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Effect of protein oxidation on the structural characteristics of hazelnut protein isolate

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Abstract

The oxidation and structural properties of hazelnut protein prepared from a low-temperature cold-pressed hazelnut meal were evaluated using three oxidative modification methods: AAPH (2,2'-azo(2-methylpropionamidine)dihydrochloride), MDA (malondialdehyde), and H_2O_2. The three different oxidants gradually increased the carbonyl content of the proteins, MDA from 1.91 to 8.87 nmol/m, AAPH (from 2.13 to 12.18 nmol/mg, and H_2O_2 from 2.28 to 13.72 nmol/mg, indicating that the hazelnut protein was oxidized. The carbonyl content of H_2O_2-modified hazelnut proteins was the highest, implying that the proteins were more susceptible to oxidation by hydroxyl radicals. FT-IR, intrinsic fluorescence spectra, surface hydrophobicity, and protein electrophoresis implied that oxidative modifications altered the secondary structure of hazelnut proteins, and promoted protein aggregation and cross-linking. In addition, the oxidative modification resulted in a larger particle size distribution of hazelnut proteins and a decrease in the zeta potential absolute value, indicating a decrease in the hazelnut proteins stability and the formation of soluble aggregates. Overall, incubation with AAPH, MDA, and H_2O_2 significantly affected the structure of hazelnut proteins, demonstrating that hazelnut proteins in food processing systems are susceptible to structural and property changes due to different oxidation products.

Keywords: hazelnut protein; peroxyl radicals; malondialdehyde; hydroxyl radicals; structure.

Practical Application: Effect of lipid oxidation products on the structural properties of hazelnut proteins.

1 Introduction

Hazelnuts (Corylus avellana L.) are among the most popular nuts globally (Alasalvar et al., 2009), valued for their pleasant taste and high nutritional value provided by valuable bioactive substances. Hazelnut is the most widely distributed and economically important member of the Betulaceae family in China. Hazelnut is primarily composed of oil (58–64%), protein (11–16%), and carbohydrates (15–18%) (Alasalvar et al., 2006). Hazelnuts are highly nutritious and provide unsaturated fatty acids, diet-friendly fiber, protein, vitamins, and antioxidants. The hazelnut meal, containing around 58% of protein, is a byproduct of lost edible oil during production (Liu et al., 2018).

Oxidation is a covalent modification in proteins, where proteins are attacked directly by reactive oxygen species (M'Fouara et al., 1992) or indirectly by reaction with lipid peroxidation byproducts, leading to structural modification (Sun et al., 2011b). ROS consists of free radicals or reactive nonradical species, such as superoxide anion, singlet oxygen, hydrogen peroxide (H_2O_2), perhydroxyl radical, and hydroxyl radical from unsaturated carbonyls and aldehydes (Lü et al., 2010). ROS indirectly induces protein oxidation mainly through lipid peroxidation and non-enzymatic glycosylation reactions,ROS is responsible for the oxidative stress in different pathophysiological conditions (Nimse & Pal, 2015). The products of the lipid peroxidation reaction can easily react with proteins, causing both sensory changes and loss of nutritional value (Duman & Kurban, 2022). Lipid peroxidation is a significant cause of protein oxidation. Hydroperoxides are the primary initial product of lipid oxidation. Still, they are extremely unstable and easily decomposed. The oxygen-oxygen bond of hydroperoxides breaks, producing peroxyl and hydroxyl radicals, and the carbon-carbon bond on both sides of the alkoxy group breaks, producing aldehydes. Lipid peroxidation causes disturbances and changes in the fine structure of proteins and loss of their integrity, mobility, and functionality (El-Beltagi & Mohamed, 2013). Oxygen-derived free radicals such as peroxyl radicals (ROO•) and hydroperoxyl radicals (HOO•) have a role in fatty acid peroxidation and have received significant attention in oxidative stress research. Meanwhile, peroxyl and alkoxyl radicals are the most present radicals in fatty acid peroxidation products. In addition, malondialdehyde (Skoie et al., 2019), a secondary oxidation product of lipid peroxidation, has a high oxidative activity and can further induce protein oxidation. E. Dorta and co-workers reported that MDA and 4-hydroxy-non-2-enal (4-HNE), among others, form covalent linkages with proteins by reacting, precisely, with ε-amino groups from protein-bound lysines (Estévez et al., 2019).

It has been reported that free-radical intermediates of lipid peroxidation could react with proteins and the reaction resulted in protein–protein cross-linked polymers via free-radical chain polymerization. Hydrophilic peroxyl radicals (ROO·) generated from AAPH (2,2′-azobis(2-methylpropionamidine)dihydrochloride thermolysis induce oxidative modifications on protein, leading to extensive protein aggregation (Dorta et al., 2019). Reaction

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of peroxyl radicals constitutes propagation process in protein chain oxidation reactions by abstracting hydrogen atoms from protein . Hydroxyl radicals can induce protein modifications, leading to structural changes in the protein (Rahman et al., 2020). Moreover, Hydroxyl radicals oxidation induced whey protein oxidative cross-linking, leading to changes in protein secondary structure (Kong et al., 2013). Meanwhile, Lin Wang and his colleagues showed that MDA-induced modifications could influence the water distribution in fish MP gels, mainly due to protein structural changes (Wang et al., 2016). Protein oxidation causes protein cross-linking and aggregation, resulting in an increase in carbonyl and disulfide bonds, a decrease in active sulfhydryl groups, and a loss of functionality such as protein solubility (Qi et al., 2022). Some studies have found that protein oxidation affected the conformational and functional properties of food proteins, such as meat protein and soybean protein. The changes in the spatial configuration and groups of these proteins affect the edible quality of hazelnut. Therefore, this study aimed to use three different modalities for the oxidative modification of hazelnut proteins, AAPH, MDA, and H_2O_2, to investigate the effects of oxidation, zeta potential, particle size, and structural characteristics of hazelnut proteins.

2 Experimental

2.1 Samples and materials

Cold-pressed hazelnut meal cake (Liaoning, China), AAPH (2,2'-azobis(2-methylpropionamidine)dihydrochloride) was purchased from APExBIO (Houston, USA), 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB) and 1,1,3,3-tetramethoxypropane (TMP, purity 98%) were purchased from Xisisi (Tianjin China), 1-aniline-8-naphthalene-sulfonate (ANS) was purchased from Yuanye (Shanghai China), glycine and guanidine hydrochloride were purchased from Solebro Technology Co.(Beijing China), tris(hydroxymethyl)aminomethane (Tris) was obtained from BIORIGIN(Beijing China), 2,4-dinitrophenylhydrazine (DNPH) was obtained from Aladdin (Shanghai, China). All other chemicals were of analytical reagent grade and obtained in China.

