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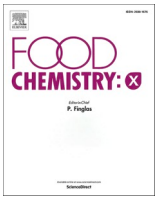

Tailoring soy protein/corn zein mixture by limited enzymatic hydrolysis to improve digestibility and functionality

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ABSTRACT

This study aimed to modify plant protein mixture to improve their functionality and digestibility by limited hydrolysis. Soy protein isolate and corn zein were mixed at the ratio of 5:1 (*w*/w), followed by limited hydrolysis using papain from 15 to 30 min. The structural characteristics, *in vitro* digestibility, and functional properties were evaluated. Also, DPPH radical scavenging activity was determined. The results indicated that the molecular weight of different modified samples was largely reduced by limited hydrolysis, and the proportion of random coil was significantly increased. Furthermore, the solubility, foaming, emulsifying and water-holding capacity of hydrolyzed protein mixture were significantly improved, which were close to those of whey protein isolate. *In vitro* digestibility after 30-min limited hydrolysis was remarkably elevated. In addition, the hydrolyzed protein mixture exhibited a higher antioxidant activity than those of untreated proteins. Overall, limited hydrolysis of protein mixture led to improved digestibility, functionality and antioxidant activity.

1. Introduction

The world's population is estimated to reach >9 billion by 2050, leading to a highly increased demand for animal protein to satisfy the needs of a growing population (Detzel et al., 2022). However, the global trend for animal-based protein is expected to exert a negative impact on the environment, including generation of more greenhouse gas as well as consumption of more land and water resources (Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). The transition from animal-based protein to plant-based protein could alleviate these negative impacts and contribute to more efficient and sustainable food production. Such transition involves the application of proteins derived from plant sources such as soy, pea, corn and wheat to develop plant-based alternatives to meat, egg, milk and fish (Tziva, Negro, Kalfagianni, & Hekkert, 2020).

Plant protein can serve as significant precursors of bioactive peptides (Tan, Nawaz, & Buckow, 2021). However, plant proteins are currently underutilized mainly due to unsatisfactory functional properties, digestibility and relatively low amount of certain essential amino acids (on a single-source basis). From the nutritional perspective, reasonable complementation of different plant proteins can improve their utilization efficiency by balancing the essential amino acid composition (Joehnke, Lametsch, & Sorensen, 2019). In addition, plant proteins tend to exhibit the lower digestibility, compared with animal proteins. The presence of protease inhibitors, tannins as well as low susceptibility of plant proteins to proteases can result in reduced digestibility and bioavailability *in vivo* (Sá, Moreno, & Carciofi, 2020).

Modification of plant proteins is a promising strategy that can be employed to improve their digestibility, functionality and bioactivity (*e.*

Abbreviations: SPI, soy protein isolate; WPI, whey protein isolate; OPA, o-phthaldialdehyde; DH, degree of hydrolysis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 5,5′-dithiobis-2-nitrobenzoic acid, DTNB; EDTA, Ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; FC, foaming capacity; FS, foam stability; EAI, emulsifying activity index; ESI, emulsifying stability index; DPPH, diphenylpicrylhydrazine.

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g. antioxidant and antihypertensive activity) (Nikbakht Nasrabadi, Sedaghat Doost, & Mezzenga, 2021). Enzymatic hydrolysis has been reported to act as one of the most commonly used methods to improve protein digestibility (Marciniak, Suwal, Naderi, Pouliot, & Doyen, 2018). Therefore, protein complementation (combination of two or more individual proteins to fulfill the required demand for essential amino acids) coupled with limited enzymatic hydrolysis may be promising to serve as an effective approach for modifying plant proteins to improve their digestibility, functionality and bioactivity.

Soy protein is regarded as a "complete" protein due to the balanced essential amino acid composition for human diet (Akharume, Aluko, & Adedeji, 2021). Even though soy protein is abundant in Lys, the contents of sulfur-containing amino acids are relatively low (Gorissen et al., 2018). In general, soy protein possesses some functional properties, such as emulsifying, gel formation, foaming, and water and oil absorption properties (Sui, Zhang, & Jiang, 2021), which can play a pivotal role in formulating soy protein-based foods. To obtain the targeted functional properties as well as desirable flavor, soy protein can be also modified by physical, chemical and enzymatic treatments. Among the different methods, limited hydrolysis is a promising strategy to enhance the functionality of soy protein (Song et al., 2018).

