



Structural changes and exposed amino acids of ethanol-modified whey proteins isolates promote its antioxidant potential

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ABSTRACT

Whey protein isolates (WPI) were treated with different ethanol level (20, 40, 60, and 80%, v/v) to promote structural unfolding and subsequent aggregation. In general, protein aggregation gradually increased with increasing ethanol level in a dose-dependent manner, which was implied by notably increased turbidity and gradually decreased solubility. The formation of aggregates, which were confirmed by the results of circular dichroism spectrum and total sulphydryl content, were promoted mainly through disulfide bonds and intramolecular hydrogen bonds. Moreover, ethanol treated WPI (E-WPI) had significantly enhanced antioxidant activities over native WPI, which was mainly attribute to the higher contents of specific amino acids (such as hydrophobic amino acids, aromatic amino acids, and sulfur-containing amino acids), and E-WPI prepared with moderate ethanol concentration (40% in our present study) exhibited the highest antioxidant activities. These results reveal that antioxidant activities of WPI can be increased by ethanol treatment and are possibly achieved through molecular unfolding of native WPI.

1. Introduction

Whey protein isolate (WPI), a crucial source of food proteins, is mainly composed of β -lactoglobulin, α -lactalbumin, bovine serum albumin, and immunoglobulins. In the past decades, WPI has been widely added as a key ingredient in various food products due to its excellent functional properties, as well as its favorable nutritional contribution (Segat et al., 2014). Apart from its functional and nutritional aspects, many studies have indicated that WPI has potential antioxidant ability which could efficiently offer health-promoting benefits to consumers by impeding oxidative stress (Veskoukis et al., 2020). Moreover, O'keeffe and Fitzgerald (2014) reported that the antioxidant ability of WPI was mainly due to its hydrophobic and aromatic amino acids which had higher free radical quenching abilities. However, the rigid compact globular protein structures of native WPI are maintained through intramolecular disulfide bonds, and hydrophobic residues are generally buried. Recently, various processing strategies, such as enzymatic hydrolysis (Adjonu et al., 2013), high hydrostatic pressure treatment (Iskandar et al., 2015), microbiological fermentation (Gammoh et al.,

2020) and conjugation or polymerization with polyphenols (Quan et al., 2019) have been applied to potentially enhance the antioxidant ability of WPI. Additionally, among these strategies, there has been consensus that enzymatic hydrolysis and subsequent fractionation was the optimal way to increase the antioxidant activity of WPI. Peng et al. (2009) also indicated that with short peptides fractionated from alcalase hydrolyzed-WPI, the molecular weight ranged from 0.1 to 2.8 kDa showed the strongest free radical scavenging capabilities. However, after undergoing enzymatic hydrolysis with a severe degree of hydrolysis or fractionating hydrolysates with even lower molecular weights, the functional properties (especially for emulsifying and foaming capacities) of the resulting protein hydrolysates significantly decreased or even disappeared under certain conditions (Liu et al., 2010). This made the protein hydrolysates unsuitable for application in different food formulations. Thus, it is essential for the food industry to develop some novel strategies to modify or unfold the compact molecular structures of WPI, as well as notably enhance its antioxidant activity.

More recently, there is evidence that ethanol may denature globular proteins and lead to obvious alterations in molecular conformations,

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thus changing the functional characteristics of the molten globule (Nikolaidis et al., 2017). Thomas and Dill (1993) indicated that ethanol tend to disrupt hydrophobic interactions or hydrogen bonds and induce the proteins to unfold or aggregate, which may be attribute to the weaker polarity and lower dielectric constant of ethanol than water. Moreover, Liu et al. (2019) indicated that ethanol-induced changes of molecular conformations of soy β -conglycinin was irreversible. Nikolaidis and Moschakis (2018) also found that whey proteins could maintain an evident degree of denaturation even after ethanol removing, which clearly indicated that ethanol-induced denaturation/aggregation of whey proteins was irreversible. Furthermore, the level of ethanol has a great effect on the state of protein unfolding or aggregation. For instance, Peng et al. (2020) indicated that with increasing concentrations of ethanol (from 20% to 80%, v/v) there was a constant increase in emulsifying ability and surface activity of soy β -conglycinin. In our previous work, we obtained that protein molecular unfolding and subsequent aggregation induced by different concentrations of ethanol during pretreatment not only effectively alter the structural and morphological properties of WPI, but also obviously improve the functional characteristics (such as emulsifying characteristics and foaming ability) (Feng et al., 2021). However, so far, no information was available about the effects of denaturation and aggregation induced by ethanol pretreatment on the antioxidant activities of WPI. Therefore, the objective of this present study was to investigate the effect of treatment with different ethanol level (20, 40, 60, and 80%, v/v) on the antioxidant activities of WPI, not only along with the assistance of the determination of protein aggregation or conformational transitions, but also further verified under the changes of the amino acid profiles.

2. Materials and methods

2.1. Materials and chemicals

WPI (Item No. 9410) was obtained from Hilmar ingredients Co. Ltd. (CA, U.S.A.). Ethanol (purity >99.9%) was purchased from Tianli Chemical Reagent Co. Ltd. (Tianjin, China). 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Yuanye Bio-Technology Co. Ltd. (Shanghai, China). Pyridine was obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). Fluorescein sodium and 5',5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

2.2. Preparation of ethanol-treated WPI (E-WPI)

The E-WPI samples were prepared according to the same method in our previous work (Feng et al., 2021). Briefly, intact WPI powder was gently dispersed into deionized water and stirred magnetically at 500 rpm for 4 h to obtain a fully hydrated stock WPI solution (15.0%, w/v). The pH of the stock WPI solution was adjusted to 7.0 via 2.0 M sodium hydroxide, and then the stock WPI solution was centrifuged at 8,000 g for 20 min to remove any insoluble substances. Then, the stock WPI solution was divided into five parts and added with different mounts of ethanol to make the final ethanol concentration of each solution varied from 0 to 20, 40, 60, and 80% (v/v), respectively. The final protein concentration of each solution was kept constant at 3.0% (w/v). After that, each of the solution was stirred magnetically at 500 rpm for 4 h in a glass beaker which covered with a sealing film (to avoid the evaporation of ethanol), and then the ethanol in each solution was evaporated using a pressurized nitrogen blowing concentrator (NDK200-1N, Chengzao Instruments Co. Ltd., Shanghai, China) with lower gas flow rate (the nitrogen just blown over the surface of each solution to avoid foaming

for 2–3 h at room temperature (25 °C). After that, each resultant solution was lyophilized via freeze dryer (Pilot 3–6M, Biocool Experimental Equipment Co. Ltd., Beijing, China). Finally, all the E-WPI samples were ground into powder using a coffee grinder (DL-MD18, Donlim Electric Appliance Co. Ltd., Guangzhou, Guangdong, China) and stored at room temperature (25 °C) in zippered bags for further analysis.

