Introduction

*Pleurotus eryngii* is an edible and medicinal mushroom, which is a tetra-polar basidiomycete belonging to the *Pleurotus* sp.\(^1\) It has been widely used as an admired functional and medicinal food in China, particularly owing to its features such as unique stipe, pleasant aroma, and good culinary qualities. It also contains countless bio-active substances like protein, polysaccharides (e.g., α- and β-glucan), peptide, and dietary fiber, among others,\(^2\) and it may possess potential bio-active functional and nutritional properties such as anti-proliferate, antioxidant, and anti-tumor activities,\(^3\) thus leading to higher production of *P. eryngii* quickly and majorly in Asia, Europe, and America.\(^4\) In recent years, the domestic and foreign investigators of the *P. eryngii* have focused primarily on the biological properties and bio-active substances,\(^5\) such as polysaccharides (e.g., β-glucan), with its potential
immune regulation\textsuperscript{[6]} and anti-tumor\textsuperscript{[7]} and antioxidant activities,\textsuperscript{[8]} and sterols (e.g., estrogen-like) with its protection against the bone loss, among others.\textsuperscript{[9]} Protein serves as one of the significant food components and provides more functional and nutritional value. According to the previous researches, different kinds of protein in the stomach and intestinal digestion might present different digestive properties.\textsuperscript{[10]} However, there are only a few reports about the physicochemical and digestive properties of \textit{P. eryngii} protein and its digestive products and antioxidant properties.

Generally speaking, the physicochemical properties include rheological properties, surface hydrophobicity ($\text{H}_0$), secondary structure, and thermal stability. The rheological property plays an important role in processing and maintaining the texture stability of food, which is reflected by the apparent viscosity in general. And it affects the sensory quality and functionality of resultant products. Testing for rheological property can provide countless valuable information for food composition, internal structure, and molecular morphology, such as elastic or storage modulus ($G'$), viscous or loss modulus ($G''$), and phase angle ($\tan \alpha$). $\text{H}_0$ and secondary structure testing can offer some important information about water-holding capacity and breaking force of protein. The previous studies reported that the fish protein, extracted by using alkaline method, exhibits strong breaking force and low deformation.\textsuperscript{[11]} pH value is a key factor in the processing of protein as it has a significant effect on rheological properties.\textsuperscript{[12]} Salts dominant in manufacturing gels and its concentration can enhance the polymerization process by promoting the cross-linkage or electrostatic interactions in the protein system.\textsuperscript{[13]} Protein after stimulating in stomach and intestinal manifests different antioxidant activity. However, there are few reports about the effect of heat treatment and ultrasound treatment on physicochemical and digestive properties of \textit{P. eryngii} protein.

Heat treatment has extensive application in the food processing industry to promote food functional properties.\textsuperscript{[14]} It also reported that food through pre-heat treatment could decrease enzyme activity and enhance food sensory characterization like flavor, texture, and color properties.\textsuperscript{[15]} Protein treated by heat treatment before enzymatic hydrolysis can rearrange the inter-linkage between protein molecules, particularly hydrophobic bonds, thus resulting in changes of protein structure and digestibility properties.\textsuperscript{[16]} The ultrasound technology serves as an important non-thermal physical treating technology, which features many applications in food processing.\textsuperscript{[17]} More importantly, the ultrasound technology enables to bring about acoustic cavitation, which results from the mechanical interaction with strong shear force by bubbles burst instantly in liquid.\textsuperscript{[18]} The ultrasound technology was accepted to promote the protein functional properties at large and to generate bio-active peptides. It is also reported that the ultrasound treatment can increase the rate of protein hydrolysis with enzyme and the bio-activity of hydrolysis products dramatically.\textsuperscript{[19]} The ultrasound treatment can change protein secondary structure as well as protein physical and chemical properties, thus bringing more protease restriction sites.\textsuperscript{[20]} It is reported that whey protein treated by heat and ultrasound could improve its functional properties.\textsuperscript{[21]} But few systematic researches have concentrated on the effects of heat treatment and ultrasound treatment on physicochemical properties like surface hydrophobicity, sulphydryl content, and secondary structure.

The relationship between physicochemical and digestive properties of \textit{P. eryngii} protein is not fully explored. Therefore, the present project aimed to investigate the physicochemical and digestive properties of \textit{P. eryngii} protein and antioxidant activity of digestive products. The mechanism was researched in terms of the changes in protein physicochemical properties, digestive products antioxidant of \textit{P. eryngii} protein.

**Materials and methods**

**Materials**

\textit{P. eryngii} was kindly provided by the Edible Fungus Engineering Technology Center (Taigu, China) and processed into 150 particles for later use. Sigma-BSA (A3912-10 g), Sigma-lipase (5 g), Sigma-mucin (100 g), and Sigma-bile (25 g) were purchased from Shanghai North Connaught Biological Technology Co., Ltd.
**Preparation of *P. eryngii* protein**

Protein was extracted by combining cellulose enzymatic hydrolysis and acid precipitation after alkaline extraction from *P. eryngii* and concentrated *P. eryngii* powder and distilled water according to a certain liquid to material ratio of 1:55, and 1.5% cellulase was added (based on the raw material dry powder), and then the solution was placed in water bath at a constant temperature of 40°C for 100 min, and then the solution was inactivated at 80°C for 10 min. Next, the pH was adjusted to 12, and the reaction was carried out at 55°C for 2.5 h after cooling. After centrifugation at 4,000 r/min for 15 min, the supernatant was collected. The pH of the supernatant was adjusted to the isoelectric point of *P. eryngii* (3.6) with 0.1 mol/L HCl. After centrifugation at 4,000 r/min for 10 min, the protein was precipitated and dissolved with distilled water. The NaOH of mol/L was adjusted to pH 7.0 to obtain the *P. eryngii* protein solution. Then, the protein solution was dealt with liquid nitrogen and vacuum freeze-dried to obtain *P. eryngii* protein power.\(^{[22]}\)