2.2 Hazelnut protein isolate

Fifty grams of defatted hazelnut meal powder were mixed with water at a 6:1 ratio to obtain a primary slurry and adjust the pH to 8.0 with NaOH. After stirring for 2 h, the resulting solution was centrifuged at 8000 g for 20 minutes at 4 °C to remove the insoluble precipitate. Then the pH was adjusted to 4.5 with 2.0 M hydrochloric acid, and the precipitate was collected by centrifugation at 8000 g for 20 minutes at 4 °C. The precipitate was dissolved in deionized water in a ratio of 1:5, the pH was adjusted to 7.0 with 2.0 M NaOH, and stirred until the protein was completely dissolved. The dissolved protein was freeze-dried and stored at 4 °C.

2.3 Preparation of MDA stock solution

MDA was prepared by TMP hydrolysis following the method of Adams and Chen (Adams et al., 2008; Chen et al., 2013). 10.0 mL of 5.0 mol/L hydrochloric acid was added to 31.6 mL of distilled water, and then 8.4 mL of TMP was mixed thoroughly and quickly transferred to a light-proof 40 °C water bath for oxidation for 30 min. Agglomerates were prevented by stirring at intervals, and the TMP hydrolysis treatment was quickly followed by adjusting the reaction solution to 1 L with 6 mol/L phosphate buffer pH 7.6. The MDA concentration was quantified by the molar absorption coefficient of 31,500 L/(mol-cm), and the reaction stock solution was prepared prior to use to ensure the oxidative activity of MDA.

2.4 Hazelnut protein isolate modification with MDA

Control and MDA-modified SPI were prepared according to the method described by (Wu et al., 2009a). Hazelnut protein was dissolved in a 0.01 mol/L phosphate buffer solution (30 mg/mL, containing 0.5 mg/mL NaN3) at pH 8.0. The hazelnut protein solution was mixed with 0, 0.1, 0.5,1.0, 5.0, and 10.0 mmol/L malondialdehyde solutions, respectively, and then shaken in a water bath shaker at 25 °C for 24 h in the dark. Unreacted malondialdehyde was removed by dialysis for 72 h, with deionized water replacement every 6 h. Finally, the malondialdehyde-oxidized hazelnut protein was obtained by freeze dried to obtain the malondialdehyde oxidized hazelnut protein.

2.5 Modification of hazelnut protein isolate with AAPH

Control and MDA-modified soybean protein isolates(SPI) were prepared according to (Wang et al., 2018). Hazelnut protein was dispersed in 0.01 mol/L Ph 7.4 phosphate buffer (30 mg/mL, containing 0.5 mg/mL NaN3). AAPH was added to make the final concentrations of 0, 0.1, 0.5, 1.0, 5.0, and 10.0 mmol/L, respectively, and the reaction was carried out at 37 °C for 24 h, protected from light. Then the solution was dialyzed in deionized water at 4 °C for 24 h to remove the free AAPH, and finally freeze-dried to obtain peroxyl radical oxidized hazelnut protein, which was stored at 4 °C until use.

2.6 Preparation of hydroxyl radical oxidation system

Hydroxyl radicals were generated using the Fe/H_2O_2/Asc (iron/hydrogen peroxide/ascorbic acid) system, mainly by the FeCl3, Asc, and H2O2redox reaction. FeCl3 and Asc concentrations in the system were fixed to 0.1 mmol/L each, and the H_2O_Q concentrations were chosen as 0.1, 0.5, 1.0, 5.0, and 10.0 mmol/L.

2.7 Hazelnut protein isolate modification with H_2O_2

Hazelnut protein (30 mg/mL) was added to the hydroxyl radical oxidation system and then oxidized at 37 °C for 1 h. The reaction was terminated by adding EDTA to a final concentration of 1 mmol/L. The protein solution was centrifuged at 8000 g for 10 min. The precipitate was added to deionized water at a ratio of 1:5, the pH was adjusted to 7.0 with 1M NaOH, and the protein solution was finally freeze-dried to obtain the oxidized protein.

2.8 Evaluation of oxidative changes

Carbonyls

The carbonyl content was determined by reaction with DNPH using a UV765 spectrophotometer (China) (Sun et al.,

2011a). Protein samples (25 mg) were suspended in 10 mL of Tris-HCL buffer of 0.05 mol/L pH 8.0. The supernatant was used to determine the protein content by the colorimetric method of Thomas Brilliant Blue. Then, in a 5 mL capped polypropylene centrifuge tube, 0.35 mL of oxidized hazelnut protein solution and 1 mL of 2M HCl (containing 10M 2,4-dinitrophenylhydrazine) were mixed. Samples without 2,4-dinitrophenylhydrazine were used as a control. The sample was mixed with 0.45 mL of 40% trichloroacetic acid in a water bath at 20 °C for 2 h and then left for 0.5 h. The sample was centrifuged at 10000 g for 20 min at 4 °C. The precipitate was eluted three times with ethanol/ethyl acetate solution (1:1, v/v) and then centrifuged at 10000 g for 20 min at 4 °C. The precipitate was dissolved in 1 mL of 0.1 M pH 8.0 Tris-HCl containing 6M guanidine hydrochloride. The 367 nm absorbance was measured, and HCl was used as a blank. The results were calculated using a molar extinction coefficient of 22,000 M-1 cm-1 and expressed as nanomoles of carbonyl group per milligram of protein.

Free sulfhydryl and disulfide bonds content

The sulfhydryl and disulfide groups content in hazelnut proteins was determined by the modified Ellman method (Huang et al., 2006). Protein samples (25 mg) were suspended in 10 mL of Urea Tris-Gly solution buffer of 8 M pH 8.0. The supernatant was used to determine the protein content in the solution by the Thomas Brilliant Blue colorimetric method. Four milliliters of hazelnut protein solution and 160 μL of pH 8.0 Tris-Gly solution buffer (containing 4 mg/mL DNTB) were mixed. No DNTB was added as a control. The absorbance was measured at 412 nm, and the free sulfhydryl content was calculated as a molar extinction coefficient of 13600 L/(mol-cm).

For total sulfhydryl content determination, 4 mL of hazelnut protein solution was mixed with 0.2% β-mercaptoethanol for 2 h. Then, 8 mL of 12% trichloroacetic acid was added to react at room temperature for 1 h and centrifuged at 10000 g for 10 min. The precipitate was washed three times with 12% trichloroacetic acid solution and dissolved in 6 mL Tris-Gly solution buffer with no DNTB as blank. The absorbance was measured at 412 nm, and the total sulfhydryl content was calculated by the molar extinction coefficient of 13600 L/(mol. cm).