Prior to measurement of the structural characteristics and antioxidant activities of E-WPI samples, different lyophilized E-WPI powders were fully dispersed into sodium phosphate buffer (10.0 mM, pH 7.0) to prepare different sample solution (25.0 mg/mL), and then stored overnight at 4 °C. Before further determination, each E-WPI solution was diluted by 10.0 mM sodium phosphate buffer (pH 7.0) to reach the optimal concentration.

2.3. Secondary structure measurement

Far-ultraviolet circular dichroism (Far-UV CD) spectra of different E-WPI sample was measured via a CD spectropolarimeter (MOS-4SO, Biologic Co., Ltd., France) according to the methods of Jiang et al. (2009) with some modification. Briefly, each E-WPI solution with a concentration of 0.5 mg/mL was scanned from 190 to 260 nm in 1.0 mm light path quartz cuvettes. The scan rate and interval were fixed at 60 nm/min and 2.0 nm, respectively. All spectra were obtained under room temperature conditions (25 °C) with 3 times scanning. The contents of secondary structure (including α -helix, β -sheet, β -turn, and random coil) for each E-WPI sample was calculated by using CD Pro. software.

2.4. Total sulfhydryl (SH) and free SH contents

The total and free SH contents of E-WPI were determined by using our previous method of Feng et al. (2022). Briefly, 0.5 mL of each E-WPI sample solution (10.0 mg/mL) was mixed with 4.5 mL of reaction buffer (0.09 M glycine, 0.086 M Tris, 4.0 mM EDTA, and pH 8.0) with (total SH) or without (exposed SH) 8.0 M urea. Then, 50.0 μ L of Ellman's reagent (4.0 mg/mL DTNB in Tris-glycine buffer) was added and mixed evenly via a vortex mixer (3030A, Scientific Industries, INC., Bohemia). After that, each resulting suspension was incubated for 30 min in a dark room at room temperature (25 °C) followed by centrifugation for 15 min at 10,000 g. The absorbance of each supernatant was measured at 412 nm via spectrophotometer (T6, Purkinje General Instrument Co. Ltd., Beijing, China) with the reagent buffer serving as blank. The protein content of each sample was measured and the total and exposed SH contents were calculated as follows:

$$\text{Total or exposed SH contents } (\mu\text{mol SH / g}) = \frac{73.53 \times A_{412} \times D}{C}$$

where A_{412} represents the absorbance at 412 nm. D represents the dilution factor. C represents protein concentration (mg/mL). 73.53 is derived from $10^6 / (1.36 \times 10^4)$; 1.36×10^4 represents the molar extinction coefficient of 13,600 $\text{M}^{-1}\text{cm}^{-1}$. 10^6 represents the conversion factor.

2.5. Turbidity

The turbidity of different E-WPI solution (20.0 mg/mL) was measured by 600 nm absorbance readings using a UV-vis spectrophotometer at room temperature (25 °C).

2.6. Protein solubility

The solubility of E-WPI samples were measured by using the method of Nishanthi et al. (2018) with some modifications. Briefly, each E-WPI solution (20.0 mg/mL) was centrifuged for 15 min at 10,000 g at room temperature (25 °C). After that, the Biuret method was used to determine the protein concentrations of the supernatants and whole

solutions. Then, the protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{C_2}{C_1} \times 100$$

where C_2 represents the protein concentration of the supernatant, and C_1 represents the total protein concentration of the whole solution.

2.7. Reducing power

The reducing power of E-WPI samples was measured by using the method of Oyaizu (1986) with some modifications. Briefly, 0.5 mL of series concentrations of E-WPI sample solutions (5.0, 10.0, 15.0, 20.0, and 25.0 mg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1.0%, m/v) in 10.0 mL centrifuge tubes. After incubating at 50 °C for 20 min, 2.5 mL of 10.0% trichloroacetic acid (w/v) was added to each tube. After centrifuging for 15 min at 1000 g at room temperature (25 °C), each supernatant (2.5 mL) was mixed with 0.5 mL of ferric chloride (0.1%, w/v) and 2.5 mL of distilled water. Finally, after 10 min of incubation under room temperature (25 °C), the absorbance of each mixture was measured at 700 nm. The changes in absorbance were used as an indicator of reducing power.

2.8. ABTS radical scavenging activity

The ABTS radical scavenging activity of E-WPI samples was performed according to the procedure of Ma et al. (2018) with some modifications. Briefly, ABTS radicals were produced by mixing ABTS solution (7.0 mM) with potassium persulfate solution (2.45 mM) in equal volume and incubated at room temperature (25 °C) in a dark room for 12 h. Then, the above ABTS^{•+} solution was adjusted with 20.0 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.70 ± 0.02 at 734 nm. After that, 20.0 μL of series concentrations of E-WPI sample solutions (5.0, 10.0, 15.0, 20.0, and 25.0 mg/mL) were added to 3.0 mL of the diluted ABTS^{•+} solution and the mixtures were incubated in a dark room at room temperature (25 °C) for 6 min. Finally, the absorbance of the mixtures at 734 nm were recorded and the ABTS radical scavenging activity (%) was calculated as follows:

$$\text{ABTS radical scavenging activity (\%)} = \left(1 - \frac{A_s - A_i}{A_c}\right) \times 100$$

where A_s is the absorbance value of samples with ABTS^{•+} solution; A_i is the absorbance value of samples without ABTS^{•+} solution; A_c is the absorbance value of the ABTS radical solution without samples.