**Physicochemical properties of *P. eryngii* protein and the effect of treatments on protein**

Rheological property: Rheological property of the protein was measured by using a DHR-3 rheometer (TA Instruments, New Castle, DE). A total of 25 mL prepared protein solution was poured into the cylinder tube, and paraffin oil was added to the sample solution surface to prevent volatilization of water. Each sample solution was subjected to a four-part test: (1) Shear rate-viscosity test. Parameter settings: the shear rate range was 0.1–200 s\(^{-1}\), and under the shear rate of 200 s\(^{-1}\) for 1 min, it was restarted from 200 to 0.1 s\(^{-1}\). Subsequently, the temperature increased from 25°C to 45°C at 5°C/min. (2) Angular frequency-viscosity test. Parameter settings: the angular frequency range was 0.5–100 s\(^{-1}\), and the controlled strain was changed to 0.5%. The automatic stress was selected, and the initial stress was set at 0.8 (Pa s). (3) Temperature scanning test. Parameter settings: the angular frequency was controlled at 10 rad/s, and the controlled strain was changed to 0.5%. The temperature program was set at the temperature range from 25°C to 65°C at 1°C/min, under 65°C for 1 min, and the temperature was restarted from 65°C to 25°C at 1°C/min. G\(^{'}\), G\(^{''}\), and tan\(\alpha\) were measured. The flow behavior of protein was manifested with the Power Law model:

\[
\tau = Kr^n
\]

where \(\tau\) is the shear stress (Pa), \(K\) is consistency coefficient (Pa sn), \(r\) is the shear rate (s\(^{-1}\)), and \(n\) is the flow behavior index.

Surface hydrophobicity (H\(_0\)): According to the method of Wang et al. with minor changes, the surface hydrophobicity (H\(_0\)) of protein was measured adopting ANS (8-Aniline-1-Naphthalenesulfonic acid) with the hydrophobic fluorescence probe.\(^{[23]}\) The different protein samples were dissolved in 0.02 M phosphate buffer (PBS, pH 7.0) and centrifuged at 8,000 g for 15 min at 4°C and collected the supernatant. The protein solution was prepared with PBS to obtain the final concentrations of 0.1, 0.2, and 0.3 M. Each protein sample (2.5 mL) added was mixed well with 8 mM ANS 12.5 \(\mu\)L at 25 ± 0.5°C. Absorption intensity was measured at excitation of 390 nm and emission of 470 nm using a multifunctional enzyme-labeled instrument (Spectra MX I3X). The protein sample intrinsic emission fluorescence spectrum was determined by using a multifunctional enzyme-labeled instrument (Spectra MX I3X) according to Liu et al. with some changes.\(^{[24]}\) Protein sample solutions with concentration of 0.02 mg/mL were prepared in 0.02 mol/L PBS (pH 7.0). Subsequently, the protein sample solutions were analyzed at excitation spectra of 280 nm and emission spectra from 300 to 650 nm at a constant slit of 5 nm for all samples analyzed. The initial slope of the relative fluorescence intensity versus protein concentration (mg/mL) plot was analyzed by linear regression analysis and used as an index of H\(_0\).

Sulfhydryl content and the disulfide bond contents: The R-SH of the protein solution was determined as described in Omama et al.\(^{[25]}\) and Zhou et al.\(^{[26]}\) Protein solution (2.5 mL) and EDTA adding (7 mM) in 25 mL of tris-glycine buffer (0.1 mol/L pH 8.0); then, the solution was...
mixed well using the Kinematica (FT-2500E). Sample solution (1mL) adding 4 mL tris-glycine buffer and 100 μL Ellman’s reagent (10 mM DTNB) mixing well at a room temperature (25°C ± 1°C) for 20 min to measure the R-SH content at 412 nm adopting a multifunctional enzyme-labeled instrument (Spectra MX I3X). The solutions without Ellman’s reagent were set as a reference, and the R-SH content was calculated using the following equation. In total, the sample solution (1 mL), 8 M urea (4 mL) and Ellman’s reagent (100 μL) were added. Afterward, the resulting solution was mixed well at 4°C for 2 h to measure the T-SH content. The solutions were measured at 412 nm by using a multifunctional enzyme-labeled instrument.

$$SH = \frac{A_{412} \times D}{C \times V \times \varepsilon} \times 10^{-6}$$

where $A_{412}$ is the absorbance value, $D$ is the dilution factor, $C$ is the protein concentration of the sample, $V$ is the assimilating protein solution volume, and $\varepsilon$ is the extinction coefficient (13,600/M/cm).

Secondary structure: Fourier-transform infrared (FT-IR) spectroscopy absorption spectrum of the protein sample from 4,000 to 400 cm$^{-1}$ was obtained using an FT-IR technology. The protein sample was produced by pressing in KBr windows (2 mg protein to 200 mg KBr) on a Carver press 4T pressure. The amide I bond in the region of 1,600–1,700 cm$^{-1}$ was connected with stretching vibration of C=O bond and C-N of peptide bond. Secondary structural components of the protein were analyzed by the second-derivative analysis of the IR-SD after dealing with the linear baseline correction, the Fourier self-deconvolution, and the deconvoluted spectrum.

Thermal property: The thermal property of P. eryngii protein was measured adopting differential scanning calorimetry (DSC) (Lab Sys evo) according to the method of Zheng et al. with minor modifications. The protein sample was weighed in Al$_2$O$_3$ crucible and heated from 20°C to 500°C at 10°C/min, and an empty Al$_2$O$_3$ crucible was viewed as a reference. The peak temperature and total calorimetric enthalpy were calculated adopting the DSC data analysis software.