Zein is a renewable co-product of corn starch and corn syrup production used for food, feed, and fuel (Kasaai, 2018). Zein stands out from other food proteins due to its unique amino acid composition. It contains a large number of sulfur-containing and hydrophobic amino acids, but lacks Lys and Trp (Gorissen et al., 2018). Furthermore, zein has a high proportion of branched-chain amino acids, including Leu, Ile and Val (Gorissen et al., 2018). Although zein has good functionality, including emulsifying, film-forming and water-holding capacity due to its unique amino acid composition (Glusac & Fishman, 2021), it has poor solubility in water as well as low digestibility, which limits its application in the food industry. Therefore, it is necessary to modify it in order to improve its solubility. Among different approaches, enzymatic modification of zein has become a key fashion for high-value utilization of zein (Glusac & Fishman, 2021).

In recent years, substitution of animal proteins by plant proteins has gained popularity (Hinderink et al., 2021). Whey protein as an important food ingredient is characterized by good functional properties and high nutritional value. By complementation of different plant proteins with appropriate mixture ratios, the essential amino acid composition of plant protein mixture can be close to whey protein, an excellent animal-derived protein. In addition, considering the resistance to proteolysis, it is crucial to modify plant protein mixture by limited enzymatic hydrolysis (using food-grade protease) to improve digestibility. This study aimed to modify plant protein mixture by limited enzymatic hydrolysis to improve its functionality and digestibility. The structural, functional and DPPH radical scavenging properties of protein hydrolysates obtained from a protein mixture containing soy protein isolate (SPI) and zein were evaluated. By protein complementation combined with limited hydrolysis, it is expected to develop plant-based protein ingredients with good digestibility and functional properties.

2. Materials and methods

2.1. Materials

Pepsin from porcine gastric mucosa (3000 U/mg) and papain (500 kU/g) were purchased from Solarbio (Beijing, China). Casein was obtained from Aobox Biotechnology Co. Ltd. (Beijing, China). Whey protein isolate, WPI (protein content, 80.5%), soy protein isolate, SPI (protein content, 90.1%) and zein (protein content, 92.0%) were obtained from Shanghai Yuanye Biotechnology Co., Ltd. *O*-phthalaldehyde (OPA) was bought from Aladdin (Shanghai, China). All other chemicals and reagents used in this study were of analytical grade.

2.2. Amino acid composition analysis

The amino acid composition of protein samples was analyzed with the aid of an automatic amino acid analyzer (L-8900, Hitachi, Tokyo, Japan). Protein samples (SPI, zein, WPI and SPI/zein mixture) of 10 mg were hydrolyzed with 6 mol/L HCl at 110 °C for 24 h. Afterwards, the digest was adjusted to pH 7.0 by adding 4 mol/L NaOH, and then transferred to sample vials for analysis.

2.3. Enzymatic hydrolysis of soy protein/corn zein complex

According to the essential amino acid composition of WPI and ratio calculation, SPI and zein were mechanically mixed at the ratio of 5:1 (*w*/w) in the L18-Y933 high-speed blender (Jinan, Shandong, China). The protein mixture was dispersed using Milli-Q water at the concentration of 5% (w/w) and the pH of protein mixture solution was adjusted to 7.0 by adding 1 mol/L NaOH. Subsequently, enzymatic hydrolysis was initiated by adding papain (1%, w/w) to the protein solution with continuous stirring at 60 °C. Papain was utilized to catalyze hydrolysis due to its comprehensive application for improving the functionality of plant proteins (Liu et al., 2022; Liu et al., 2022). Enzymatic hydrolysis was terminated by heat treatment in boiling water for 20 min to inactivate enzyme, and all the digested samples and withdrawn (including both supernatant and pellets) at 15, 30, 45 and 60 min, and cooled to room temperature (25 °C), followed by lyophilization to obtain the modified samples. The lyophilized powder was stored at −18 °C. According to our preliminary test, enzymatic hydrolysis for 45–60 min led to the excessive degradation of proteins, which can deteriorate the functionality. Hence, limited hydrolysis for 15 and 30 min was employed for subsequent analysis.

