isolate oxidized aggregates. The surface of protein was modified by free thiol groups and hydrophobic groups, and disulfide bonds and hydrophobic bonds were formed between molecules, promoting the formation of β_1 structures and inducing the cross-linking of small aggregates to form large, thermally induced aggregates. When the microwave treatment time reached 70 s, the Th T fluorescence intensity of WOSPI-g decreased. This may have been because the formation of fibrous aggregates had entered a stable period, and the generation rate had slowed down. At the same time, the increase in microwave treatment time led to an excessive thermal effect, inducing further aggregation and embedding of some β_1 structures within the fibrous aggregates, making it difficult for Th T to bind with them. These factors jointly provoke a diminution of Th T fluorescence intensity of WOSPI-g.

Inherent fluorescence

The fluorescent spectra were used to characterize changes of polarity of aromatic amino acid microenvironment of oxidized aggregates after microwave treatment, which allowed the prediction of modifications in the tertiary structure of aggregates (Wang et al., 2022). From Fig. 1C, it can be seen that compared with SPI, the intrinsic fluorescence intensity of OSPI is significantly reduced, indicating the occurrence of oxidative aggregation in OSPI. After microwave treatment, the intrinsic fluorescence intensity of WOSPI gradually decreases, and the maximum absorption wavelength (λ Max) increases first and then decreases, reaching its maximum value at 30 s. This indicates that the fluorescence quenching reaction of Tryptophan residue occurred, and the polarity of the surrounding microenvironment first increased and then decreased. This may be because the electric field effect caused by microwave treatment makes the Brownian motion between soy protein isolate molecules strengthen, and the intermolecular collision increases, and then the dynamic fluorescence quenching effect occurs between the Excited state soy protein isolate oxidation aggregates, leading to the decline of fluorescence molecules (Afkhami et al., 2023). With the further extension of microwave treatment time, the thermal effect was enhanced, leading to thermal aggregation between protein, that was consistent with molecular weight distribution finding. Wang's study showed that the thermal effect caused by microwave application can modify soy protein conformation and form large spherical protein aggregates under the action of hydrophobic forces, embedding various groups originally present on protein surface (Wang et al. 2022). Therefore, microwave application favored the rearrangement of OSPI polar groups and the reconstruction hydrophobic environment, where more fluorescent chromophores entered the interior of aggregates, leading to a gradual decrease in fluorescence intensity.

Surface hydrophobicity (H_0)

H_0 index is a key parameter, widely used to evaluate structuro-functional changes of protein as well as emulsification propertiy. Fig. 2A revealed that hydrophobicity surface index of OSPI was significantly reduced, when comparing to SPI. This indicated that oxidation promoted soybean protein isolates aggregation phenomenon, closed or hid hydrophobic regions, and formed low surface hydrophobicity oxidized aggregates. The study by Liu, Cai, Wu, Lin, and Hua (2014) demonstrated that during the protein oxidation process, exposed hydrophobic residues such as tryptophan residues inside the protein were covalently modified, and hydrophobic interactions induced protein binding and aggregation. The formation of carbonyl groups also decreased surface hydrophobicity of protein oxidation aggregates. With the prolongation of microwave processing time, the surface hydrophobicity index of WOSPI increased first, then decreased. This was because when microwave processing time increase, a non-thermal effect, which initially played a dominant role, induces collision between WOSPI molecules and vibration of the molecules themselves, resulting in the rupture of non-covalent bonds represented by hydrophobic bonds. This caused the destruction of the aggregate structure and exposed hydrophobic zones on protein surface. Furthermore, appropriate low-intensity thermal effects also promoted the relaxation of protein structure, exposing originally buried hydrophobic groups, thereby increasing hydrophobicity surface. As microwave processing time is further extended, the thermal effect became stronger and dominant. The polar groups such as carbonyl groups in WOSPI gradually increased, and through hydrophobic interactions, the molecules underwent cross-linking and re-embed hydrophobic groups into the interior of protein. This result was in agreement with the trend of inherent fluorescence.