Effect of heat treatment and ultrasound treatment on physicochemical properties of protein: The protein was treated by heat at 75°C for 30 min and by ultrasound at 200 W for 30 min and then research the effect of heat treatment and ultrasound treatment on surface hydrophobicity, sulfhydryl content and the disulfide bond content, secondary structure, and thermal property.

**Digestive properties of P. eryngii protein and the effect of treatments on protein**

*In vitro* digestive procedure: The *in vitro* digestive model presents a three-step processing including the mouth, stomach, and small intestine according to Versantvoort et al. Digestion juices were prepared defiantly following Table 1, and the *in vitro* digestive processing is described in Figure 1. Before each experiment, the digestive juice is incubated at 37°C for 15 min. The *in vitro* digestive is stimulated after adding saliva to the protein solution. Subsequently, gastric juices and intestinal fluids are mixed well in order to initiate the *in vitro* digestive processing in the stomach and intestine. Admittedly, the supernatant was collected after centrifugation so as to analyze digestive properties.

*In vitro* digestibility of the protein: The VDP was recorded using the method of the trichloroacetic acid (TCA-precipitation). The protein content was measured by using Coomassie brilliant blue method. The *in vitro* digestibility of protein was defined as follows: $VDP = (C_0 - C_1)/C_0 \times 100\%$, where $C_0$ is referred to the TCA-precipitation protein content before the digestion (mg) and $C_1$ is referred to the TCA-precipitation protein content after the digestion (mg).

Peptide contents of digestive juices: The peptides of protein after digesting in stomach and intestine were measured using the method of Lowery. The digestive solution 4 mL was collected and added to an equal volume of 10 g/L TCA, and then, the solution was centrifuged at 6,000 r/min for 15 min. The content of peptides in the supernatant was determined using the Lowry method.

Digestive juices DH: According to the method of ortho-phthaldialdehyde (OPA) by Mulcahy et al. with some changes, DH were determined by using the OPA method.
products were diluted to 100 times with ultrapure water. Subsequently, the reaction solutions were measured at 340 nm using a multifunctional enzyme-labeled instrument (Spectra MX I3X). In order to obtain the content of AAG of protein according to the reference to 1-leucine standard curve (concentration range from 0.2 to 1.2 mM).

The digestive solution (2 mL) was transferred into a 10-mL volumetric flask and then re-added distilled water. The above solution was leached via 0.45 μm syringe filter and then transferred into a preparing injection bottle. At the same time, the standard sample was prepared as 5 mg/mL. The

Table 1. Composition of digestive juices (500 mL) used for in vitro incubation of protein sample.

| Mouth | Stomach | Duodenum |
|-------|---------|----------|
| Saliva | Gastric juice | Duodenal juice | Bile |
| KCl | 0.45g | NaCl | 1.375 g | NaCl | 0.35 g | NaCl | 2.63 g |
| KSCN | 0.10 | NaH₂PO₄ | 0.135 g | NaHCO₃ | 1.70 g | NaHCO₃ | 2.38 g |
| NaH₂PO₄ | 0.45 | KCl | 0.410 g | KCl | 0.28 g | KCl | 0.18 g |
| NaSO₄ | 0.275 | NH₄Cl | 0.155 g | MgCl₂ | 0.025 g | 37% HCl | 0.075 mL |
| NaCl | 0.15 | CaCl₂·2 H₂O | 0.2 g | MgCl₂ | 0.025 g | 37% HCl | 0.075 mL |
| NaHCO₃ | 0.845 | 37% HCl | 0.650 mL | 37% HCl | 0.09 mL | BSA | 0.90 g |
| Urea | 0.20 | Urea | 0.045 g | Urea | 0.05 g | Bile | 10 g |
| Uric acid | 5.75 mg | Glucuronic acid | 0.01 g | BSA | 0.50 g | CaCl₂·2H₂O h2O | 0.10 g |
| Mucin | 12.5 mg | Glucose | 0.325 g | Pancreatin | 2.00 g | Lipase | 0.50 g |
| Peroxidase | 1.25 UI | Glucosamine-HCl | 0.165 g | Lipase | 2.00 g | Lipase | 0.50 g |
| NaNO₂ | 3.45 mg | Ascorbic acid | 8.8 mg | CaCl₂·2 H₂O | 0.10 g |
| | | BSA | 0.5 mg | | |
| | | Pepsin | 1.25 g | | |
| | | Mucin | 1.5 g | | |
| | | β₂-glucosy | 5 µL | | |
| | | FeSO₄·7 H₂O | 5.6 mg | | |

Figure 1. In vitro digestive model.
amino acid analysis operating condition: mobile phase – sodium citrate buffer solution, flow rate – 0.4 mL/min, injection volume – 20 μL, separation temperature – 57°C, reaction column temperature – 115°C, detection wavelength-valine detection wavelength is 440 nm, and other amino acid detection wavelength is 570 nm. The peak area was used as the test result, and the amino acid concentration was calculated using an external standard method.