Hazelnut protein Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectroscopy of hazelnut protein was performed by an IRTracer-100 spectrometer (Shimadzu, Kyoto, Japan). The resolution was set to 4 cm−1, and the cumulative scan was 64. The secondary structural components were assigned in the amide I band region (1700-1600cm−1). The data were processed by the PeakFit software v.4.12 (SPSS Inc., Chicago, IL, USA), involving baseline correcting, smoothing, and curve-fitting.

Surface hydrophobicity

Hazelnut protein was mixed with 0.01 M pH 8.0 phosphate buffer, and the protein concentration was determined using a colorimetric assay with Thomas Brilliant Blue. Known concentrations (0.005, 0.025, 0.05, 0.25, 0.5 mg/mL) of hazelnut protein solutions were prepared, Then 4 mL different gradients of protein dilutions were mixed in 50 μL ANS solution (8 mM in 0.01 M pH 7.0 phosphate buffer). The hazelnut protein fluorescence spectrum was measured at the 395 nm excitation wavelength and 475 nm emission wavelength. The curve was fitted with fluorescence intensity and protein concentration, the initial slope of which was used as an index of protein surface hydrophobicity (H0).

Intrinsic fluorescence measurement

The endogenous fluorescence of unoxidized and oxidized hazelnut proteins was measured in phosphate buffer (0.01 M pH 7.0) using a fluorescence spectrometer at Ex 28 0 nm (slit width was 2.5 nm), Em 300-5000 nm (slit width was 2.5 nm) with a scan speed of 20 nm/s. Phosphate buffer was used as a blank solution for all samples.

Measurement of zeta potential and particle size distribution (PSD)

Oxidized hazelnut protein was dissolved in phosphate buffer (0.01 M pH 7.0) to prepare a 1 mg/mL protein solution. Then, the hazelnut protein solution's particle size distribution and zeta potential were measured by a Nicomp 380 DLS nanoparticle size analyzer (Malvern, UK) at 25 °C.

SDS–PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% separation gel and 5% gel concentrate in a discontinuous buffer system. Ten microliters of hazelnut protein (10 mg/mL, 10 mM phosphate buffer, pH 7.0) were added to the loading buffer and incubated at 100 °C for 5 min in a dry bath. Each sample was added to the lanes. The current was 12A at the beginning of electrophoresis and changed to 24A after the sample entered the separation gel. After electrophoresis, the samples were stained with Thomas Brilliant Blue G250 staining solution (0.1% Thomas Brilliant Blue G250, 10% acetic acid, 45% methanol) for 30 min and then decolorized using methanol/acetic acid/water (1:1:8) for 12 h, changing the decolorizing solution three times halfway.

2.9 Statistical analysis

Statistical analyses were performed using the statistical package SPSS 26.0 (SPSS Inc., Chicago, IL) for one-way ANOVA. Data are expressed as means ± standard deviations (SD) of triplicate determinations unless specifically mentioned.

3 Results and discussion

3.1 Oxidative modifications of hazelnut protein isolations

Carbonyls content

Protein carbonyl content was used to evaluate the hazelnut proteins oxidation because protein carbonyl content is the most common oxidation indicator of protein oxidation. The modification of oxidized protein carbonate content in hazelnuts by MDA, H_2O_2, and AAPH is given in Figure 1.

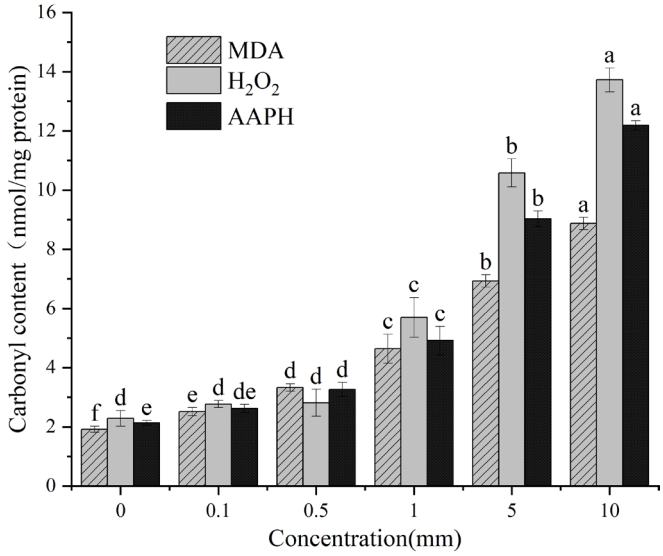

Figure 1. Protein carbonyl of hazelnut protein incubated with different MDA, AAPH, and H_2O_2 concentrations. The results are expressed as the mean value ± standard deviation. Mean values followed by the different letters are significantly different at $P < 0.05$.

In this work, the control protein carbonyl content was 1.91, 2.28, 2.13 nmol/mg; increasing concentrations in all three systems resulted in significant increases in hazelnut protein carbonyl content to 8.87, 13.72, 12.18 nmol/mg ($p < 0.05$), which increased by 364.39%, 501.75% 471.83%, respectively. This is similar to the trend of peroxyl radicals and MDA-modified soy and rice bran proteins (Li et al., 2021; Wu et al., 2009b). In addition, the hazelnut oxidized protein was similar to that of hydroxyl radicals-modified muscle myofibrillar protein (Park et al., 2007). Peroxyl radicals can induce the protein peroxyl radicals formation from the protein's main peptide chains and side-chain groups, subsequently leading to the formation of carbonyl compounds through amidation and other pathways. The malondialdehyde molecule contains 2 carbonyl groups, one of which reacts with the primary amine in the protein molecule to form an enamine compound, further introducing the carbonyl group. Moreover, hydroxyl radical attack on peptide bonds in the protein backbone or side chains produces carbonyl groups. We found that AAPH- and H_2O_2-modified hazelnut proteins had higher carbonyl content than those modified by MDA. This might be due to the different sensitivity of the conversion of different groups to carbonyl groups. It has been proposed that NH or NH2 groups transformed into carbonyl groups during protein oxidation were more sensitive to •OH (Stadtman, 1990). Moreover, ferric and ferrous iron combined with hydrogen peroxide can effectively form carbonyl groups from meat proteins (Park et al., 2006). Oxidation may partially disrupt the molecular morphology of hazelnut proteins, exposing more sites of action.