$$\text{EAAI} = \sqrt[9]{\frac{(\text{mg of lysine in 1 g of text protein}) \times (\text{etc. for the other 8 essential amino acids})}{(\text{mg of lysine in 1 g reference protein})}}$$

2.9. DPPH radical scavenging activity

The DPPH radical scavenging activity of E-WPI samples were measured according to the method described by Liu et al. (2010) with some modifications. Briefly, 2.5 mL of series concentrations of E-WPI sample solutions (5.0, 10.0, 15.0, 20.0, and 25.0 mg/mL) were mixed with 2.5 mL of DPPH radical solution (0.1 mM in absolute ethanol). After 30 min of incubation in a dark room at room temperature (25 °C), the absorbance of each mixture at 517 nm was measured. The DPPH radical scavenging activities (%) were calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_s - A_i}{A_c}\right) \times 100$$

where A_s is the absorbance value of samples with DPPH radical solution; A_i is the absorbance value of samples without DPPH radical solution; A_c is the absorbance value of the DPPH radical solution without samples.

2.10. Metal chelating activity

Metal (Cu^{2+} and Fe^{2+}) chelating activities of different E-WPI samples were determined according to the method of Kong and Xiong (2006). For Cu^{2+} -chelating assay, 1.0 mL of CuSO_4 solution (2.0 mM), 1.0 mL of pyridine solution (10.0%, v/v) and 20.0 μL of pyrocatechol violet solution (0.1%, w/v) were thoroughly mixed in a test tube. After added with 1.0 mL of each E-WPI sample solution with different concentrations (5.0, 10.0, 15.0, 20.0, and 25.0 mg/mL), the absorbance of each mixture was measured at 632 nm via a UV-vis spectrophotometer at room temperature (25 °C) with 1.0 cm light path length cuvettes. For Fe^{2+} -chelating assay, 1.0 mL FeCl_2 (20.0 μM) and 1.0 mL of ferrozine (0.5 mM) were thoroughly mixed in a test tube. After added with 0.5 mL of each E-WPI sample solution with different concentrations (5.0, 10.0, 15.0, 20.0, and 25.0 mg/mL), the absorbance of each mixture was measured at 562 nm via a UV-vis spectrophotometer at room temperature (25 °C) with 1.0 cm light path length cuvettes. The metal chelating activities (%) were calculated as follows:

$$\text{Metal chelating activity (\%)} = \left(1 - \frac{A_s - A_i}{A_c}\right) \times 100$$

Where A_s represents the absorbance of the E-WPI samples after reaction, A_i represents the absorbance of the intact WPI sample, A_c represents the absorbance of the reagent blank.

2.11. Oxygen radical absorption capacity (ORAC) assay

The ORAC of E-WPI samples were carried out according to our previous method of Feng et al. (2022).

2.12. Amino acid profiles

The amino acid compositions of different E-WPI were measured according to the method of Kim et al. (2020). The amino acid content was expressed as g/100 g product. Additionally, the essential amino acid index (EAAI) was used to evaluate the protein quality, which was calculated as follows:

The human essential amino acids were used as a reference protein (Rf.) for which to refer to (FAO/WHO/UNU, 1985).

2.13. Statistical analysis

Three independent batches of E-WPI (replicates) were prepared. For each batch of E-WPI, measurements of related traits were carried out in triplicate. All data were expressed as means \pm standard deviations (SD) using the General Linear Models procedure of the Statistix 8.1 software package (Analytical Software, St Paul, MN, USA). One-way analysis of variance (ANOVA) with Tukey's multiple comparison procedure was

Table 1
Changes in WPI secondary structure compositions after treatment with different concentrations of ethanol.

Samples	Contents of secondary structure (%)			
	α -helix	β -sheet	β -turns	Random coil
Native WPI	19.15 \pm 0.05 ^B	52.45 \pm 0.66 ^A	15.73 \pm 0.15 ^C	12.67 \pm 0.15 ^C
E-WPI-20	20.76 \pm 1.53 ^{AB}	43.84 \pm 0.11 ^B	16.45 \pm 0.28 ^{BC}	18.95 \pm 0.10 ^B
E-WPI-40	22.85 \pm 1.00 ^A	28.80 \pm 0.20 ^E	17.35 \pm 0.26 ^{BC}	22.00 \pm 0.03 ^{AB}
E-WPI-60	21.37 \pm 0.87 ^{AB}	35.50 \pm 0.25 ^D	18.33 \pm 0.45 ^B	24.80 \pm 0.83 ^A
E-WPI-80	15.23 \pm 0.21 ^C	40.97 \pm 0.04 ^C	22.10 \pm 0.01 ^A	21.70 \pm 0.07 ^{AB}

Values are given as means \pm SD from triplicate determinations; for each column letters ^{A–E} indicate significant differences between samples ($P < 0.05$).

performed to evaluate the significance of the main effects ($P < 0.05$).

3. Results and discussion

3.1. CD analysis

The changes in WPI secondary structure compositions induced by different ethanol concentrations are shown in Table 1. For native WPI, the relative content of α -helix, β -sheet, β -turn, and random coil were 19.15%, 52.45%, 15.73%, and 12.67%, respectively. Not surprisingly, the secondary structure composition of E-WPI samples altered significantly after treatment with ethanol at different concentrations (20–80%, v/v). Compared with native WPI, the α -helix, β -turn, and random coil structures of E-WPI samples increased considerably, while the β -sheet structures obviously decreased ($P < 0.05$). Moreover, E-WPI-40 achieved the highest amount of α -helix and the lowest content of β -sheet ($P < 0.05$). Shiraki et al. (1995) had pointed out that ethanol pre-treatment could unfold the protein structure and contributed to the formation of α -helical structure. Peng et al. (2020) and Nikolaidis et al. (2017) also indicated similar changes with soy β -conglycinin and β -lactoglobulin, respectively. However, as the level of ethanol exceeded 40% (v/v), the differences in α -helical or β -sheet content between native WPI and E-WPI were much smaller. A similar study was reported by Peng et al. (2020), who found that high ethanol concentration (80%, v/v) would cause serious aggregation of soybean β -conglycinin, leading to the

formation of more compact structures. In conclusion, ethanol treatment might promote the conversion of the β -sheet component of WPI into α -helical structures, and Molla and Mandal (2013) suggested that ethanol-induced α -helical formation was bound up with the formation of intra-molecular hydrogen bonds.