ζ-Potential

ζ-Potential was associated with charge strength on protein surface and reproduced the strength of electrostatic repulsion or attraction (Xia et al., 2017). Fig. 2B revealed that ζ-potential values for OSPI were significantly below SPI. This was due to the reduction in the polar amino acids on oxidized protein aggregates surface and the destruction of amino acid residues, resulting in a decrease in the amount of surface charge and a subsequent decrease in the ζ-potential. As the microwave treatment time increased, ζ-potential absolute value for microwave-treated WOSPI first increased, then decreased ($P < 0.05$), reaching a maximum 30 s of microwave treatment, similar to the trend of hydrophobicity changes. This was because the presence of electromagnetic fields during short-term microwave treatment altered protein solutions electrostatic equilibrium and disrupted the structure of oxidized

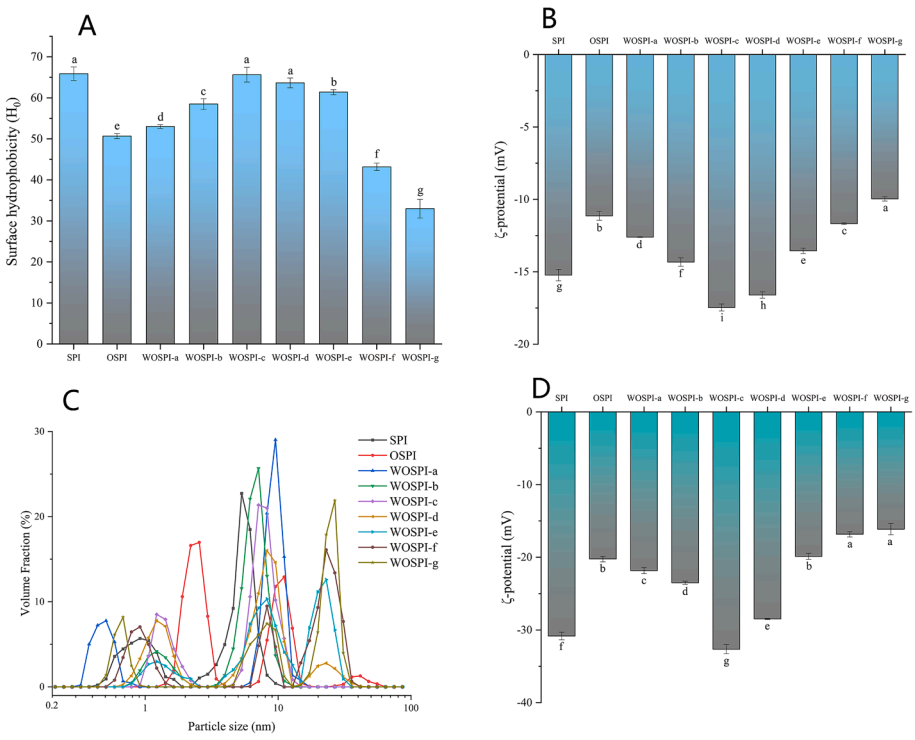

Fig.2. (A) Surface hydrophobicity, (B) ζ-potential of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). (C) Particle size distribution, (D) ζ-potential of emulsion of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). The symbols SPI, OSPI and WOSPI are the same as the legend of Fig. 1. The different lowercase or uppercase letters in the graphs indicate that the results are significantly different ($p < 0.05$).

aggregates. This caused them to dissociate into smaller molecules and expose hydrophobic groups and polar amino acids on the surface. Therefore, the increase in the number of charged amino acids on the surface of WOSPI enhanced the electrostatic repulsion between molecules and increasing ζ-absolute value of the electric potential. However, when the microwave processing time exceeded 30 s, polarized dipole molecules continuously oscillated in microwave electromagnetic field, generating enormous heat, acting directly on OSPI to increase intermolecular forces and aggregation. Charged amino acid residues were destroyed or embedded, due to a possible diminution of ζ-potential absolute value. Research conducted by Huang et al. (2022) also showed that excessive thermal effects resulting from prolonged microwave treatment could weaken electrostatic repulsion and cause aggregation. And the formation of large protein thermal aggregates led to a decline ζ-potential absolute value.

The free sulfhydryl group (-SH) and disulfide bonds (S—S) contents

The function and conformation of soy protein are closely associated with disulfide bond (SS) and free sulfydryl (SH). The mutual conversion between free SH and SS played q crucial role in protein aggregation, and was the basis for the functional activity of SPI. Table 1 showed that the total SH and free SH content of OSPI significantly decreased when comparing with SPI, while the SS content significantly raised. This suggested that oxidation application favored the formation of more compact soy protein aggregates by forming SS. The formation of SS may be associated to oxidative modification of free SH on cysteine residues in SPI by ROO· produced by AAPH. This led to the reversible oxidation reaction generating sulfonic acid and thiol substances, which affected the equilibrium constant for the reversible exchange reaction between free SH and SS, thereby facilitating disulfide bonds the formation (Morzel et al., 2006). As the microwave application time increased, total SH content of WOSPI generally decreased, while the free SH content and SS content exhibited a trend of first increasing and then decreasing and first decreasing and then increasing, respectively. This may be linked to breaking of covalent / non-covalent bonds between protein molecules induced by short-term microwave treatment, which led to the -SH/-SS

Table 1
Sulfhydryl and disulfide bond content of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s).