Antioxidant activities of digestive juices: The DPPH radical scavenging activity of protein digestive products was determined as described in Ebru Pelvan Pelitli et al. with modifications. The test tube A₀ (control group), 3.0 mL distilled water and 2 mL DPPH·solution were added. To test tube A (sample group), 3.0 mL sample solution and 2.0 mL DPPH·solution were added. To test tube A (sample control group), 3.0 mL sample solution and 2.0 mL absolute ethanol were added. Followed by adding the reaction mixture and mixing well, it reacted in dark condition at room temperature for 30 min, and subsequently, the reaction solutions were determined at 517 nm for A₀, A, and B by using a multifunctional enzyme-labeled instrument (Spectra MX I3X). The DPPH radical scavenging activity was calculated using the following equation.

\[
\text{DPPH RSA} = \left( 1 - \frac{A - B}{A_0} \right) \times 100\%
\]

Reducing power activity assay: According to the method of Pelitli et al., the reducing power activity of protein digestive products was measured. An protein digestive product solution 2 mL was mixed with 2 mL PBS (pH 6.6) and 2 mL of 1 g/L potassium ferricyanide. The mixture was reacted at 50°C for 20 min and then added 2 mL of 10 g/L trichloroacetic acid. Then, 2.0 mL of supernatant was chosen and mixed with 2.0 mL of distilled water and 0.4 mL of 0.1 g/L ferric chloride after centrifugation at 2,000 g for 10 min. Finally, the mixture was set at room temperature for 10 min and measured at 700 nm using a multifunctional enzyme-labeled instrument (Spectra MX I3X). The control group was the equivalent volume of distilled water rather than of the protein digestive product sample.

Superoxide anion radical activity: Superoxide anion radical was measured according to the method of Smirnoff N with some changes. All of the superoxide anion radical of protein digestive products were reacted. To test tube A₀ (control group), 1.0 mL distilled water, 0.1 mL Pyrogallol of 60 mM, and 3.0 mL Tris-HCl of pH 8.2 were added. To test tube A (sample group), 1.0 mL sample solution, 0.1 mL Pyrogallol of 60 mM, and 3.0 mL Tris-HCl of pH 8.2 were added. To test tube B (sample control group), 3.1 mL sample solution and 1.0 mL sample solution were added. Followed by adding the reaction mixture and mixing well, it reacted at room temperature for 5 min, and subsequently, the reaction solutions were determined at 320 nm for A₀, A, and B by using a multifunctional enzyme-labeled instrument (Spectra MX I3X). The superoxide anion radical activity was calculated using the following equation.

\[
\text{Scavenging} = \left( 1 - \frac{A - B}{A_0} \right) \times 100\%
\]

Hydroxyl radical-scavenging activity: The hydroxyl radical-scavenging activity was measured according to the method of Dai et al. with minor modification. The hydroxyl radical was initiated by a Fenton reaction in a condition including FeSO₄ and H₂O₂. All of the hydroxyl radical-scavenging activity of protein digestive products were reacted. To test tube A₀ (control group), 1.0 mL distilled water, 0.3 mL salicylic acid of 20 mM, and 1.0 mL FeSO₄ of 1.5 mM were added. To test tube A (sample group), 1.0 mL sample solution, 0.3 mL salicylic acid of 20 mM, and 1.0 mL FeSO₄ of 1.5 mM were added. To test tube B (sample control group), 1.3 mL distilled water and 1.0 mL sample solution were added. Followed by adding the reaction mixture and mixing well, it reacted at 37°C for 30 min, and the absorbance was measured at 510 nm. The hydroxyl radical-scavenging activity was calculated using the following equation.
Effect of heat treatment and ultrasound treatment on digestive properties of the protein

The proteins were treated by heat at 75°C for 30 min and by ultrasound at 200 W for 30 min and then research the effect of heat treatment and ultrasound treatment on surface hydrophobicity after stimulating in stomach and intestinal of the protein, peptide contents and available amino groups, and antioxidant activities of digestive juices.

Statistical data analysis

All experimental data in this study were analyzed using Prism 5.0 and one-way analysis of F-test. Differences between variables with a P value of <0.05 were viewed as significant. All data were shown as mean ± standard deviation. In this study, all measurements were performed in triplicate.

Results and discussion

Physicochemical properties of Pleurotus eryngii protein

Rheological properties: Rheological properties of the protein solutions substantially affected the mechanical and film properties. We studied the rheological behavior of protein solutions to investigate the machine-processed effects of protein concentration on rheology. Figure 2a illustrates the viscosity (η) of different protein concentrations (3 g/100 g (raw materials/H₂O)–9 g/100 g (raw materials/H₂O)) at 25°C, and the viscosity of protein solutions formed at different concentrations was dependent on frequency. Higher viscosity was obtained at lower frequencies compared with those obtained at higher frequencies because, at longer experimental time scales, more interaction between proteins allows them to become stress-free during periodic deformation. As shown in Figure 2a, five distinct areas were found in the rheological curves: an apparent increase, two moderate increases, and two small increases between 3 g/100 g raw materials and 5 g/100 g raw materials, 5 g/100 g raw materials and 9 g/100 g raw materials, and 9 g/100 g raw materials and 11 g/100 g raw materials, which eventually tend to balance the state.

The hysteresis loop in the shear stress curves to shear rates is shown in Figure 1b, the hysteresis loop is in a clockwise direction, and the shear stress increases with the increase in protein solution concentrations. In the study of Liang, Zhang, and Xu, when the hysteresis loop was in a clockwise direction, the solution would form a new structure by shearing or breaking the structure. In addition, increasing protein concentrations can increase the closed loop area because the more closed the loop area, the more energy is needed to break down the structure at higher protein concentration.

Effects of temperatures on rheological property: Figure 3a shows the viscosity (η) of the 3 g/100 g raw material protein solution at different temperatures (25°C, 35°C, and 45°C). The previous researches showed that the SPI dispersion viscosity increased when heated because of the formation of new structures, hydrophobic interactions, and disulfide bridges. However, the protein solution viscosity decreased as the temperature increases in the present experiment. This result is in agreement with that of Liu, Xu, and Zhao.