3.2. Total and free SH contents

The total and free SH content of different WPI were closely related to the degree of unfolding or aggregation of proteins. As shown in Fig. 1 (A), for the native WPI, the amount of total SH was 34.04 μM /g of protein, while the content of total SH in E-WPI treated with ethanol at 20%, 40%, 60% and 80% (v/v) significantly decreased by 13.10, 34.20, 37.87 and 46.86% ($P < 0.05$), respectively. A possible explanation for the decrease of total SH groups is that as the level of ethanol increased, ethanol-induced protein unfolding or aggregation might enhance the reactions of intra- or inter-molecular SH/SS interchange or SH oxidation (Segat et al., 2014), leading to the formation of disulfide bonds. The formation of disulfide covalent polymers has been illustrated by the SDS-PAGE results in the study of Feng et al. (2021). In addition, it can be seen from Fig. 1 (A) that the amount of free SH in E-WPI samples showed a trend of first increasing and then decreasing, reaching a maximum in E-WPI-40. The protein structure gradually unfolds when the level of ethanol was less than 40% (v/v), exposing more content of free SH. At this time, the rate of disulfide bond formation might be lower

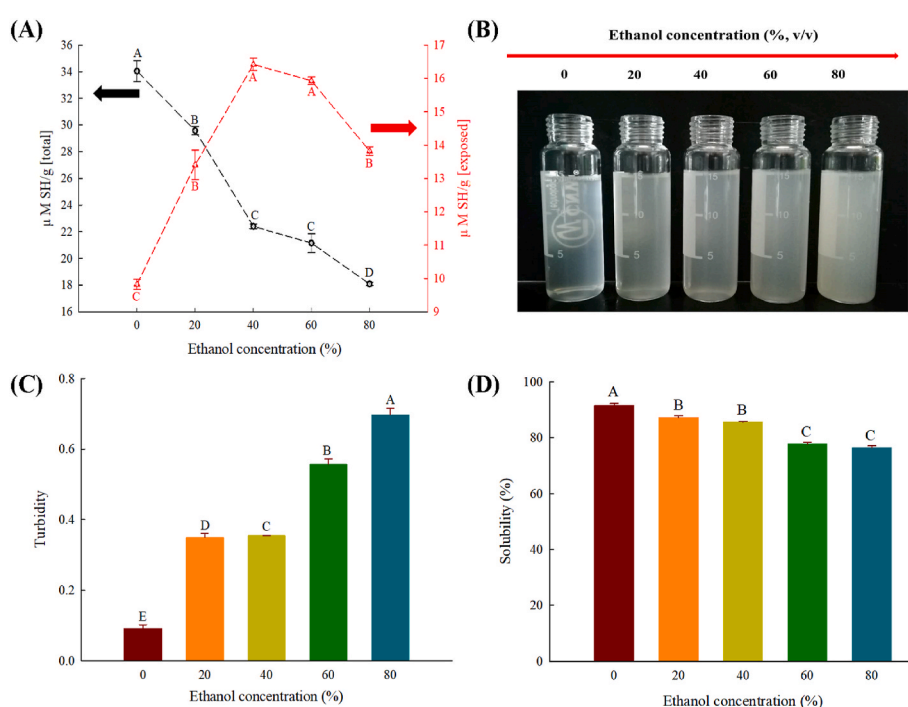


Fig. 1. Total and exposed sulfhydryl (SH) contents (A), visual appearance of solutions (20 mg/mL) (B), turbidity (C) and solubility (D) of WPI samples after treatment with different concentrations of ethanol. The different uppercase letters (A–E) indicate significant differences between samples ($P < 0.05$).

than the rate of sulfhydryl exposure, thus the free sulfhydryl groups increased significantly ($P < 0.05$). However, as the level of ethanol increased to 60 or 80% (v/v), the content of free SH decreased, which may be attributed to the reorganization of the protein and some free SH groups being buried. Meanwhile, according to the study of Lambrecht et al. (2016), they pointed out that ethanol-induced protein structure unfolding was conducive to the formation of disulfide bonds, and the SH-SS exchange reaction rates increased as ethanol concentration increased. Moreover, disulfide bonds could be formed between the interaction of free SH groups and cysteine (Cys) residues of whey proteins, which led to the decrease of free SH. Furthermore, another possible explanation for the decrease in free sulfhydryl groups could be the change in the pH environment. Casal et al. (1988) found that the pH of aqueous-ethanol solutions could exceed 7.5 as the ethanol level is higher than 55%. The formation of disulfide bonds via SH oxidation and SH/SS interchange reactions could be accelerated in an alkaline environment (Chen et al., 2019).

3.3. Turbidity

The turbidity of protein dispersions is positively correlated with aggregation behavior after treatments or processing operations. In our present study, the visual appearance and turbidity of different E-WPI solutions (20.0 mg/mL) is shown in Fig. 1 (B) and 1 (C), respectively. As shown, with an increase in the level of ethanol from 0 to 80% (v/v), the sample solutions were more turbid and the turbidity of E-WPI solutions increased significantly ($P < 0.05$). This suggests that ethanol-induced aggregation of WPI is ethanol concentration-dependent. Turbidity reached its maximum (from 0.096 to 0.687) when the level of ethanol increased to 80% (v/v). This was likely attributed to severe aggregation and the increase in the aggregate quantity under high ethanol concentrations, where larger sized particles could scatter more light, thus causing more solution opacity. Moreover, Ye et al. (2019) observed the same change in appearance for WPI suspensions upon desolvation at different ethanol concentrations (0–50%, v/v). Yoshikawa et al. (2012) also found that a solution of BSA and SH-modified BSA showed turbidity under treatment with 60–70% aqueous ethanol solution due to protein aggregates. Similarly, an increased turbidity of soy β -conglycinin solutions after ethanol treatment had been reported in a study by Peng et al. (2020), which stated that the solution even appeared milky when the ethanol concentration was 80% (v/v). Liu et al. (2019) indicated that ethanol treatment disrupted the hydrophobic regions of the protein, and the protein aggregates formed by molecular covalent and noncovalent interactions (such as disulfide bonds and hydrophobic interactions) could cause increased protein solution turbidity.