2.4. Determination of degree of hydrolysis (DH)

The DH was determined using OPA method based on our previous work (Fu, Liu, Hansen, Bredie, & Lametsch, 2018). The absorbance was recorded at 340 nm after 10-min incubation in the dark. Leucine was used as the standard curve, and the free amino group content was calculated according to the standard curve, and the regression equation was obtained: $y = 0.433\times + 0.0513$, $R^2 = 0.9992$. DH was calculated according to the following formula.

$$\mathrm{DH}\ (\%) = \frac{NH_2 \times V_1}{NH_{21} \times V_2} \times 100\%.$$

where NH_2 is the concentration of free amino acids in the hydrolysate (mmol/mL); V_1, the volume of the hydrolysate (mL); NH_{21}, the concentration of free amino acids in the protein mixture solution (mmol/mL); and V_2, the volume of the protein mixture solution (mL).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis of protein samples was performed on the 5% stacking gel and 10% separating gel according to a previously reported method (Jan et al., 2016). In brief, protein dispersions (10 mg/mL) were mixed with loading buffer at the ratio of 1:1 (*v*/v), and of 10 μL protein samples (zein, SPI, protein mixture, WPI, and hydrolysates of 15–60 min) were loaded. Electrophoresis was carried out at 60 V for stacking gel and at 90 V for separating gel until the tracking dye migrated to the gel bottom. The gel was stained in Coomassie brilliant blue R-250 solution (0.25%, *w*/w), followed by destaining using a solution that contained a mixture of 10% (v/v) acetic acid and 45% (v/v) ethanol. Subsequently, the gels were scanned on an Image Scanner (Bio-Rad, Richmond, USA).

2.6. Infrared spectrum analysis

The powder of protein sample was mixed with KBr powder, uniformly ground, and pressed to form sheets in a tableting mode. Scanning was performed using a Fourier transform infrared spectrometer (Nicolet 670, Thermo Fisher, USA). The measurement wavelength was 4000–400 cm^{-1}, scanning resolution was 4 cm^{-1}, and the number of scans was 32. The secondary structure was further subjected to quantitative analysis using a nonlinear curve fitting procedure according to the method of Grewal, Huppertz, and Vasiljevic (2018).

2.7. Measurement of free amino groups and total free thiol groups

The free amino groups were determined using OPA method described in section 2.4. The total free thiol group contents of different protein samples were determined according to Ellman's method using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reagent (Standal et al., 2018). The protein sample was diluted to approximately 2 mg/mL by PBS (0.1 mol/L, pH 8.0). Protein solution (l mL) was mixed with 2 mL of urea-SDS solution containing 8 mol/L urea, 3% SDS, 1 mmol/L EDTA, followed by adding DTNB reagent (4 mg/mL) of 50 μL. The mixture was further incubated at room temperature (25 °C) for 30 min. The absorbance of the resultant sample was measured at 412 nm. The equation for calculating the total free thiol group is as follows.

$$\textit{Total free thiol group}\ (\mu mol/g) = \frac{73.53 \times A_{412} \times \mathrm{D}}{C}$$

where A_{412} denotes the absorbance at 412 nm; D corresponds to dilution ratio; C is the protein concentration (mg/mL).

2.8. In vitro protein digestibility

The *in vitro* simulated gastro-pancreatic digestion was conducted according to a previously reported static method with slight modifications (Brodkorb et al., 2019). The electrolyte simulated gastric fluid and electrolyte simulated intestinal fluid were first prepared. As for the gastric step, samples were diluted with simulated gastric fluid stock electrolyte solution at the ratio of 1:1 (*v*/v), followed by continuous stirring for 5 min. Thereafter, pH was further lowered to 2.5 using HCl (4 mol/L), and the peptic hydrolysis was initiated by the addition of 2000 U/mL of pepsin at 37 °C. After 2-h peptic digestion, the slurry was subjected to the pancreatic phase of digestion. The simulated intestinal fluid was added, followed by adjusting to pH 7.5 by adding 2 mol/L sodium carbonate. Pancreatin (0.8 g/L) and bile salts (10 mmol/L) were subsequently added and simulated intestinal digestion was performed at 37 °C for 2 h. The digestion was terminated by adding 1 mL of trichloroacetic acid solution (10%, *w*/w) to inactivate the enzyme. The sample was subsequently centrifuged at 10000*g* for 10 min. The supernatant was employed to measure the nitrogen content by the micro Kjeldahl method. The protein digestibility was calculated using the equation below.

$$\text{Protein digestibility}\ (\%) = \frac{N_0 - N_t}{N_{tot}} \times 100$$

where N_0 and N_t are the nitrogen content in TCA precipitate in the sample before and after digestion, and N_{tot} is the total nitrogen content in the sample.