Sample	Total sulfhydryl (nmol/mg)	Free sulfhydryl (nmol/mg)	Disulfide bond (nmol/mg)
SPI	15.64 ± 0.07^{a}	11.72 ± 0.16^{a}	1.96 ± 0.03^{e}
OSPI	13.24 ± 0.18^{b}	7.88 ± 0.18^{c}	2.68 ± 0.04^{c}
WOSPI-a	13.21 ± 0.12^{b}	7.67 ± 0.09^{c}	2.77 ± 0.08^{c}
WOSPI-b	13.08 ± 0.14^{b}	8.18 ± 0.11^{b}	2.45 ± 0.05^{d}
WOSPI-c	12.78 ± 0.05^{c}	7.16 ± 0.04^{d}	2.49 ± 0.12^{d}
WOSPI-d	12.37 ± 0.10^{d}	6.75 ± 0.06^{e}	2.81 ± 0.09^{c}
WOSPI-e	12.01 ± 0.13^{e}	5.59 ± 0.11^{f}	3.21 ± 0.07^{b}
WOSPI-f	11.63 ± 0.06^{f}	4.67 ± 0.07^{g}	3.48 ± 0.12^{a}
WOSPI-g	10.30 ± 0.20^{g}	3.46 ± 0.14^{h}	3.42 ± 0.09^{a}

Note: Comparisons were carried out between values of the same row; values with different letter(s) indicate a significant difference at $p \leq 0.05$.

exchange reaction and the conversion of disulfide bonds to free SH. As a result, protein structure unfolded, and the oxidative aggregates underwent dissociation. Meanwhile, non-reversible reactions of protein SH may have occurred, generating non-disulfide sulfur-containing compounds. With the further extension of microwave processing time, OSPI molecules encountered directional alignment of dipole moment generating intermolecular cross-linking and aggregation phenomenon. At the same time, the thermal aggregation effect was enhanced, inducing the formation of disulfide bonds through covalent interactions between free SH. Hu, Cheung, Pan, and Li-Chan (2015) also underlined that free SH in aggregates could be oxidized to form SS, promoting the re-aggregation of dissociated soy protein aggregates. However, when the microwave application time reached 70 s, no significant change was exerted in the SS content. This may have been due to the high-intensity thermal effect on the protein, which enhanced the production of non-disulfide sulfur-

containing compounds, and simultaneously led to strong hydrophobic aggregation. This embedded unreacted free SH inside the protein, disrupting the reversible transformation equilibrium and slowing down the increase of SS content.

Transmission Electron Microscopy (TEM)

TEM is considered a valuable tool to characterize the structural changes in protein aggregation by observing the changes in the protein's skeleton structure (Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007). As shown in Fig. 3, the TEM image of SPI did not exhibit any obvious aggregation regions or dendritic structures. In contrast, OSPI displayed a highly aggregated region, and the outer layer of the core had an irregular cluster shape, indicating that the oxidation induced the formation of an aggregated form. Upon microwave treatment for up to 30 s, WOSPI-a, b, and c showed a decreasing trend in the aggregation core and a reduction in the size of the aggregated molecule. The dendritic structures on the exterior of the large molecule aggregates were significantly reduced and transformed into branched structures, indicating that the protein's network structure was disrupted due to the short duration of microwave treatment. As a result, the dense center of the aggregated region was shattered, leading to the dissociation and rearrangement of protein skeleton to varying degrees. When the microwave treatment exceeded 40 s, an aggregation pattern began to emerge in the TEM image of WOSPI-e, indicating that the prolonged treatment led to thermal effects that resulted in hydrophobic bonds and disulfide bonds formation, causing soy protein isolate to aggregate and form a soft fibrous structure (de Pomerai et al., 2003). Subsequently, as the microwave treatment time increased further, the TEM image of WOSPI-f showed a grape-like and network structure, which exhibited a more aggregated center and numerous branched structures connected to each other compared to WOSPI-e. This suggested that the electric field and thermal effects of microwave treatment intensified protein denaturation and when reaching 70 s of application, the network structure in TEM image disappeared, leaving only large molecular aggregates interconnected by small molecular aggregates. This suggested that under prolonged microwave treatment, small molecular oxidized aggregates more quickly aggregated to form larger heat-aggregated molecules.