3.2 Free sulfhydryl and disulfide bonds content

As shown in Figure 2, MDA modification decreased free sulfhydryl groups from 24.39 to 10.31 nmol/mg, and increased the disulfide groups from 11.71 to 14.41 nmol/mg in hazelnut protein. AAPH modification decreased free sulfhydryl groups

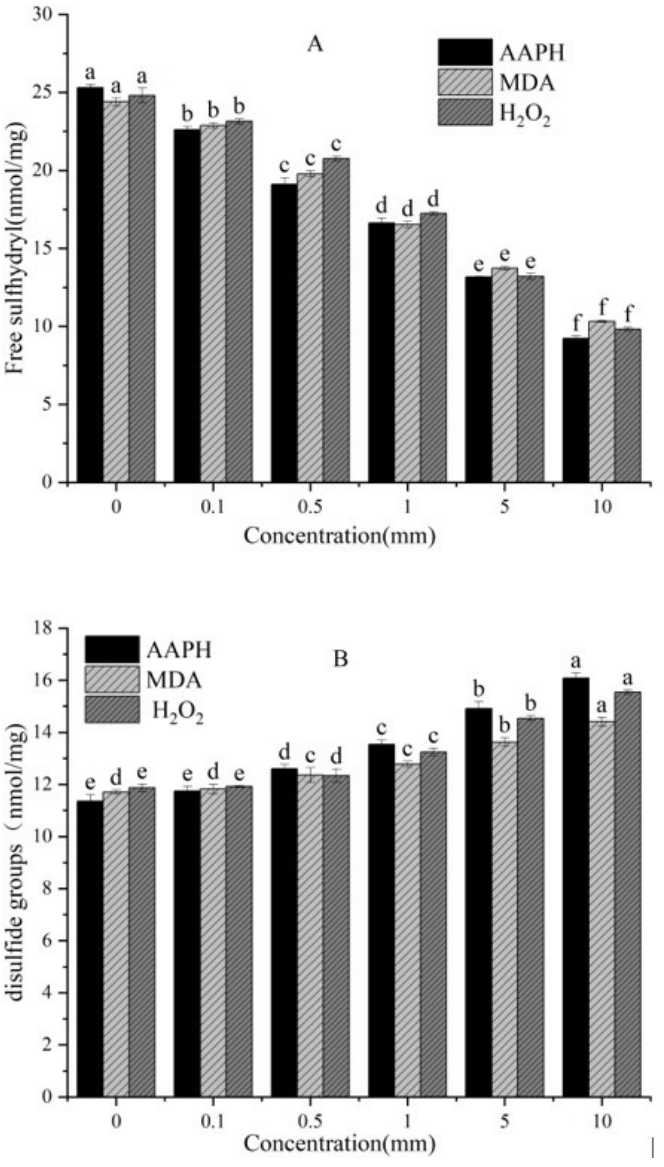

Figure 2. Free sulfhydryl and disulfide groups of hazelnut protein were incubated with different concentrations of MDA, AAPH, and H_2O_2. Free sulfhydryl (A). Disulfide groups (B). The results are expressed as the mean value ± standard deviation. Mean values followed by the different letters are significantly different at $P < 0.05$.

from 25.29 to 9.23 nmol/mg and increased disulfide groups from 11.35 to 16.07 nmol/mg. H_2O_2 modification decreased the free sulfhydryl groups content from 24.79 to 9.82 nmol/mg, and increased the disulfide groups content from 11.87 to 15.54 nmol/mg.The free sulfhydryl groups and disulfide bonds in all modified-hazelnut proteins showed the same trend. Moreover, protein oxidation could break the equilibrium of the interchange reaction between free sulfhydryl and disulfide bonds, leading free-sulfhydryl groups to form irreversible or reversible products under different oxidative conditions (Li et al., 2019; Waszczak et al., 2015). The decrease in free sulfhydryl groups was significantly greater than the increase in disulfide bonds, indicating that both reversible and irreversible oxidation of free

sulfhydryl groups of hazelnut proteins occurred. The reduction of free sulfhydryl groups is mainly due to the oxidative modification inducing both disulfide bonds and irreversible oxidation products. It indicates that peroxyl radicals, hydroxyl radicals and MDA are highly reactive with sulfhydryl radicals.

3.3 Surface hydrophobicity

Changes in surface hydrophobicity can estimate protein unfolding and folding. The obtained protein surface hydrophobicity might be attributed to the balance between the exposure of hydrophobic amino acid side chains and hydrophobic interaction (Li et al., 2021). In Figure 3, the surface hydrophobicity of hazelnut protein increased from 837.35 to 1055.17, and then decreased, and then decreased to 615.45 with the addition of AAPH, peaking at 0.5 mM AAPH, similar to previous reports (Traverso et al., 2004). The surface hydrophobicity of the MDA-modified hazelnut protein increased from 842.15 to 1039.31 and then decreased to 579.04 and peaked at 0.5 mM, similar to the AAPH-modified hazelnut protein. Then the surface hydrophobicity of the H_2O_2-modified hazelnut protein increased from 830.36 to 970.42 and then decreased to 558.76 and peaked at 0.1 mM H2O2. Aggregation and unfolding of protein happened synchronously under oxidative stress. Protein oxidation may simultaneously expose hydrophobic groups to the protein surface through partially unfolded proteins and promote protein cross-linking through hydrophobic interactions (Zhou et al., 2015). Our results indicate that under AAPH and MDA-induced oxidative stress, the unfolding of hazelnut proteins is dominant at concentrations of 0.5 mM. Similarly, the H_2O_2-induced unfolding of hazelnut proteins dominates at 0.1 mM. The exposure of hydrophobic amino acid groups leads to an increase in surface hydrophobicity. At the same time, the aggregation of hazelnut proteins increased with oxidant concentration, leading to decreased surface hydrophobicity.