2.9. Determination of protein solubility

The soluble protein concentration was determined according to a previous method (Hall, Jones, O'Haire, & Liceaga, 2017) using casein as the standard. Briefly, 20 mg of samples were dissolved in 2 mL Milli-Q water and pH of the solution was adjusted to 7.0 by 1 mol/L NaOH. The mixture was subjected to stirring for 30 min at 25 °C, followed by centrifugation at 10,000*g* for 10 min. The protein concentration of the resulting supernatants was measured based on the micro Kjeldahl method. Protein solubility was calculated as the percentage with the following equation:

$$\textit{Solubility}\ (\%) = \frac{A}{B} \times 100$$

where A is the protein content of the supernatant, and B is the protein content of the solution.

2.10. Measurement of foaming capacity and foam stability

Foaming capacity and foam stability were measured according to Intarasirisawat, Benjakul, Visessanguan, and Wu (2012). Protein sample solution (0.5%, *w*/w) of 20 mL was homogenized by a high-speed homogenizer Ultra-Turrax model T25 (IKA, Staufen, Germany) at the speed of 12,000 r/min for 2 min. Thereafter, the total volume was measured at 0 and 30 min after whipping. The foaming capacity (FC) was expressed as foam expansion at 0 min, while foam stability (FS) was expressed as foam volume remaining 30 min after whipping. The foam expansion was calculated according to the following equation.

$$FC = \frac{H_1}{H_0} \times 100\%$$

$$FS = \frac{H_2}{H_1} \times 100\%$$

where H_0 is the volume of protein solution before whipping, H_1 is the volume immediately after whipping, and H_2 is the volume after whipping and standing for 30 min.

2.11. Measurement of emulsifying activity and emulsifying stability

The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined according to a previous method (Pearce & Kinsella, 1978) with a slight modification. The protein solution and soybean oil are mixed at a ratio of 3:1 (*v*/v), and homogenized for 1 min with a high-speed homogenizer Ultra-Turrax model T25 (IKA, Staufen, Germany) at 10000 r/min. Fifty microliters of emulsion (the bottom of the homogenized emulsion) were withdrawn and diluted with 5 mL of 0.1% SDS solution. The absorbance was measured at the wavelength of 500 nm for 0 min (A_0) and 10 min (A_{10}) after emulsion formation. EAI (m^2/g) and ESI (min) were calculated according to the equations below.

$$\mathrm{EAI} = \frac{2 \times 2.303 \times A_0 \times N}{C \times \varphi}$$

where N is dilution factor, C is protein concentration and ϕ is oil volume fraction.

$$\mathrm{ESI} = \frac{A_{10}}{A_0} \times \mathrm{t}$$

where t means time interval (min).

2.12. Determination of water holding capacity

Water holding capacity of different protein samples was evaluated based on a meat model system according to Cumby, Zhong, Naczk, and Shahidi (2008). Different protein samples at the concentration of 1% (*w*/w) were thoroughly mixed with 8.5 g of ground pork and 1.5 g of Milli-Q water. A control was prepared without use of protein sample in the same procedure. After being vacuum-packed, the mixture was left at 4 °C for one hour. The samples were further heated to 95 °C for 1 h in a water bath, followed by cooling using tap water. The filter papers were employed to remove drip water from meat and the resultant meat was further weighed. The drip loss was obtained by calculating the weight

loss after cooking. Water holding capacity for different protein samples was expressed as the decrease of drip loss (%) against a control.

2.13. DPPH radical scavenging activity

DPPH radical scavenging activity was used to evaluate antioxidant potential based on our previous method (Luo, Yao, Soladoye, Zhang, & Fu, 2021). The DPPH reagent (0.2 mmol/L) of 1 mL was mixed with protein sample solution (1 mg/mL) of 1 mL, followed by incubation at room temperature in the dark for 30 min. The absorbance was subsequently measured at 517 nm (A_{sample}). The control group was performed following the same conditions by replacing sample solution with absolute ethanol ($A_{control}$). DPPH radical scavenging activity of protein sample was calculated based on the equation below.

$$\text{DPPH radical scavenging rate } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

2.14. Statistical analysis

All the experiments were carried out in triplicate and the obtained data were expressed as the mean ± standard deviation by Excel 2019. Statistical analysis was performed using one-way analysis of variance (ANOVA) using SPSS20.0. Duncan's multiple range test was used for analyzing mean comparison ($P < 0.05$).