Particle size of emulsions

The particle size of protein emulsions can characterize the oil droplets distribution in emulsions and impact of protein aggregation on emulsion properties. The particle size distribution of emulsions prepared with OSPI changed to a three-peak distribution. Compared with emulsions prepared with SPI, the peak for small particle sizes shifted to right and increased, while largest particle shifted to right but decreased, and a small peak formed at even larger particle sizes (Fig. 2C). Analysis of protein surface hydrophobicity and endogenous fluorescence indicated that this was possibly linked with the formation of insoluble oxidative aggregates in SOI induced by oxidation, generating a diminution in the amount of interfacial protein that could migrate to oil–water junction. As a result, there were fewer proteins available for emulsification of the same volume of oil and thus causing an elevation in OSPI emulsion particle size (Liang et al., 2016). In addition, combined with zeta-potential analysis, the reduction in electrostatic repulsion between proteins resulted in a decrease in the repulsive force between emulsion droplets. The collision of small droplets with each other formed larger droplets, which also caused an increase in emulsion particle size. When

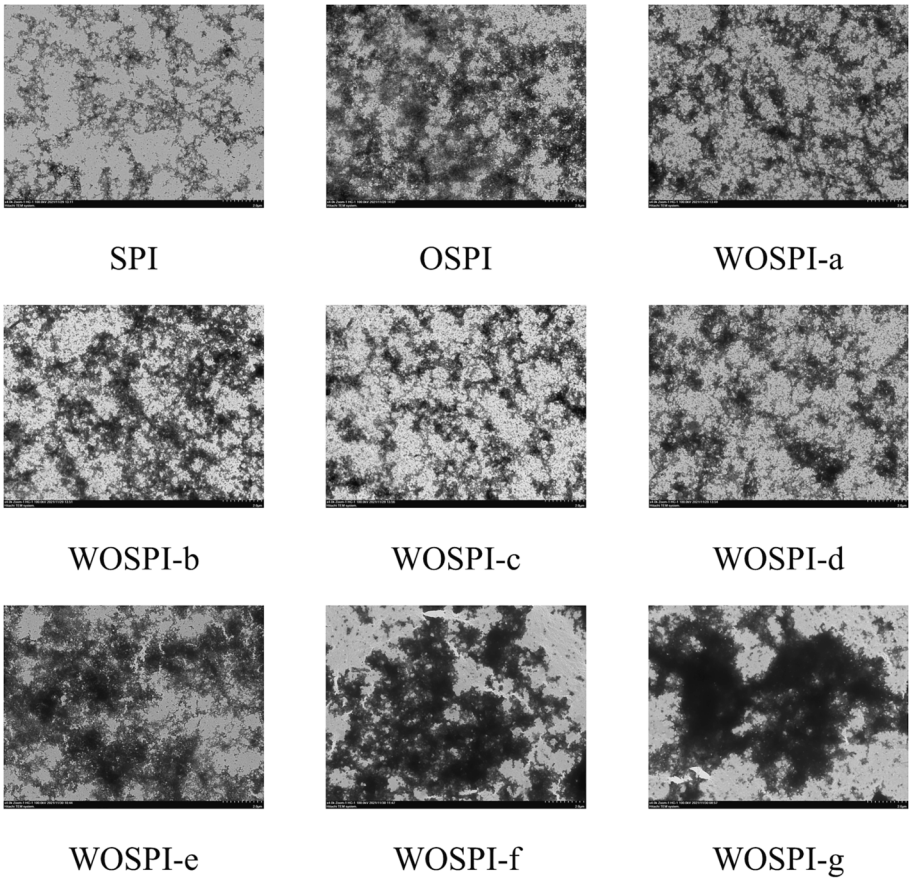

Fig.3. TEM images of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). The symbols SPI, OSPI and WOSPI are the same as the legend of Fig. 1.

microwave treatment was applied for 10–30 s, the peak for small particle sizes in particle size distribution of emulsions prepared with microwave-treated oxidized SPI (WOSPI-a,b,c) increased, while the peak for large particle sizes decreased. This was because the short-term microwave treatment accelerated the collision of SPI molecules and their own vibration, causing changes in protein conformation. The surface activity of SPI increased, the electrostatic repulsion between emulsion droplets increased, and the energy required for protein adsorption to the oil–water interface decreased. This facilitated its adsorption, the formation of interfacial membrane structure, and a decrease in emulsion particle size. As the duration of microwave treatment continued to increase, the thermal effect gradually increased, and thermal aggregation of SPI occurred. The protein structure became more compact, making it difficult for protein to stretch and bind. Furthermore, due to the formation of insoluble thermal aggregates, the electrostatic repulsion between proteins decreased, causing a decrease in the repulsive force between emulsion droplets. This led to the collision and combination of small droplets into larger ones, increasing emulsion particle size (Amid & Mirhosseini, 2013).