3.4. Solubility

As a functional index of protein, solubility can effectively measure protein denaturation and aggregation. The solubility of different E-WPI is exhibited in Fig. 1 (D). The solubility of E-WPI samples were obviously lower than that of native WPI ($P < 0.05$), and the decrease was more significant as ethanol concentration increased, especially at the ethanol concentration of 80% (v/v) (from 91.55 to 76.47%). These changes in protein solubility of different E-WPI were consistent with the results of the turbidity analysis, further confirming that protein aggregation caused by decreased solubility led to increased turbidity. These results are similar with that of Peng et al. (2020), who observed that the solubility of ethanol treated soy β -conglycinin decreased obviously ($P < 0.05$) as the level of ethanol increased from 60 to 80% (v/v). Meanwhile, an ethanol-induced decrease in protein solubility also occurred with lysozyme, the main reason for reduced solubility is the low dielectric constant of ethanol, and it has been proposed that the adverse interaction between polar groups of the protein and the organic solvent coaxed the protein to precipitate or crystallize (Yoshikawa et al., 2012). Moreover, Feng et al. (2021) indicated that ethanol-induced protein

unfolding or aggregation could lead to more hydrophobic side chains being exposed thereby increasing surface hydrophobicity of WPI, decreased protein solubility was closely related to increased surface hydrophobicity. Furthermore, ethanol-induced α -helical structures would mask the polar amide groups of WPI, which might also reduce the solubility of the protein (Shiraki et al., 1995). However, in a report by Chang et al. (2019), the reduced solubility of lentil protein isolate caused by ethanol treatment (35–75%, v/v) was more obvious under low ethanol concentration conditions. The difference between this change and ours was mainly due to the difference in protein composition and the initial degree of denaturation or aggregation. Additionally, although the solubility of a protein is closely related to its usage in the industry, it should be noted that the solubility of protein is a result of its balanced surface hydrophobicity and hydrophilicity, which could further promote the functional properties of the protein itself (Tang, 2017). In the study of Peng et al. (2020), they indicated that ethanol-treated soy β -conglycinin with decreased solubility could be effectively applied to prepare high internal phase emulsions with excellent stabilities. Our previous work also indicated that compared with native WPI, E-WPI prepared with an intermediate ethanol concentration (40%, v/v) showed the highest emulsifying activity, emulsifying stability, foaming capacity, and surface hydrophobicity, which implied that ethanol-treated WPI had their unique advantages in food industry, except for its decreased solubility (Feng et al., 2021).

3.5. Reducing power

Reducing power can effectively evaluate the ability of proteins to donate electrons or protons, and there is a positive correlation between reducing power and the global antioxidant capacity of antioxidants (Cho, 2020). As shown in Fig. 2 (A), the absorbance of each E-WPI sample at 700 nm increased as protein concentration increased, and each E-WPI showed obviously higher absorbance than native WPI ($P < 0.05$). This indicates that E-WPI had a higher antioxidant activity. For example, when the protein concentration was 20.0 mg/mL, the reducing power of native WPI was 0.088, while the reducing power of E-WPI increased to 0.108, 0.133, 0.129, and 0.119 with increasing ethanol concentration (20–80%, v/v), respectively. Again, the highest value was shown at an intermediate ethanol concentration (40%, v/v). According to the results, the effect of ethanol treatment on the reducing power of WPI might be related to changes in protein structure and conformation. Proper ethanol pretreatment caused protein unfolding or some degree of reorganization, leading to exposure of histidine (His), methionine (Met) and glycine (Gly). These amino acids are usually acted as antioxidants that reduce Fe^{3+} to Fe^{2+} and exhibit powerful antioxidant activities (Marcuse, 1962). However, high ethanol concentration induced serious aggregation that was not conducive toward allowing these functional amino acids to exert their reducing effects.

3.6. ABTS radical scavenging activity

The ABTS assay is a common method for evaluating the antioxidant ability of proteins. As shown in Fig. 2 (B), the ABTS radical scavenging activity of all samples were obviously enhanced ($P < 0.05$) as the protein concentrations increasing from 5.0 to 25.0 mg/mL. Compared with native WPI, the ABTS radical scavenging activity of E-WPI-20 increased, but not obviously ($P > 0.05$). However, as the level of ethanol increased to 40, 60 or 80% (v/v), the increase in radical scavenging activity became extremely significant ($P < 0.05$, except for E-WPI-80 at the protein concentration of 5.0 mg/mL). Moreover, as ethanol concentration increased, the scavenging activity for ABTS free radicals of WPI first increased and then decreased, reaching maximums at 40% (v/v) ethanol concentrations. According to the study of Alizadeh and Aliakbarlu (2020), the increased radical scavenging activity was a consequence of WPI molecular unfolding after ethanol treatment, and the unfolding of globular proteins was frequently accompanied by an increase in surface