3.4 Intrinsic fluorescence measurement

The intrinsic fluorescence spectrum can reflect the changes in protein tertiary structure and conformation, and an intensity decrease can partly reflect tryptophan and tyrosine oxidation (Wang et al., 2022). In the folded state, tryptophan residues were generally located within the hydrophobic environment of the protein, having a high quantum yield and, therefore, a high fluorescence intensity. They become exposed to a hydrophilic environment in the unfolded state, therefore reducing fluorescence

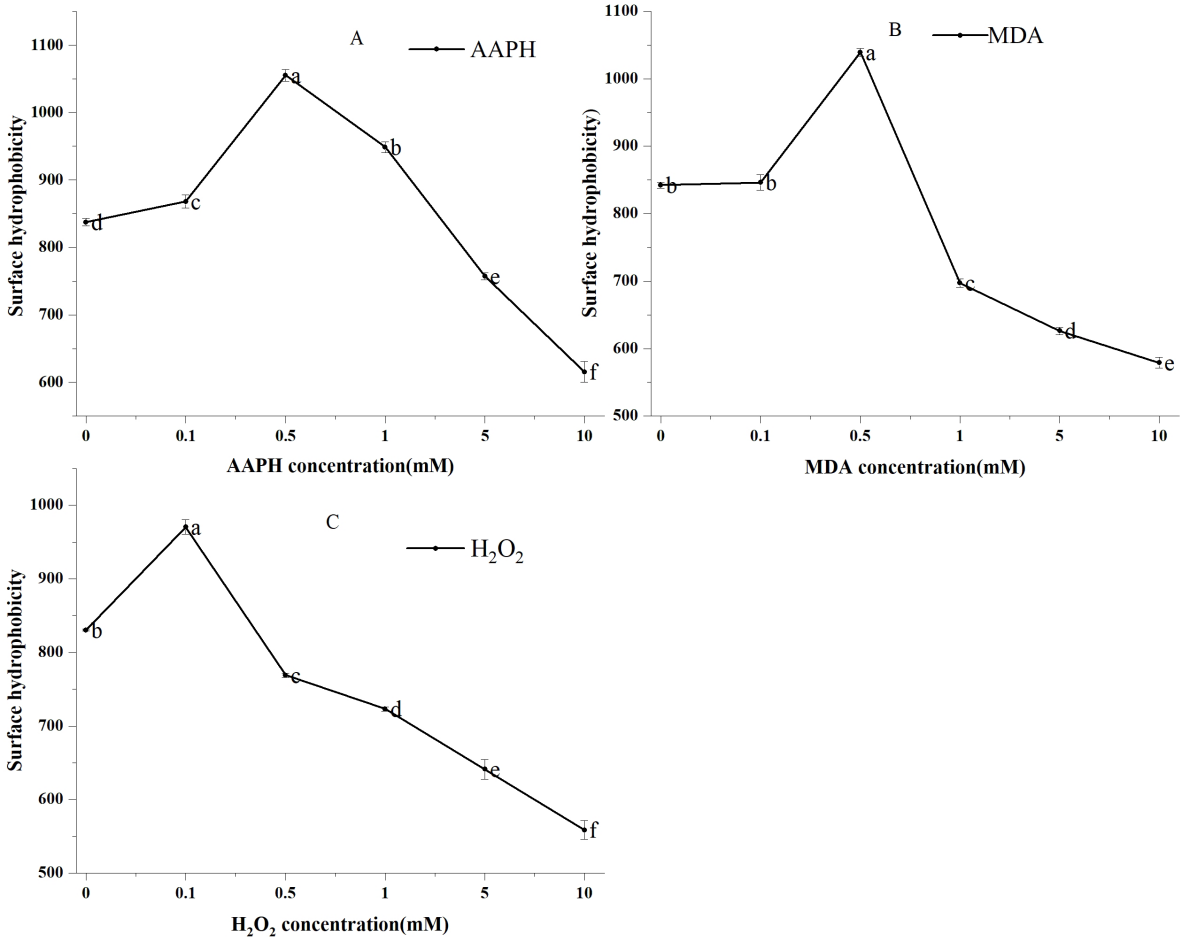

Figure 3. Effect of MDA, AAPH, and H2O2 treatment on surface hydrophobicity of hazelnut. The concentrations were 0, 0.1, 0.5,1.0, 5.0, and 10.0 mM. The results are expressed as the mean value ± standard deviation. Mean values followed by the different letters are significantly different at $P < 0.05$. A (Oxidized by AAPH); B (Oxidized by MDA); C (Oxidized by H_2O_2).

intensity (Wang et al., 2021). The intrinsic fluorescence spectrum of AAPH oxidized hazelnut protein is presented in Figure 4A. As AAPH concentration increased, the maximum intrinsic fluorescence intensity decreased (from 595.5 to 478.4 nm). One possibility is that peroxyl radicals oxidation transfers the energy inside the hazelnut protein leading to the protein unfolding, and tryptophan quenching by exposure to the polar environment. In Figure 4B, as MDA concentration increased, the maximum intrinsic fluorescence intensity gradually decreased (from 594.4 to 51.1 nm), accompanied by the continuous blue shift of the peak position from 327.0 to 313.0 nm. Also, carbonyl–amine condensation reactions result in the protein aggregates that could bury the tryptophan residues, enhancing the non-polarity of tryptophan residues' microenvironment, thus contributing to the blue shift of maximum fluorescence peak (Sponton et al., 2015; Wu et al., 2021). In Figure 4C, the maximum intrinsic fluorescence intensity gradually decreased from 587.1 to 360.5 nm with H2O2 concentration. The decreased fluorescence intensity may be due to the ability of protein aggregates formed by oxidative cross-linking of hydroxyl radicals to occlude some of the tryptophan residues. Our results show that MDA induces the strongest oxidation of hazelnut proteins, followed by H_2O_2, and finally AAPH.

3.5 Zeta potential and particle size distribution (PSD)

The particle size distribution reflects the degree of protein cross-linking and aggregation. Our results show that oxidation changes the particle size distribution of hazelnut protein into large size (1000 nm) and small size (< 100 nm). Figures 5A and 5C show that the particle size distribution curves of oxidized hazelnut proteins all shifted toward larger sizes with AAPH and H_2O_2 concentration. However, in Figure 5B, for the particle size distribution, hazelnut gradually shifted to a smaller particle size than the control within MDA ≤ 0.1 mM. In contrast, at concentrations between 0.5 and 10 mM, it shifted to larger particle size. It has a similar trend to the studies that have been reported (Wu et al., 2009b; Ye et al., 2013). This may be that oxidation denatures and aggregates hazelnut proteins, so the protein particle size increases, resulting in soluble oxidized aggregates of hazelnut proteins.