ζ-Potential of emulsions

This parameter can characterize surface charge of droplets and is related to the electrostatic repulsion between droplets. It reflects the overall stability of the emulsion system with larger absolute values (Sui et al., 2017). Fig. 2D revealed that ζ-potential absolute value of emulsions prepared with OSPI was considerably lower in contrast to that prepared with SPI emulsions. It indicated that oxidation treatment considerably diminished the electrostatic repulsion of emulsions prepared with proteins and led to the deterioration of emulsion properties. This was because oxidation made rearrangement difficult at oil–water interface, decreasing amount of interfacial proteins that can bind to the oil–water interface. Moreover, charged amino acid groups on protein surface molecules were buried due to oxidized aggregates formation, consequence of a decrease in electrostatic repulsion. With an increase in microwave treatment period time, ζ-potential absolute value of emulsions prepared with WOSPI first increased and then decreased. This was because appropriate microwave treatment could break the disulfide bonds and hydrogen bonds of oxidized soy protein aggregates, generating the dissociation of protein aggregates into smaller molecules. As a result, molecular flexibility and surface charge of proteins were increased due to the exposure of internal hydrophobic and polar groups (Puppo et al., 2005). This reduced the steric hindrance effect, leading to an increase ζ-potential absolute value of resulting emulsion. However, as microwave treatment time increased, small oxidized aggregates that have dissociated initially may re-aggregate due to thermal aggregation to form larger aggregates. This could buried the charged amino acid groups inside the aggregates and reduced the electrostatic repulsion of proteins and the ζ-potential absolute value of emulsions.

Laser scanning confocal microscopy (LSCM)

This approach characterize distribution and microstructure of emulsive particles formed by soy protein oxidation-induced aggregates. The protein-oil interfacial membrane and the aggregation state of the composite system were also observed. As shown in Fig. 4, the emulsion droplets prepared from soy protein without oxidation treatment were uniformly distributed and appeared in a spherical shape. Compared with the emulsion prepared from SPI, the emulsion prepared from OSPI increased significantly particle size and aggregation. This indicated that oxidation treatment resulted in an increased degree of protein aggregation and a decrease in solubility. Consequently, OSPI could not fully dissolve and unfold during the emulsification process, affecting the formation and stability of the interfacial membrane, resulting in the formation of a red oil droplet aggregation region. With the increase of microwave application time, the emulsion droplets formed by WOSPI became more uniform in size, indicating that appropriate microwave application could influence the highly ordered WOSPI to undergo disintegration, accompanied by molecular unfolding and structure expansion, exposing hydrophobic and hydrophilic residues. This significantly enhanced flexibility of protein molecules, improved adsorption, expansion, and rearrangement properties of WOSPI at the interface, thereby improving oil–water interface stability (Zheng et al., 2020). However, when the microwave application time exceeded 30 s,