Similar to Figure 2a, Figure 3a shows the shear thinning behavior, and the protein solution viscosity reduces rapidly in the low-shear-rate range (<10 S⁻¹). The apparent viscosity decreases with increasing temperature, resulting in more molecule chain motion at a higher temperature to obtain more energy. Figures 2a and 3a show that the protein solution shear thinning behavior is the same as that of the common polymer solution, which dissolves P. eryngii proteins to form a steady protein solution.
In order to study the effects of temperature on the rheological behavior of P. eryngii protein solution, the 3 g/100 g raw material protein solution was selected. The shear stress curves and shear rates are shown in Figure 3b, which shows thixotropic behavior at different temperatures. As shown in the figure, the shear stress decreased with increasing temperature. The protein solution’s thixotropic property was lower at higher temperatures. This result indicated that the energy required to break down the thixotropic structure was lower at higher temperatures. This result is different from corn starch \[38\] but is similar to soy protein isolate solution,\[37\] which means that we can form gels in these systems and need more energy to destroy the structure.

Effects of pH on rheological property: The measurements were performed at different pH values (2.0, 3.5, 5.0, and 7.0) for the 15 g/100 g (raw materials/H\(_2\)O) protein solution at 70°C. As shown in Figure 4, the mechanical spectrum curves can be observed. Differences can be observed at each pH. According to the previous results for the processing of gelation, the gel strength is lower in each case. In Figure 4, \(G'\) is always higher than \(G''\) at both pH levels; however, it has higher dependence of the modulus on frequency. We can observe a cross point at pH 2.0 and 5.0 (Figure 4a,b), which means that a quite subtle gel was formed.\[12\] The electrostatic interactions might play important roles in the protein system at pH 2.0 and 5.0, such as departing isoelectric point (pH 3.6), and the cross point was not observed at pH 3.5 and 7.0. At pH 7.0, \(G'\) and \(G''\) are very higher compared with those in other pH levels, which showed an evident increase in the protein solution. The protein solutions showed that \(G'\) is twice higher than that in other cases, which means that it cannot form a gel. At pH 3.5, \(G'\) and \(G''\) are almost parallel, which indicates the formation of gel.
Effects of pH on sulfhydryl and disulfide bond contents are shown in Table 2. The content of sulfhydryl bonds (R-SH and T-SH) increases and the content of disulfide bonds increases with pH increase. These results indicated that the content of disulfide bond might be associated with the aggregation of protein due to the formation of chain-chain. In other words, the increase in the disulfide bond might lead to network formation. The connections between physicochemical and rheological properties are shown in Table 3. As summarized above, the sulfhydryl and disulfide bond contents were significantly related to the rheological properties of proteins. The sulfhydryl content was correlated with the reduction in $\eta$, meaning that the protein might be held closer with higher sulfhydryl bond content and be less coacervation with a higher disulfide bond.

The Power Law model demonstrates flow behavior of each protein sample, and the $n$ manifests the deviation degree from Newtonian fluid. From Table 4, the $n$ value was lower near the protein isoelectric point and increased deviating from the isoelectric point at different pH concentrations, and it is reported that the viscosity of Newtonian fluid retains constant with strengthening shear rate when $n = 1$, and the less the $n$ value, the more the degree of deviation from Newtonian fluid, which indicates that the fluid viscosity is more decided by shear rate. At the same pH concentrate, the $n$ value decreased with increasing temperature; this result might be that by improving the moving ability of protein–protein molecules, the viscosity dependence to shear rate decreases, and the higher the temperature, the more the energy enabled to the breakage of entanglements.

Effects of Ca$^{2+}$ on rheological property: Figure 5 shows the comparison between the evolution of $G'$ at 0.5 Hz for the 15 g/100 g raw material protein solution with different temperatures by adding...
different concentrations of Ca\(^{2+}\) (0.01, 0.03, and 0.05 mol/L). Dynamic rheology has been used to describe the heat-induced gelation of proteins, which is usually present in G’ because G’ plays a representative role in the recovered per cycle of sinusoidal shear deformation.\(^{[40]}\) In all cases, G’ decreased sharply as the temperature was raised from 25°C to 65°C. In the recovered proteins, G’ was initially increased at approximately 25°C, emerged a peak at 28°C, and reduced to minimum at 65°C without Ca\(^{2+}\). However, the temperature of the emerging peak was decreased. When the Ca\(^{2+}\)

![Figure 4](image-url)

**Figure 4.** Modulus of 15 g/100 g (raw materials/H\(_2\)O) protein solution at different pH at 70°C. (a) Modulus of 15 g/100 g (raw materials/H\(_2\)O) protein solution at pH 2.0. (b) Modulus of 15 g/100 g (raw materials/H\(_2\)O) protein solution at pH 5.0.

**Table 2.** Effect of pH on the sulfhydryl and disulfide bond contents.

| pH   | R-S      | S-S      |
|------|----------|----------|
| 2.0  | 1.49 ± 0.34\(^a\) | 1.27 ± 0.23\(^a\) |
| 3.5  | 1.42 ± 0.29\(^a\) | 1.63 ± 0.32\(^a\) |
| 5.0  | 1.62 ± 0.41\(^a\) | 1.61 ± 0.28\(^a\) |
| 7.0  | 2.52 ± 0.38\(^b\) | 0.92 ± 0.41\(^a\) |

Note: \(^a\) is control standard; \(^b\) and \(^c\) mean significant difference (\(P < 0.05\)).