Zeta-Potential is an indicator of the surface charge properties of particles in solution. Zeta potential represents the amount of charge on the protein surface and reflects the stability of the aggregates. The absolute value of potential and the stability of the aggregates are positively correlated. Decreasing the absolute value of the zeta potential might reduce the spatial stability of

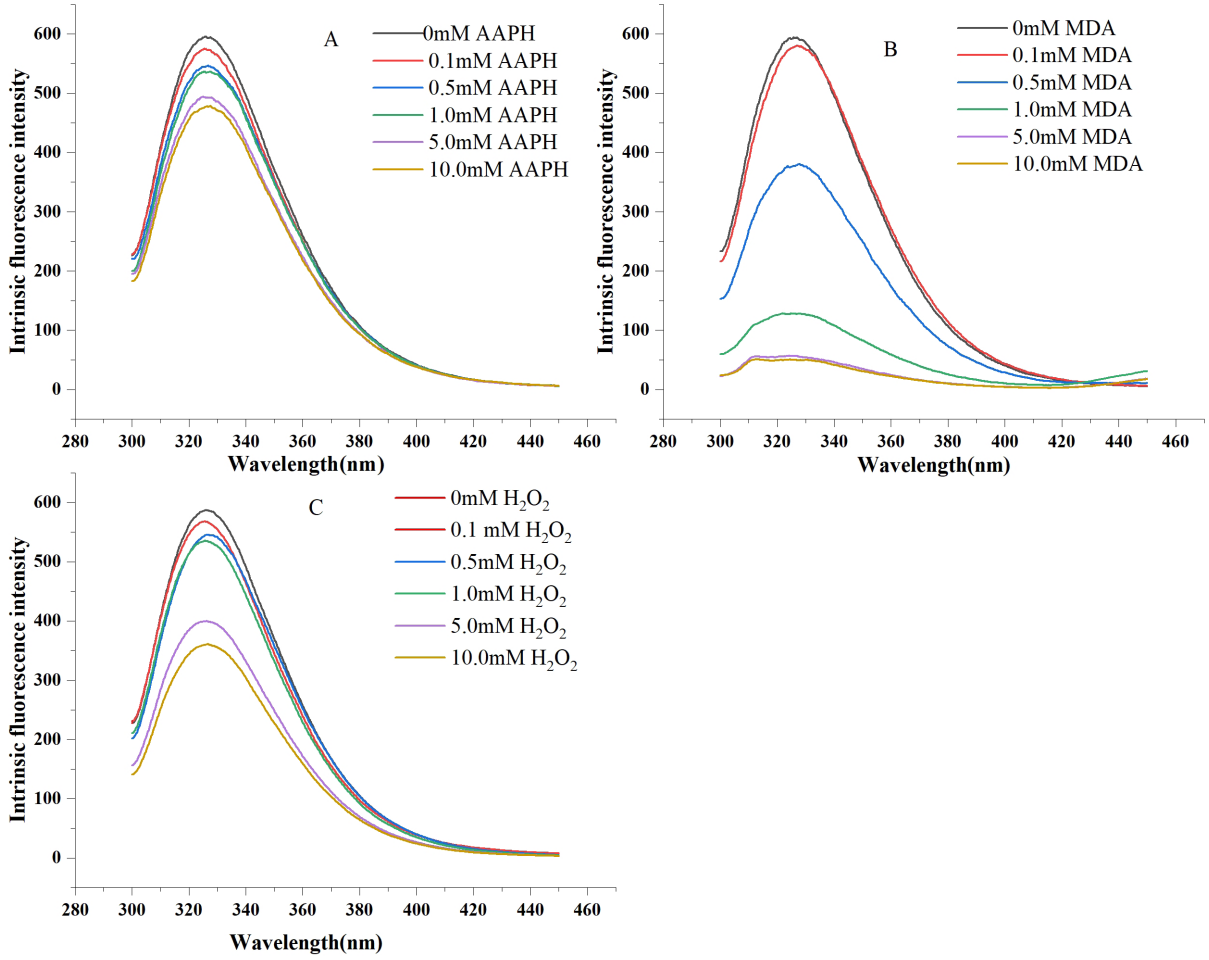

Figure 4. Impact of AAPH, MDA, and H_2O_2 on intrinsic fluorescence spectra: hazelnut protein modified by AAPH (A), hazelnut protein modified by MDA (B), hazelnut protein modified by H_2O_2 (C).