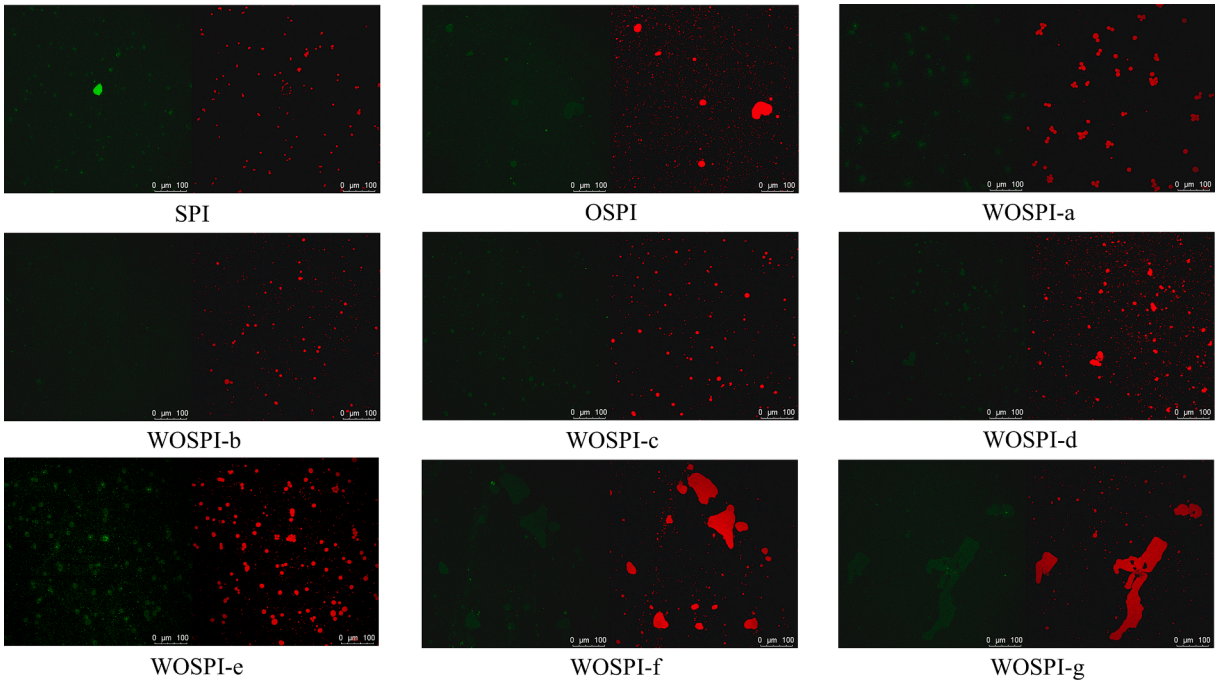

Fig.4. LSCM images of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). The symbols SPI, OSPI and WOSPI are the same as the legend of Fig. 1.

the emulsion droplets showed agglomeration and an increase in particle size, gradually losing their spherical shape and becoming irregularly shaped large particles. This indicated that prolonged microwave treatment caused the disintegrated WOSPI to undergo re-aggregation, forming large protein and insoluble protein aggregates. During emulsion preparation process, WOSPI could not sufficiently bind with the oil–water interface through expansion and interfacial rearrangement, resulting in the inability to form a stable interfacial membrane and reducing its emulsification ability (Wang et al., 2022).

Percentage of adsorbed proteins (AP%)

Protein content on oil–water interface of an emulsion played a crucial role in determining its stability. The higher a protein adsorption on interface, a stronger the protein's capacity to adsorb oil–water interface will be (Izmailova et al., 1999). As shown in Fig. 5A, the interface protein content in the emulsion prepared with OSPI significantly decreased compared to that of SPI. This was probably linked to the oxidation treatment, which stimulated the aggregation of soy protein isolate, forming a dense protein oxidation aggregate structure, with hydrophobic fraction. And it promoted the formation of aggregates with large particle sizes and complex structures so that more hydrophobic groups were embedded into the proteins. This phenomenon reduced the affinity of the protein to the interface and the rate of protein molecular rearrangement, resulting in a decrease in the protein adsorption capacity at the interface. Following the increase of microwave treatment time, the interface protein content in the emulsion prepared with WOSPI trended to increase and then to decrease. Combining with the analysis above, this was because appropriate microwave treatment caused insoluble oxidation aggregates to dissolve and form soluble aggregates, exposing the hydrophobic and hydrophilic protein groups. Its interesting to underline that solubility was enhanced, which promoted protein and oil phase interaction, thereby increasing protein content at oil–water interface (Falade et al., 2021). However, prolonged microwave application promoted denaturation of soy protein isolate, gradually forming thermal aggregates, embedding hydrophobic groups, and decreasing flexibility. At the same time, some insoluble thermal aggregates were generated, reducing protein solubility and adsorption capacity at oil–-water interface. This resulted in a downward trend in interface protein content.

Interfacial tension

Interface tension can characterize the surface activity of proteins at oil–water interface and is affected by types, components, structures, and concentrations of proteins adsorbed at the interface (Amine et al., 2014). Comparing with SPI, interfacial tension of OSPI significantly increased (Fig. 5B). This was because oxidation treatment caused subunits of protein molecules to aggregate, forming insoluble oxidized aggregates with larger particle size. Moreover, the specific surface area of protein decreased and steric hindrance increased. Liu et al. (2014) found that oxidation increased interfacial tension of soy protein, leading to poorer foaming and emulsifying properties. With increasing microwave treatment time, the interfacial tension of OSPI showed a trend of first decreasing and then increasing, reaching a minimum at a treatment time of 30 s. This might be because microwave treatment can promote the breaking of disulfide bonds and hydrogen bonds in insoluble oxidized aggregates, disrupting the highly ordered antiparallel β_1-fold structure and causing molecular unfolding. This increased the specific surface area of the molecule and exposed internal hydrophobic and hydrophilic groups. As a result, the interfacial adsorption properties of OSPI were improved, and interfacial tension decreased. However, exceeding 50 s of microwave treatment, the small-molecule oxidized aggregates that were originally dissociated underwent thermal aggregation under the heat