3. Results and discussion

3.1. Amino acid composition analysis

The information on the amino acid composition of SPI, zein and WPI is listed in **Table S1**. In general, the amino acid composition of different protein samples (both supernatant and pellets in the digest) exhibited discrepant profiles of amino acids. In general, the nutritional value of WPI was quite high due to its high content of essential amino acids (including Lys and Thr) and branched-chain amino acids (Leu, Ile, Val). It was worth noting that the content of Lys in zein was only 0.18%, as the limiting amino acid in zein, whereas a relatively high content (6.12%) of Lys in SPI was observed. The content of Leu in SPI was relatively low (8.82%), while the content of Leu in zein was extremely high (20.47%). Furthermore, SPI and zein shared a high similarity of Glu proportion with the proportions of 20.18% and 21.99% respectively, which were close to that of WPI. Therefore, the complementation of protein mixture containing SPI and zein at certain mass ratios may contribute to a new alternative to WPI. Considering the branched-chain amino acids and essential amino acids of the protein mixture (SPI and zein), the ratio of SPI to zein was calculated to be 5:1 (*w*/w), which was close to the ratio of essential amino acids in WPI. Similarly, it has been shown that the partial replacement of whey by plant proteins (SPI or pea protein) is effective in improving the amino acid composition and sustainability of food products (Alves, Martha, Casanova, & Tavares, 2022; Kornet et al., 2021).

3.2. SDS-PAGE of different protein samples

The impact of limited hydrolysis on the electrophoretic profile of different protein samples was analyzed by SDS-PAGE (Fig. 1). As expected, SPI, zein and WPI exhibited typical protein patterns. Compared with unhydrolyzed proteins, enzymatic hydrolysis of protein mixture (both supernatant and pellets in the digest) by papain from 15 to 60 min led to degradation of most of high molecular weight (MW) proteins. Furthermore, the smearing of protein bands was observed in hydrolyzed samples from 15 to 60 min, indicating the formation of peptides with different MW during limited hydrolysis. The proteins with the MW close to 30 kDa can be observed due to the degradation of high MW proteins. With the extended hydrolysis for 60 min, most proteins were intensively