**Table 3.** Connection between physicochemical and rheological properties.

|       | \(\eta\) | G’       | G”       |
|-------|----------|----------|----------|
| R-S   | -0.829\(^*\) | 0.919\(^**\) | 0.946\(^**\) |
| T-S   | -0.613   | 0.933\(^**\) | 0.946\(^**\) |
| S-S   | 0.955\(^**\) | -0.652   | -0.680   |

Note: \(^*\) means significant difference (\(P < 0.05\)); \(^**\) means extremely significant difference (\(P < 0.01\)).
concentration was 0.01 mol/L, the peak corresponding to the temperature was approximately 36°C, and when the Ca\(^{2+}\) concentrations were 0.03 and 0.05 mol/L, the peaks corresponding to the temperature were both approximately 42°C; \(G'\) increased at first, which might be related to the crosslinking of proteins, and then, it subsequently decreased, which might be due to the slight denaturation of proteins. Above of that \(G'\) of proteins gradually increased with increasing calcium ionic strength because the system was much more complex with the bio-polymer competition for Ca\(^{2+}\), and when the calcium ionic strength was lower, the interaction between Ca\(^{2+}\) and proteins was relatively weak, leading to lower \(G'\). By contrast, the binding force between the calcium ions and proteins was strong, allowing the protein system to substantially depend on calcium ions.\[41\]

**Surface hydrophobicity (\(H_0\))**

The surface hydrophobicity (\(H_0\)) plays one of the leaders in conformation and interaction of protein molecule so as to evaluate the change in protein structure and reflect the number of hydrophobic amino acids on the protein surface.\[42\] As can be observed from Figure 6, protein through heat treating and ultrasound treating was characterized by higher \(H_0\) than non-treating protein significantly (\(P < 0.05\)). This result was similar to that of the previous research like bovine serum albumin\[43\] and black bean protein isolate.\[44\] This increase in \(H_0\) of protein might mean that the hydrophobic amino acids were exposed to the surface from inside of protein molecules because of generating the cavitation phenomenon triggered by ultrasound and the unfolding of protein molecules under heat treating.\[45,46\]

**Contents of sulfhydryl and disulfide bond**

The reactive sulphydryl content (R-SH) has a significant effect on food protein functional property. The change in R-SH of protein result in sonication is shown in Table 5. As observed from Table 5, heat treatment increases significantly the R-SH content of protein (\(P < 0.05\)); this might be due to heat movement inducing heat treatment, thus exposing more R-SH.\[46\] Ultrasound treatment increased significantly (\(P < 0.05\)) the R-SH and T-SH contents of protein and decreased significantly (\(P < 0.05\)) the S-S content, and these results mean that ultrasound treatment might promote protein unfold and break the disulfide bonds, making more decrease in the content of SH, and protein through ultrasound treating could expose potential buried sulphydryl groups into the protein surface due to the cavitation phenomenon.\[45\]

### Table 4. The Power Law model of protein in different conditions.

|       | \(K\)   | \(N\)   | \(R^2\) |
|-------|---------|---------|---------|
| 25°C  |         |         |         |
| pH = 2.0 | 0.8566  | 0.3718  | 0.9812  |
| pH = 3.0 | 1.6412  | 0.3253  | 0.9942  |
| pH = 3.5 | 1.9249  | 0.3898  | 0.9953  |
| pH = 4.0 | 1.7519  | 0.3628  | 0.9906  |
| pH = 4.5 | 0.0631  | 0.4882  | 0.9120  |
| 35°C  |         |         |         |
| pH = 2.0 | 0.5340  | 0.4327  | 0.9938  |
| pH = 3.0 | 0.9111  | 0.3935  | 0.9883  |
| pH = 3.5 | 1.3751  | 0.4151  | 0.9912  |
| pH = 4.0 | 1.2127  | 0.3958  | 0.9920  |
| pH = 4.5 | 0.0616  | 0.4774  | 0.9241  |
| 45°C  |         |         |         |
| pH = 2.0 | 0.4888  | 0.4114  | 0.9903  |
| pH = 3.0 | 0.6400  | 0.4014  | 0.9650  |
| pH = 3.5 | 0.9386  | 0.4266  | 0.9929  |
| pH = 4.0 | 0.8326  | 0.4065  | 0.9837  |
| pH = 4.5 | 0.0662  | 0.4514  | 0.9175  |
Figure 5. Evolution of storage modulus (G') and loss modulus (G'') at 0.5 Hz for 15 g/100 g (raw materials/H₂O) protein solution with different temperatures by adding different concentrations of Ca²⁺.
Secondary structure

FT-IR spectroscopy is used to determine the secondary structure of the protein in a wide variety of situation, and the amide I region of protein FT-IR is connected with the C=O and C-N stretching vibration. The FT-IR spectra of protein in the region of 400–4,000 cm$^{-1}$ are shown in Figure 7, and the results presented that the absorption region and peak shape of protein were not dramatically different between the control group and treated group including heat treating and ultrasound treating; however, we can observe that the absorption strength of peak of protein increased through heat treating and ultrasound treating. The fitted peaks of protein are presented in Figure 8 by analyzing the deconvolution and the overlapping, and the specific distribution of the secondary structure of protein is shown in Table 6; the heat and ultrasound pretreatment increased the content of $\alpha$-helix and decreased the content of $\beta$-sheet and random coil in protein, while these decreased by heat treating and increased by ultrasound heating in $\beta$-turn of protein compared with control group. The changes in the secondary structure of protein might be owing to the acoustic cavitation brought by ultrasound like shear force, shock waves, and others and the rearrangement of the inter-linkage between molecules by heating pretreatment (Girgih A T, Chao D & Lin L, 2015), which might have disrupted the order of local amino acids, thus leading to protein molecule rearrange. These results were identical with Liu et al. while contradictory with Jin et al., which found that the zein treated by ultrasound and the secondary structure of protein presented that the ultrasound treatment increased the content of $\beta$-sheet. The result was different from that of other studies due to the discrepancy in ultrasound environment like the ultrasound frequency and the method of heat treating.