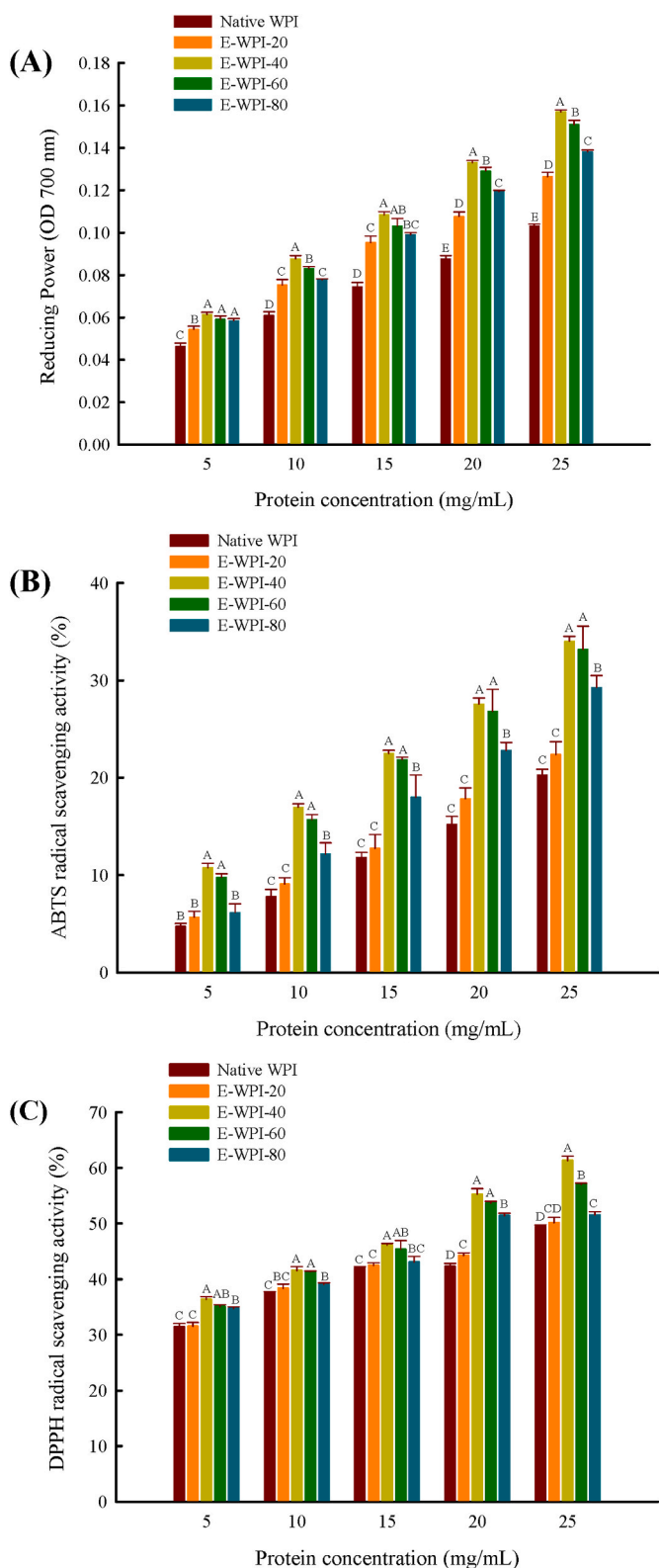


Fig. 2. Reducing power (A), ABTS radical scavenging activities (B) and DPPH radical scavenging activities (C) of WPI after treatment with different concentrations of ethanol. The different uppercase letters (A–E) indicate significant differences between samples for each protein concentration ($P < 0.05$).

hydrophobicity. Arranz et al. (2019) indicated that the antioxidant ability of whey protein was closely related to its hydrophobicity and aromatic amino acids, which could provide protons to stabilize electron-deficient free radicals. Furthermore, our radical scavenging activity results are consistent with our reducing power results and reaffirm that the antioxidant ability of WPI was enhanced after treatment with ethanol.

3.7. DPPH radical scavenging activity

DPPH is a stable free radical showing maximum absorbance at 517 nm in ethanol and is usually scavenged when encountering a proton-donating substance (H^+), thus the scavenging ability of DPPH free radicals can indicate the antioxidant activity of proteins. In our present investigation, the DPPH radicals were reduced to yellow compounds after adding different WPI samples, which suggests that DPPH radicals were scavenged. As shown in Fig. 2 (C), it is similar with the trends of ABTS free radical scavenging activity, the DPPH radical scavenging activity of E-WPI were higher than that of Native WPI. For example, under the protein concentration of 20.0 mg/mL, as the level of ethanol increased from 0 to 20, 40, 60 and 80% (v/v), the DPPH scavenging ability of samples increased from 42.32 to 44.26, 55.26, 53.67, and 51.38%, respectively. The native WPI had the lowest antioxidant activity due to its compact structure, while ethanol treatment increased the DPPH radical scavenging ability of WPI due to the change of the protein structure with the destruction of the hydrophobic core of the protein and the exposure of active amino acid residues that could react with oxidants (Li et al., 2019). Additionally, it was speculated that the increase of DPPH radical scavenging activity of WPI might also in connection with the increase in sulfhydryl groups (Arranz et al., 2019; Alizadeh and Aliakbarlu, 2020), especially for the E-WPI-40. However, the antioxidant capacity of protein was closely related to its degree of denaturation or aggregation (Arzeni et al., 2012). WPI treated with high ethanol concentration (80%, v/v) resulted in serious aggregation, which might lead to the decrease in DPPH radical scavenging activity compared with the optimal concentration (40% in our study).

3.8. Metal-chelating activity

Transition metal ions (such as Cu^{2+} and Fe^{2+}) might be acted as catalysts that promoted the generation of free radicals, which subsequently prone to initiate the oxidative chain reactions (Liu et al., 2010). Proteins could act as chelating agents and effectively decrease the availability of transition metals and then retard the oxidative chain reactions in biological or food systems (Kong and Xiong, 2006). Therefore, the determination of metal-chelating activity is also a common method to evaluate the antioxidant activities of samples. As shown in Fig. 3 (A), the Cu^{2+} -chelating activity of each WPI sample increased as protein concentration increased. Compared with native WPI, the Cu^{2+} -chelating activity improved with the increasing level of ethanol, and the E-WPI-40 exhibited the highest Cu^{2+} -chelating activities ($P < 0.05$). However, the intensity of this change decreased as the level of ethanol increase above 40% (v/v). Similar changes were obtained in Fe^{2+} -chelating activity (Fig. 3B), for example, when the protein concentration was 20.0 mg/mL, the Fe^{2+} -chelating activity of native WPI was 23.87%, as the level of ethanol increasing (20–80%, v/v), the Fe^{2+} -chelating activity of E-WPI increased to 24.83, 27.44, 26.61, and 25.65%, respectively. These results might be attributed to the idea that protein treated with ethanol solutions might dissociate, causing previously hidden carboxyl groups to be exposed (Nikolaidis et al., 2017), and the increased concentration of carboxylic groups led to prooxidative free metal ions being removed (Liu et al., 2010). In addition, the ethanol-induced protein unfolding might lead to more exposure of certain metal-binding amino acids (such as His) (Kong and Xiong, 2006), thus enhancing the metal chelating activity of E-WPI.