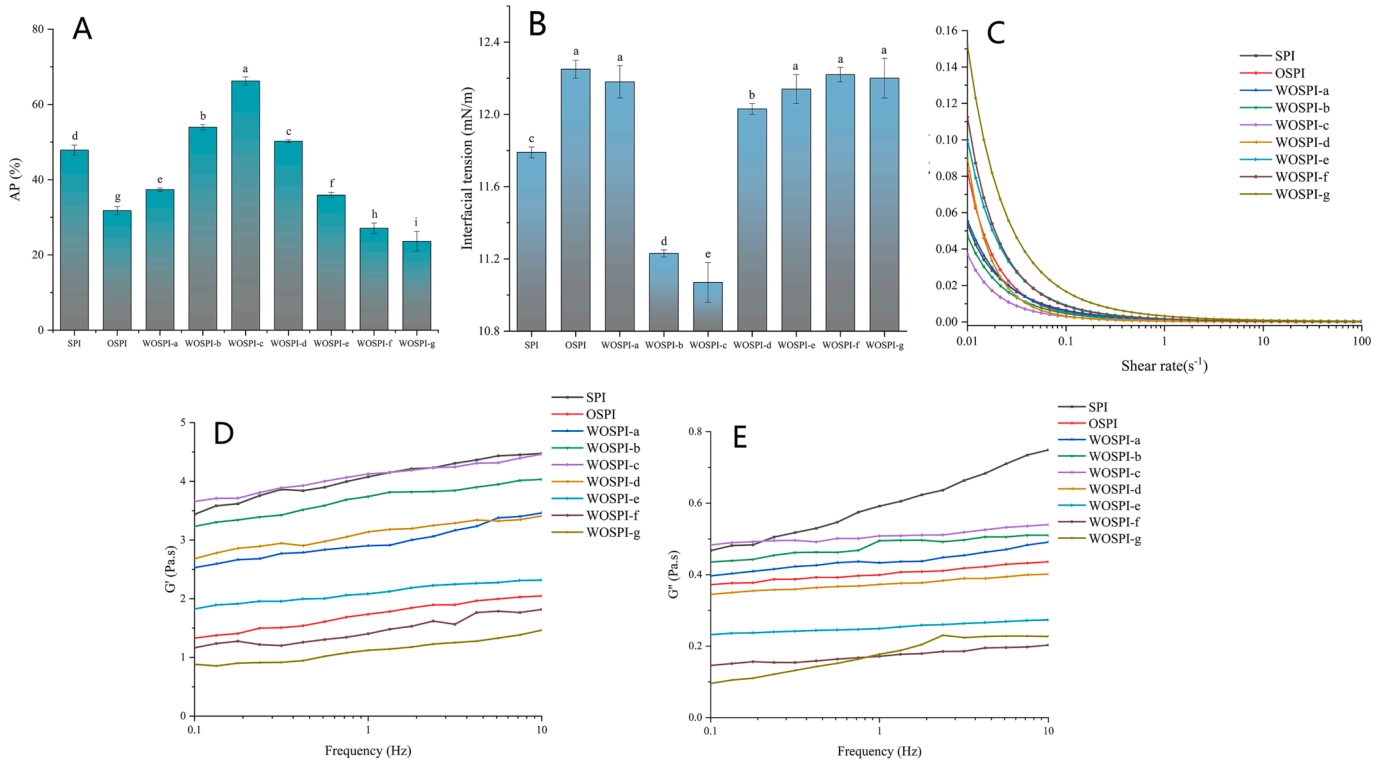

Fig. 5. (A) Percentage of adsorbed proteins of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). (B) Interfacial tension, (C) Apparent viscosity of emulsion of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s). Changes in (D) storage modulus (G′), (E) loss modulus (G″) of SPI, OSPI, and WOSPI at different microwave treatment times (0, 10, 20, 30, 40, 50, 60, and 70 s) as functions of the frequency. The symbols SPI, OSPI and WOSPI are the same as the legend of Fig. 1. The different lowercase or uppercase letters in the graphs indicate that the results are significantly different ($p < 0.05$).

generated by the microwave treatment. Extensive denaturation of protein may result in poor interfacial mechanical properties, which would be detrimental to long-term stability of emulsions. Therefore, the interfacial tension of the sample over 50 s had little change, because the emulsion droplets couldnot maintain the original round overall structure at this time.