Table 5. The contents of sulfhydryl and disulfide bond of protein treated by heat and ultrasound.

|                         | R-SH (μmol/L) | T-SH (μmol/L) | S-S (μmol/L) |
|-------------------------|---------------|---------------|--------------|
| Non-treatment group     | 0.110 ± 0.006$^a$ | 0.207 ± 0.017$^a$ | 0.048 ± 0.012$^a$ |
| Heat treatment group    | 0.175 ± 0.002$^b$ | 0.255 ± 0.002$^b$ | 0.039 ± 0.002$^a$ |
| Ultrasound treatment group | 0.292 ± 0.014$^c$ | 0.337 ± 0.008$^b$ | 0.025 ± 0.004$^b$ |

Note: a is control standard; b and c mean significant difference ($P < 0.05$).

Figure 6. The surface hydrophobicity ($H_0$) of Pleurotus eryngii protein. Note: a is control standard; b and c mean significant difference ($P < 0.05$).
Thermal properties

Protein features heat resistance at a certain temperature, and the maintenance force of spatial conformation of a protein can be changed under the situation that the outside temperature exceeds its initial denaturation temperature (Tm), thus leading to protein denaturation. Figure 9 shows the

Figure 7. The FT-IR spectra of Pleurous eryngii protein in the region of 400–4000 cm⁻¹.

Figure 8. The fitted peaks of Pleurous eryngii protein in the region of 1600–1700 cm⁻¹.

Thermal properties

Protein features heat resistance at a certain temperature, and the maintenance force of spatial conformation of a protein can be changed under the situation that the outside temperature exceeds its initial denaturation temperature (Tm), thus leading to protein denaturation. Figure 9 shows the
DSC and Tp analyses of *P. eryngii* protein treated by heat and ultrasound; as can be observed from Figure 9 and Table 7, the Tm of protein under untreated, heat treated, and ultrasound treated was A, respectively. These results might due to the different hydrophobicity of protein through different treating. The higher the hydrophobicity, the more stable the protein because the hydrophobic interaction was endothermic, thereby increasing the denaturation temperature. Moreover, the enthalpy change ($\Delta H$) of untreated protein was higher compared with treated by heat and ultrasound, which might be owing to the different content of disulfide bonds, and the more the disulfide bonds, the greater destroying energy was required. However, there are few researches concentrated on this aspect presently, and the specific mechanism is not clear yet and further research must be needed.

**Digestive properties of *P. eryngii* protein and the effect of treatments on protein**

Available amino groups (DH) of protein and *in vitro* digestibility of digestive juice (VDP): As shown in Figure 10, DH and VDP of different kinds of protein increased with digestion method (stomach digestion and small intestinal digestion). The DH and VDP of protein by trypsin in the intestinal phase were significantly higher than by pepsin in the gastric phase ($P < 0.05$), and this was because protein in the intestinal digestion was more digestive than in the gastric phase, and trypsin in the small intestinal could break peptide bonds more easier than pepsin in the stomach.\(^{33}\) According to the previous research, the increased digestibility of heated and ultrasound-treated protein was connected with an increase in protein hydrophobic surface ($H_0$) because the enhancement of $H_0$ could provide more access to proteases that laminate at hydrophobic amino acid residues, thus promoting protein digestibility of heated and ultrasound-treated protein compared to non-treated protein.\(^{49}\)
Peptides and amino acids of protein digestive juice

As shown in Figure 11, the peptide content of protein digestive juice was different via different treatment methods. The peptide content of protein digestive products through pepsin digested and intestinal digested was reduced under heat treatment and ultrasound treatment of protein, but the effect was significant under the ultrasound treatment ($P < 0.05$). The amino acid composition of protein digestive products via treating with different methods was shown in Table 8. We could observe the difference in composition and ratio of amino acids through treating with different methods (the amino acid content was calculated using standard amino acid samples). In non-treatment group and heat treatment group, five kinds of amino acids were shown, and there are 10 kinds of amino acids in the ultrasound treatment group. These results indicate that protein treated by heat and ultrasound changed the protein structure, promoting the protein digestion, which was consistent with the above results of DH and VDP. However, the systematic research was needed due to the different functions of peptides and amino acids.

Absorption intrinsic of P. eryngii protein and protein digestive products

The absorption intrinsic of P. eryngii protein and protein digestive products was measured to investigate the change in different kinds of protein spatial structures after heat-treated and ultrasound pre-heated. The intrinsic fluorescence spectrum represents the Trp residues' characteristic peaks excited at 360 nm, and the change of peak position and peak strength reflects the change of Trp residues. The intrinsic fluorescence spectrum of protein and protein digestive products was post and reflected in Figure 12, and the fluorescence intensity of protein (FIP) and protein digestive products (FIPD) was changed by treating method, and the Trp residues' characteristic peak of protein and protein digestive products was not changed. The FIP and FIDP through heat-treated and ultrasound pre-heated intensified compared to the control group and the enhancement of FIP and FIDP by heating treat surpassed by ultrasound pre-treated; this is because ultrasound pre-treated

| Table 7. The denaturation temperature (Tm) and enthalpy change ($\Delta H$) of Pleurotus eryngii protein. |
|----------------------------------------------------------|
|                        | Non-treatment group | Heat treatment group | Ultrasound treatment group |
| Tm(°C)                | 84.85 ± 0.02       | 91.43 ± 0.04       | 94.51 ± 0.03              |
| $\Delta H$(J)         | 0.543 ± 0.07       | 0.195 ± 0.02       | 0.183 ± 0.03              |