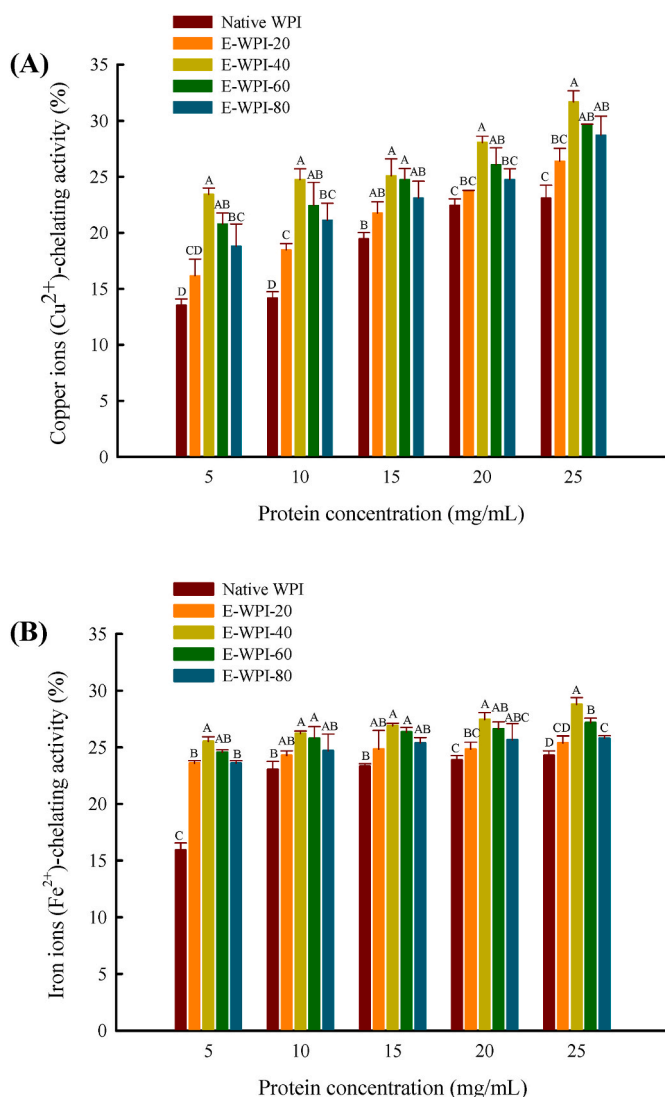


Fig. 3. Cu²⁺-chelating activity (A) and Fe²⁺-chelating activity (B) of WPI after treatment with different concentrations of ethanol. The different uppercase letters (A–D) indicate significant differences between samples for each protein concentration ($P < 0.05$).

3.9. ORAC assay

The ORAC assay is an effective method to measure the total antioxidant capacity of proteins, which used AAPH as the initiator of free radicals, combining the inhibition time and the degree of inhibition to study the ability of antioxidants to prevent free radical chain reactions from scavenging ROO• (Cho, 2020). In our present study, the fluorescence decay curves of different samples are shown in Fig. 4A. Firstly, for comparison, the fluorescence intensity of the natural fluorescence decay group (-AAPH) had little change during the measurement period, while the fluorescence intensity of the free radical action group decayed rapidly after adding the strong oxidant AAPH. Moreover, the E-WPI-20 and E-WPI-40 had higher fluorescence intensity than native WPI, and the quenching time was prolonged effectively. Meanwhile, combined with the ORAC values of each WPI sample (Fig. 4B), the total antioxidant capacities of E-WPI-20 and E-WPI-40 were obviously improved ($P < 0.05$). Not surprisingly, the maximum ORAC value was acquired when the level of ethanol was 40% (v/v) (from 15.34 to 22.14 μmol TE/g). These results may be due to the conformational changes of protein, leading to more exposure of amino acids with electronic donation activities to ROO•, thus the damage to the fluorescent probe by free

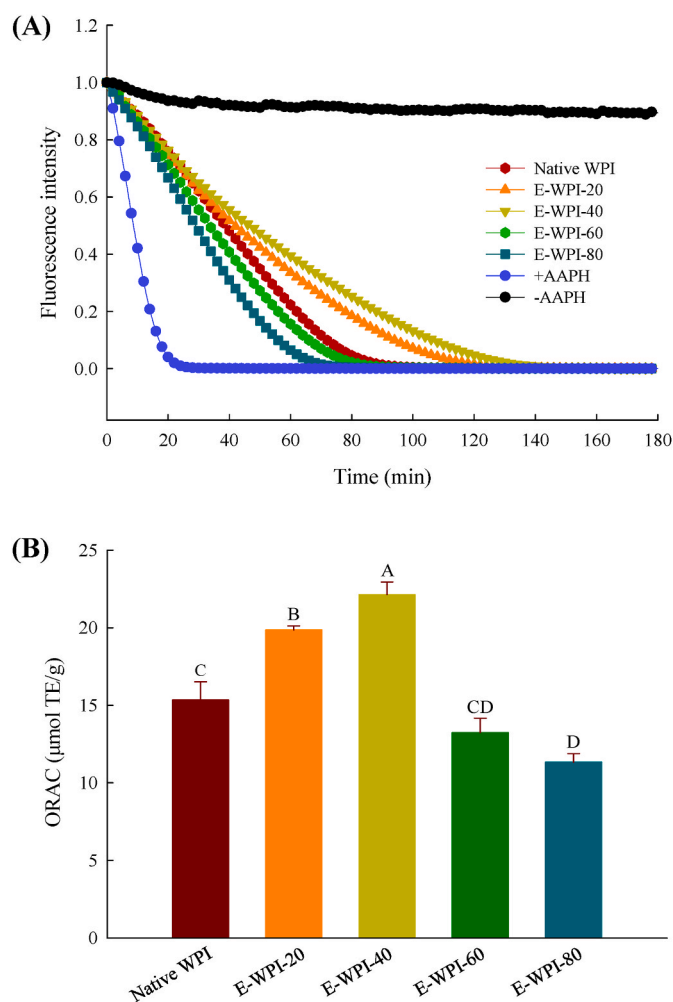


Fig. 4. Attenuation curves of fluorescence inhibition (A) and oxygen radical absorbance capacity (ORAC) (B) of WPI after treatment with different concentrations of ethanol. The different uppercase letters (A–D) indicate significant differences between samples ($P < 0.05$).

radicals would be inhibited (Dong et al., 2021). As the level of ethanol exceeded 40% (v/v), the fluorescence intensity of E-WPI decreased and the decay rate increased. Consistent with these results, the ORAC value of E-WPI also reduced significantly, especially for the E-WPI-80 ($P < 0.05$).