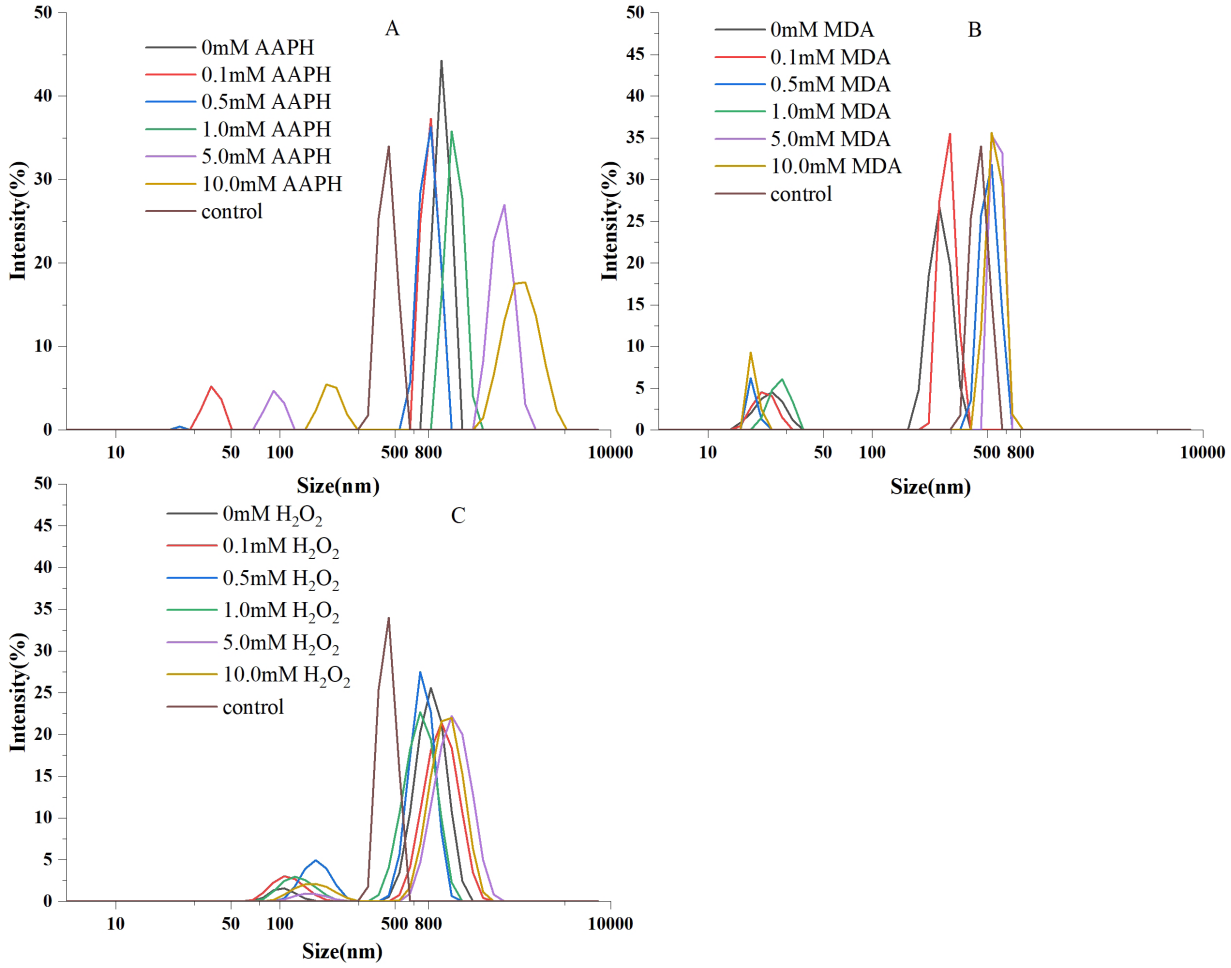

Figure 5. Particle size distributions of AAPH, MDA, and H_2O_2-modified hazelnut protein. AAPH-modified hazelnut protein (A), MDA-modified hazelnut protein (B), H_2O_2-modified hazelnut protein (C). The results are expressed as the mean value ± standard deviation. Mean values followed by the different letters are significantly different at $P < 0.05$.

the protein and promote the aggregation and polymerization of droplets, which further reduces the emulsification properties of the protein (Hu et al., 2019). The aggregation and cross-linking induce the burial of hydrophobic groups and a decrease in solubility, leading to a decrease in the amount of soluble hazelnut protein adsorbed at the interface. In Figure 6, as the concentration increases, the hazelnut protein potential absolute value decreases significantly ($p < 0.05$). Hazelnut protein-stabilized emulsions have a lower surface charge and a higher degree of oxidation, indicating that the emulsions are less stable. Lin Ye proposed that high protein oxidation decreases the solution's surface charge; therefore, increased protein oxidation will make the protein less stable (Ye et al., 2014).

3.6 SDS–PAGE

SDS–PAGE was employed to monitor structural changes of hazelnut protein subunits after oxidation. The SDS-PAGE pattern of the native hazelnut proteins is characterized by proteins in the molecular weight range of 10–80 kDa (Cucu et al., 2011). Our results' main components of hazelnut proteins are distributed around MW 70-50 kDa, 50-40 kDa, 40-30 kDa, 30-25 kDa, and

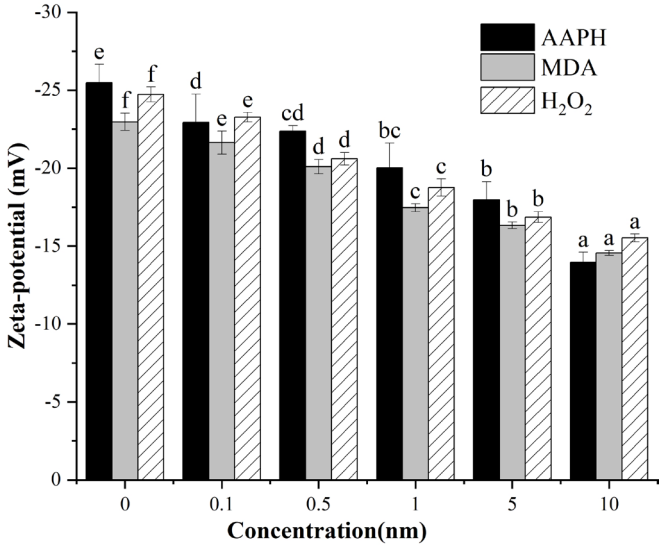

Figure 6. Zeta-potential of hazelnut protein of AAPH, MDA, and H_2O_2-modified hazelnut protein. The results are expressed as the mean value ± standard deviation. Mean values followed by the different letters are significantly different at $P < 0.05$.

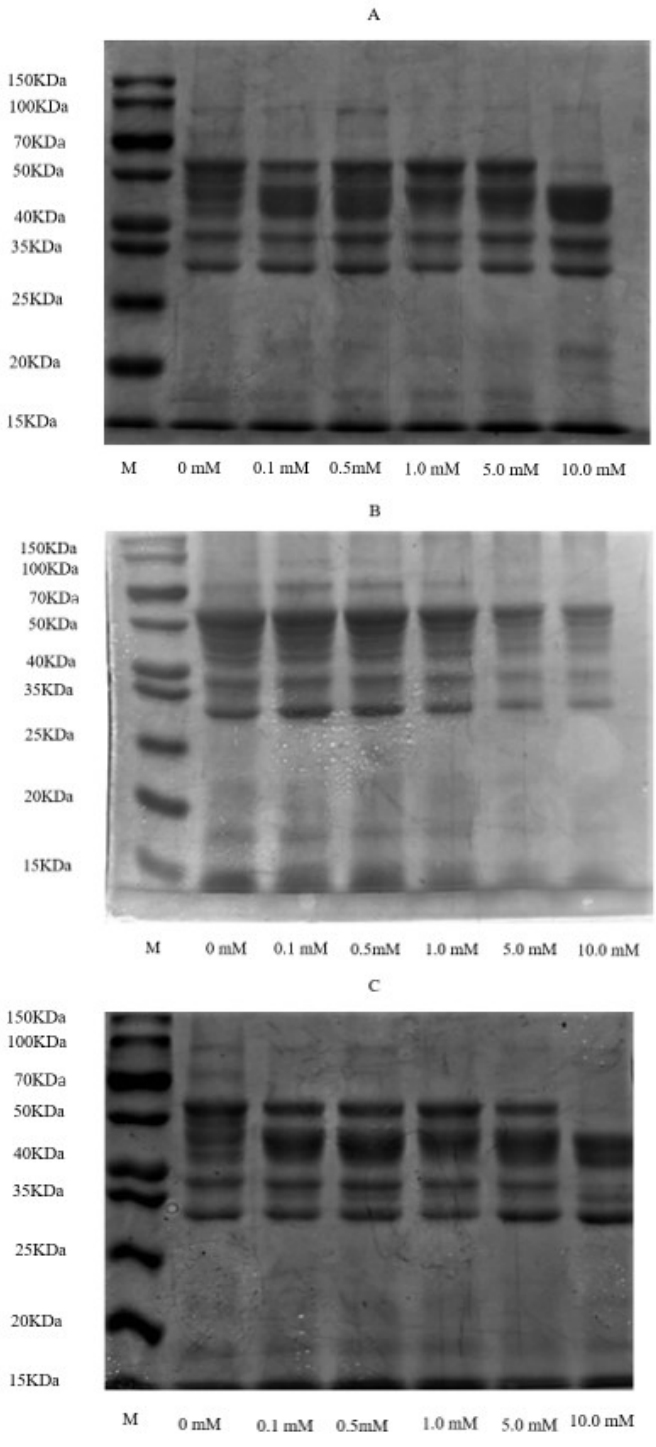

Figure 7. SDS-PAGE patterns of hazelnut as affected by AAPH, MDA, and H_2O_2. AAPH (A); MDA (B); H_2O_2 (C).