Figure 10. DH and VDP of Pleurotus eryngii protein
Note: a is control standard, b and c means significant difference ($P < 0.05$).
might destroy the interactions of protein–protein, thereby causing the structure change of protein molecules and making more Trp residues being exposed from inside to outside, lead to the FIP and FIDP increase.\[42\] In addition, we could observe that the FIDP was more higher than FIP and FIDP through intestinal digestive (FIDP-2) was more higher than stomach digestive (FIDP-1), and as observed in Figure 12, these results indicated that the micro-condition of Trp residues changed; more Trp residues were transferred from molecule inside to outside. In a word, the protein might be more digestible in the small intestine than stomach, and this might be due to trypsin intestine juice could disrupt more easily on peptide bonds than pepsin stomach juice.\[33\]

### Antioxidant activities of protein digestive products

As shown in Figure 13, it reflected majorly the antioxidant activity of protein digestive products, which presented higher antioxidant activity through in vitro digestive including pepsin-digested and intestinal-digested except DPPH- free radicals characterized with decreasing. In other words, the ability of protein and the trypsin digest for DPPH- radicals was lower than the pepsin digest. However, in contrast to Wang et al.’s research,\[50\] this result showed that DPPH- was an oil-soluble free-radical, which can make a more stable product by obtaining an electron. After protein and pepsin interacted with each other in stomach phase, more hydrophobic amino acids were appeared, making protein digestive products more accessible by DPPH-, and this was to promote electron divert from protein digestive products, thus stabilizing DPPH-. However, when the protein further digested with trypsin in the intestinal phase, the peptide bonds were cracked, which forming many shorter peptides like tripeptides and dipeptides and more amino acids, thus

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Table 8. Amino acid composition of protein digestive juice.

| Amino acids     | Non-treatment group | Heat treatment group | Ultrasound treatment group |
|-----------------|---------------------|----------------------|---------------------------|
| Threonine       | 5.08% ± 0.12        | 1.22% ± 0.11         | 2.60% ± 0.12              |
| Serine          | -                   | -                    | -                         |
| Alanine         | -                   | -                    | 2.41% ± 0.08              |
| Viline          | 30.51% ± 0.07       | 13.41% ± 0.10        | 22.26% ± 0.14             |
| Isoleucine      | 5.08% ± 0.03        | 1.22% ± 0.05         | 5.89% ± 0.12              |
| Leucine         | -                   | 60.98% ± 0.13        | 22.92% ± 0.10             |
| Tyrosine        | 28.81% ± 0.11       | -                    | 8.34% ± 0.08              |
| Phenylalanine   | -                   | -                    | 4.94% ± 0.12              |
| Histidine       | -                   | -                    | 2.00% ± 0.03              |
| Lysine          | 30.51% ± 0.05       | 23.17% ± 0.12        | 19.28% ± 0.08             |

Note: "-" means not detected.
Figure 12. The absorption intrinsic of *Pleurotus eryngii* protein and protein digestive products.

Figure 13. The antioxidant of protein digestive products.
leading to less effect of lipid-soluble DPPH- on amino acids and shorter peptides; therefore, the scavenging capacity of DPPH- free radical was different in the two complex digestive systems.\textsuperscript{[51]} This result was concurred with Liu et al.\textsuperscript{[52]} Effects of digestive on superoxide anion radical (O\textsuperscript{2-}) and hydroxyl radical (-OH) and reducing power activities of a protein are presented in Figure 13b–d. The scavenging activities of protein digestive products were stimulated to increase digesting times presented a regular profile even if treated by different methods, which scavenging superoxide anion and radical hydroxyl radical and reducing power activities were increased after treatment by pepsin for 1 h and then by pancreatin for 4 h. It was worth mentioning that the scavenging activities like superoxide anion, radical hydroxyl radical, and reducing power were higher in pepsin phase than in the intestinal phase. In other words, the antioxidant activities of protein digestive products were also remained in the intestine because the digests after further digestive with trypsin also featured better antioxidant activities. These results were consistent with Rao et al.\textsuperscript{[53]} And it reached a steady state after about 4 h in the intestinal digestive system, which the scavenging superoxide anion, radical hydroxyl radical, and reducing power activities reached. The scavenging activities of protein digestive products treated by heating and ultrasound were higher than non-treated. This result was because that ultrasound pre-treated might cause ultrasound cavitation effect, which could bring a huge shear force and destroy the interactions of protein–protein, thus changing the protein molecule structure and exposing more cleavage sites,\textsuperscript{[42]} which was conducive to the combination of protein and enzyme; therefore, the peptides after digestive presented higher antioxidant activities like superoxide anion radical, hydroxyl radical, and reducing power. Moreover, heat-treat might expose cleavage sites, resulting in the movement of protein–protein molecules, thereby promoting the antioxidant capacity of protein digestive products.

### Conclusion

Protein concentration and temperature in the solution system played an important role in the rheological properties and viscosity. The shear stress and viscosity increased as the protein concentration increased from 3 g/100 g raw materials to 9 g/100 g raw materials and decreased as temperature increased from 25°C to 45°C. In addition, pH from 2.0 to 7.0 significantly affects the content of disulfide bond and sulphydryl bond, which showed that the lowest of disulfide bond content at pH 3.5 and higher of sulphydryl bond at pH 7.0. The addition of calcium from 0.01 to 0.05 mol/L to the protein system could increase the rheology properties. The protein treated by heat and ultrasound increased significantly the surface (H\textsubscript{0}) (P < 0.05) and enhanced terminal stability of the protein. The DH and VDP under pre-treatment were higher significantly than non-treatment (P < 0.05), and the kinds of essential amino acids increased. Moreover, heat treatment and ultrasound treatment can enable to unfold structure, which promoted the formation of well-digestible power of protein with excellent functional properties. In addition, the protein digestive products feature strong antioxidant activities, but the DPPH- free radicals characterized with decreasing through pepsin-digestible and intestinal-digestible. Therefore, based on these results of this research, heat treatment and ultrasound treatment is an effective processing way that promotes the digestibility of protein and cultivates its nutritional value.

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