Apparent viscosity

For stable emulsions, the shear thinning behavior may be caused by intermolecular attraction and network structure formed by the interaction between protein molecules in the emulsion (Khouryieh et al., 2015). As shown in Fig. 5C, shear thinning phenomena were observed in emulsions prepared with OSPI and all emulsions prepared with WOSPI. Moreover, compared with SPI, OSPI showed a significant increase in initial apparent viscosity. This was because the oxidation of SPI induced oxidized aggregates formation, which could enhance protein groups intermolecular interaction in the emulsion system. Therefore, the ability of oxidized aggregates to bind other molecules was increased, leading to the emergence of more stable network structure on oil droplets surface and an elevation in the initial apparent emulsion viscosity. Research by Taherian, Britten, Sabik, and Fustier (2011) also indicated that the oxidation occurring during storage can cause protein aggregation, affecting the stability of the prepared emulsion and leading to an increase in flocculation and apparent viscosity. With an increase in microwave treatment time, the initial apparent viscosity of emulsions prepared with WOSPI decreased first, then increased. The plausible explication allow the emergence of a hypothesis that shorter microwave application times could enhance protein flexibility, resulting in a smaller interfacial tension of the formed membrane. Therefore, the initial apparent viscosity gradually decreased. However, with further prolonged microwave treatment time, OSPI underwent thermal aggregation under the gradually enhanced thermal effect, forming tightly bound thermal aggregates. These aggregates experienced steric hindrance effects with oil, making it difficult for WOSPI to fully expand at oil–water interface, causing a formation of rigid network structure on oil droplets surface. As a result, the membrane strength of oil–water interface was insufficient, resulting in severe aggregation and flocculation in emulsion and an increase in emulsion viscosity.

Viscoelastic properties

Elastic modulus as well as viscous modulus of emulsions were influenced by the protein interfacial membrane structure, molecular interactions, and thickness, which were related to the protein's adsorption and interfacial properties. The variations in adsorption and rearrangement rates of interfacial proteins could lead to some changes in the rheological properties, which affect the stability of protein-based emulsions (Wang et al., 2017). As shown in Fig. 5D-E, the elastic modulus (G') of all emulsion groups was higher than corresponding viscous modulus (G''). This indicated that protein formed viscoelastic adsorption layers on the interface, and the emulsions had a gel-like network structure dominated by elasticity. Compared to SPI, the G' and G'' of OSPI emulsions were significantly reduced, consistent with the trend of apparent viscosity changes. Following treatment time, G' and G'' of WOSPI showed an upward trend followed by a downward trend, reaching a maximum at 30 s of microwave treatment. This indicated that an appropriate microwave treatment time could increase the interfacial elastic modulus of OSPI. Combined with the previous experiments, it could be concluded that an appropriate microwave treatment caused OSPI to undergo dissociation, increasing molecular flexibility. Additionally, it exposed the hydrophobic groups inside the protein, reducing steric hindrance effect caused by large molecular oxidized aggregates. Thus, it enhanced protein's adsorption at oil–water interface, increased interfacial membrane thickness, and improved the interfacial elastic modulus. Therefore, the adsorption energy barrier at the interface was lowered and the adsorption efficiency was improved, resulting in a decrease in the interfacial tension. In contrast, when microwave application period time was prolonged, the molecule aggregates that had already been dissociated may have undergone re-aggregation through covalent and non-covalent interactions, and the ordered protein structure progressively enhanced. This dramatically affected protein's flexibility and expansion at the interface, which wasn't conducive to interfacial proteins adsorption, resulting in a diminution in elastic modulus (Sun & Arntfield, 2012).

Conclusion

This study showed that Microwave treatment had a significant effect on the aggregation and emulsifying and interface properties of oxidized soy protein. The coupling effects of the thermal and non-thermal effects of appropriate microwave treatment accelerate protein structure disruption, exposing hydrophobic residues and inducing conformational changes in both soy protein isolate (SPI) and its oxidized aggregates. Such structural modifications expose the free -SH and buried hydrophobic groups on the protein surface, which can enhance their emulsifying and interfacial properties. However, the impact of microwave treatment on these properties varies with the duration of treatment. After applying appropriate microwave treatment time (30 s), the molecular weight of OSPI decreased, the surface hydrophobicity and absolute ζ-potential value increased, the structure unfolded, and the disulfide bond content decreased. These changes improved the emulsifying status of OSPI and increased its protein adsorption capacity and interfacial tension. On the other hand, excessive microwave treatment (>30 s) had a negative impact on the structure, emulsifying and interfacial properties of OSPI. Therefore, the beneficial effects of microwave technology on the physicochemical and functional properties of oxidized soy protein cannot be ignored, and it is conducive to further expanding the application of SPI in the food industry. Additionally, microwave treatment can be used as an efficient and potential method to modify oxidized soy protein, and further efforts should be made to optimize the operating parameters involved in the microwave process to maximize the function and quality of oxidized soy protein.

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Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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