15 kDa, similar to the molecular weight range of natural hazelnut protein. In Figure 7A, in low AAPH concentration (≤ 0.5 mM), the MW 70-50 kDa band strength increased, with further oxidation (≥ 10 mM), disappeared, similar to myofibrillar protein results (Zhou et al., 2014b). In Figure 7B, the bands of all hazelnut proteins became lighter with MDA concentration, similar to (Zhou et al. 2014a). In Figure 7C, the H2O2-oxidized hazelnut proteins showed the same tendency as AAPH. The disappearance of protein bands indicates that proteins are highly cross-linked with high oxidation.

Table 1. Impact of AAPH, MDA, and H_2O_2 on α-Helix, β-Sheet, β-Turn, and Random coil contents of hazelnut protein.

Samples	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
0 mM AAPH	42.03 ± 0.03^{a}	41.18 ± 0.03^{a}	16.79 ± 0.01^{b}	--
0.1 mM AAPH	39.66 ± 0.02^{ab}	41.74 ± 0.01^{a}	18.60 ± 0.01^{b}	--
0.5 mM AAPH	38.27 ± 0.04^{b}	38.29 ± 0.02^{a}	23.44 ± 0.03^{b}	--
1.0 mM AAPH	32.69 ± 0.08^{b}	23.89 ± 0.02^{b}	22.99 ± 0.04^{b}	20.42 ± 0.02^{a}
5.0 mM AAPH	21.99 ± 0.0c^{d}	21.12 ± 0.02^{b}	36.96 ± 0.03^{a}	19.94 ± 0.01^{a}
10.0 mM AAPH	20.34 ± 0.03^{d}	19.67 ± 0.04^{b}	41.55 ± 0.09^{a}	18.44 ± 0.03^{a}
0 mM MDA	41.98 ± 0.06^{a}	37.20 ± 0.03^{a}	19.92 ± 0.03^{b}	--
0.1 mM MDA	39.45 ± 0.04^{a}	38.55 ± 0.03^{a}	22.00 ± 0.03^{b}	--
0.5 mM MDA	37.78 ± 0.03^{a}	35.42 ± 0.02^{a}	26.80 ± 0.04^{b}	17.16 ± 0.01^{a}
1.0 mM MDA	22.5 ± 0.04^{b}	20.27 ± 0.10^{b}	36.84 ± 0.10^{ab}	20.39 ± 0.03^{a}
5.0 mM MDA	21.54 ± 0.02^{b}	19.87 ± 0.02^{b}	38.76 ± 0.05^{a}	19.83 ± 0.02^{a}
10.0 mM MDA	19.14 ± 0.03^{b}	25.14 ± 0.05^{b}	36.22 ± 0.06^{ab}	19.50 ± 0.03^{a}
0 mM H_2O_2	38.24 ± 0.02^{a}	39.73 ± 0.03^{a}	22.03 ± 0.03^{b}	--
0.1mM H_2O_2	38.27 ± 0.01^{a}	35.12 ± 0.01^{a}	26.61 ± 0.01^{ab}	--
0.5 mM H_2O_2	39.43 ± 0.02^{a}	36.82 ± 0.01^{a}	23.74 ± 0.01^{b}	--
1.0 mM H_2O_2	19.14 ± 0.01^{a}	26.31 ± 0.10^{ab}	38.95 ± 0.08^{a}	15.6 ± 0.03^{a}
5.0 mM H_2O_2	20.42 ± 0.02^{a}	25.56 ± 0.10^{ab}	34.38 ± 0.11^{a}	19.64 ± 0.02^{a}
10.0 mM H_2O_2	20.41 ± 0.02^{a}	20.12 ± 0.03^{b}	33.94 ± 0.10^{a}	25.53 ± 0.11^{a}

3.7 FT-IR

The amide I band (1700−1~1600 cm−1) is the most commonly used band to reflect changes in protein secondary structure. The secondary structure of hazelnut protein is shown in Table 1, including those of vibrations of β-sheets (1600–1640 and 1670–1690 cm−1), random coils (1640–1650 cm−1), α-helixes (1650–1660 cm−1), and β-turns (1660–1670 and 1690–1700 cm−1).

AAPH significantly decreased the α-helix (from 42.03% to 20.34%) and β-sheet (from 41.18% to 19.67%) contents, and increased β-turns (from 16.79% to 41.55%). When adding 1.0 mM AAPH, the secondary structure of hazelnut protein appeared to be a random coil structure. This indicates that the secondary structures of hazelnut proteins become more diverse under protein oxidation. With the addition of MDA, the α-helices and β-sheets contents decreased significantly (from 41.98% to 19.14%, and from 37.20% to 19.87%), and an increase in β-turn (from 19.92% to 38.76%). With the addition of H_2O_2, the α-helices content decreased significantly (from 38.24% to 20.41%) and β-sheet (from 39.73% to 20.12%), and an increase in β-turns (from 22.03% to 33.94%). The secondary structure of hazelnut proteins became disordered from ordered; the hydrophobic groups form aggregates in the presence of oxidative and hydrophobic interactions. In addition, under highly oxidizing conditions, the peptide chain of hazelnut protein breaks, leading to drastic changes in the secondary structure of the protein.

4 Conclusion

In the present study, as the concentration of the oxidation system increases, the content of carbonyl and disulfide bonds

in hazelnut proteins gradually increases. In contrast, the free sulfhydryl groups content decreases. These results showed that AAPH, MDA, and H_2O_2 oxidized hazelnut proteins. The intrinsic fluorescence intensity of hazelnut protein and the λ(max) appears blueshift showed that the tryptophan residues of hazelnut protein were oxidized to form aggregates. Meanwhile, the results suggested that oxidation causes hazelnut protein aggregation and cross-linking, decreases the surface hydrophobic, zeta potential, and the α-helix, destabilizing the hazelnut protein structure. This study demonstrates the relationship between protein oxidation and structural properties of hazelnut protein, reveals the effect of different oxidation modes on the structural characteristics of hazelnut proteins, and provides ideas for obtaining different applications of hazelnut protein by controlling the oxidation of hazelnut protein in practical production.

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