3.10. Amino acid composition and protein quality

The antioxidant activity of proteins is strongly linked to its composition and quantity of amino acids. As shown in Table 2, with an increase in the level of ethanol from 0 to 80% (v/v), the sum of total amino acids of E-WPI increased from 73.31 to 73.78, 75.89, 74.22, and 74.07g/100g protein, respectively. This result indicated that ethanol pretreatment could change the composition and content of amino acids in WPI. Khrustalev et al. (2017) found that the ethanol preferred to bind N-terminal parts of alpha helices of amino acid residues, which promote the formation of both hydrogen bonds and hydrophobic interaction force. Specifically, ethanol pretreatment could obviously increase the exposure of aromatic amino acids and sulphur-containing amino acids of WPI (such as Cys, Met, tyrosine and phenylalanine). Kerasiotti et al. (2014) indicated that these amino acids were contributory to the antioxidant ability of proteins. Moreover, the content of hydrophobic amino acids (such as alanine, Phe, isoleucine, Gly, leucine, valine, proline and Met) in E-WPI samples also increased after ethanol treatment. The high

Table 2

Amino acid profiles and protein quality of WPI after treatment with different concentrations of ethanol.

Unit (g/100 g protein)	Native WPI	E-WPI-20	E-WPI-40	E-WPI-60	E-WPI-80	Ref.
Essential amino acids (EAA)						
Histidine (His)	1.00	0.97	1.03	0.99	1.00	1.5
Isoleucine (Ile)	4.59	4.61	4.73	4.63	4.64	3.0
Leucine (Leu)	8.46	8.51	8.76	8.55	8.55	5.9
Lysine (Lys)	7.13	7.21	7.43	7.25	7.22	4.5
Methionine + Cysteine (Met + Cys)	3.31	3.33	3.48	3.36	3.39	2.2
Phenyl-alanine + Tyrosine (Phe + Tyr)	3.93	3.98	4.11	3.99	3.98	3.8
Threonine (Thr)	5.13	5.17	5.30	5.20	5.18	2.3
Valine (Val)	4.13	4.15	4.25	4.17	4.17	3.9
Sum of EAA	37.68	37.93	39.09	38.14	38.13	27.1
Alanine (Ala)	4.19	4.21	4.36	4.26	4.23	
Arginine (Arg)	1.49	1.50	1.55	1.54	1.50	
Aspartic acid (Asp)	7.45	7.52	7.73	7.57	7.54	
Glutamic acid (Glu)	13.58	13.68	14.00	13.74	13.72	
Glycine (Gly)	1.00	1.00	1.05	1.01	1.00	
Proline (Pro)	4.59	4.56	4.66	4.57	4.56	
Serine (Ser)	3.33	3.38	3.45	3.39	3.39	
Sum of hydrophobic amino acids	30.64	30.79	31.66	30.92	30.88	
Sum of total AA	73.31	73.78	75.89	74.22	74.07	
EAAI	1.98	1.99	2.00	1.99	1.99	

Reference from [FAO/WHO/UNU \(1985\)](#).

content and hydrophobicity of these amino acids might contribute to the high antioxidant ability of the E-WPI ([Liu et al., 2011](#)). Furthermore, the following amino acids in E-WPI, such as His and aspartate were also more than that of native WPI, and these amino acids have been shown to have antioxidant ability ([Kong and Xiong, 2006](#); [Muley et al., 2021](#)). Interestingly, the contents of the amino acids mentioned above reached the highest in E-WPI-40. However, high level of ethanol (60 or 80%, v/v) may induce severe protein aggregation, leading to the exposed amino acid residues to be masked again, thereby reducing the antioxidant activity of E-WPI.

Additionally, the protein quality is usually judged by the composition of amino acids, and the essential amino acid index (EAAI) can reflect the quality of protein. As shown in [Table 2](#), all E-WPI samples not only contained all the essential amino acids needed by humans ([FAO/WHO/United Nations University, 1985](#)), but also sum of essential amino acids (EAA) in E-WPI samples was higher than that of human necessity (27.1g/100g crude protein). It was observed that the EAAI of all E-WPI samples was higher than native WPI, the E-WPI-40 had the highest EAAI, which further indicates that the protein quality of the E-WPI-40 was the best.

4. Conclusions

In summary, through ethanol-induced molecular unfolding and the following aggregation via disulfides and intra-molecular hydrogen bonds, a remarkable improvement in antioxidant activity of E-WPI was shown. As the level of ethanol increasing (20–80%, v/v), significant structural changes were observed and manifested within the results of CD spectra, total and free SH contents, turbidity and solubility. However, E-WPI-40 had the highest antioxidant activities, which was further verified by the results of amino acid profiles. In general, comprehensively considering the findings of our previous ([Feng et al., 2021](#)) and present work, E-WPI could be used as a potential functional ingredient both with remarkable emulsifying characters and antioxidative activities. In the future, in an effort to promote the physical and oxidative

stabilities of oil-in-water emulsions, the aforementioned E-WPI will be systematically investigated.

CRedit authorship contribution statement

Yangyang Feng: Methodology, Investigation, Writing – original draft. **Dongxue Yuan:** Software, Investigation, Validation. **Baohua Kong:** Data curation, Formal analysis. **Fangda Sun:** Visualization, Resources. **Meijuan Wang:** Investigation, Formal analysis. **Hui Wang:** Conceptualization, Investigation, Data curation. **Qian Liu:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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.

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