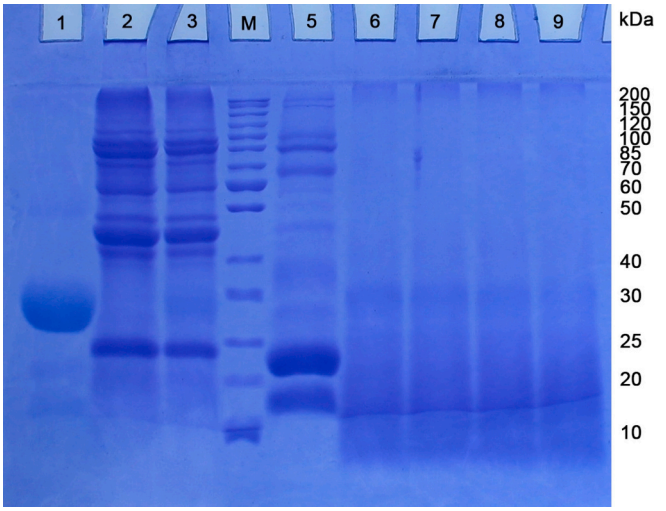


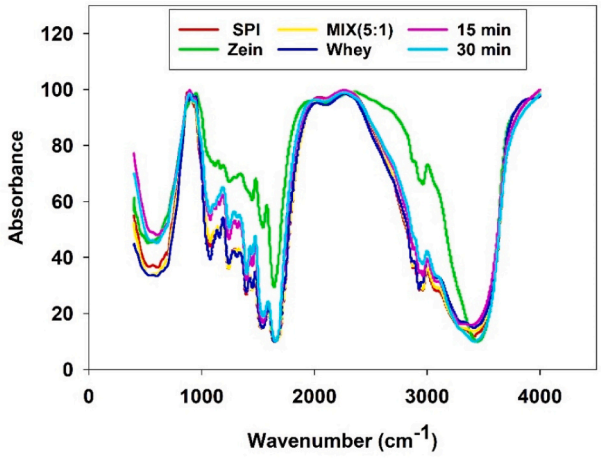
Fig. 1. Electrophoresis profile of different protein samples. Note: Lane M is standard protein. Lane 1 to 9 represents zein, SPI, protein mixture (5:1, *w*/w), protein marker, WPI, protein mixture hydrolyzed for 15 min, protein mixture hydrolyzed for 30 min, protein mixture hydrolyzed for 45 min, and protein mixture hydrolyzed for 60 min, respectively.

degraded to low MW peptides below 30 kDa (Fig. 1). The present electrophoretic results revealed that enzymatic treatment can exert a significant impact on the MW of protein. Some high MW proteins can be largely hydrolyzed to low MW proteins/peptides, which might be conducive to the improved digestibility of enzymatically modified protein mixture. The current results are in agreement with some previous studies showing that after limited hydrolysis, nearly all rice endosperm proteins (Nisov, Ercili-Cura, & Nordlund, 2020) and peanut proteins (Chen, Zhang, Zhang, Kong, & Hua, 2021) were degraded to low MW proteins. Given that enzymatic hydrolysis over 30 min can provoke prominent degradation of proteins, the hydrolyzed samples of 15 and 30 min were subsequently selected in the following study. In addition, the DH values of the hydrolyzed protein mixture were determined to be 4.8% (15-min hydrolysis) and 6.4% (30-min hydrolysis).

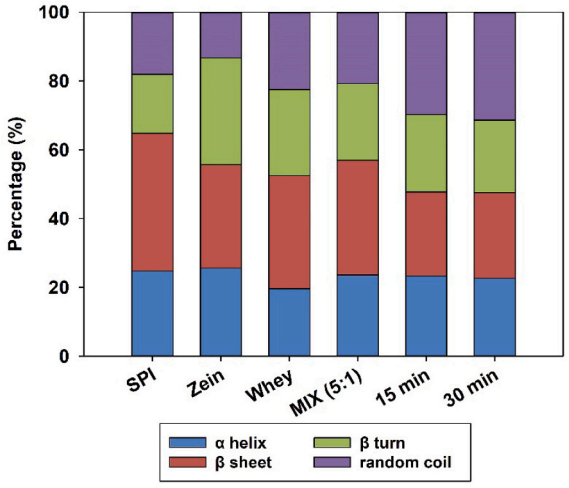
3.3. The secondary structure

FTIR can be employed to analyze the changes in the secondary structure of proteins during limited hydrolysis and provide quantitative information on the secondary structure content. The FTIR spectra of protein samples are illustrated in Fig. 2A. The pronounced changes in the spectra were observed in the region of 1000–1500 cm^{-1} and 3000 cm^{-1}. The amide I region (1700–1600 cm^{-1}) of protein in FTIR spectra exhibited deformation vibrations. The current results revealed that through limited hydrolysis of protein mixture, peptide bonds were cleaved, and the secondary structure of proteins was altered. Similarly, enzymatic hydrolysis of soybean protein by food proteases can give rise to pronounced changes in the FTIR spectra in the region of 1000–1500 cm^{-1} due to the altered secondary structure during enzymatic hydrolysis (Liu, Huang, et al., 2022; Liu, Lin, et al., 2022; Singh, Siddiqi, & Sogi, 2021). Furthermore, the major change in the peaks of protein hydrolysates in the present study was observed in the range of 3200–3300 cm^{-1}, which is similar to the enzymatically hydrolyzed proteins from milk protein concentrate (Cui, Sun, Zhou, Cheng, & Guo, 2021).

In addition, the proportions of α-helix, β-sheet, β-turns and random coil are shown in Fig. 2B. Compared with unhydrolyzed protein mixture, the proportions of α-helix, β-turn, and β-sheet were reduced from 23.58%, 22.32%, and 33.38% to 22.63%, 21.07%, 24.91%, respectively, after 30-min limited hydrolysis catalyzed by papain. Meanwhile, the percentages of random coil of protein samples were significantly increased after limited hydrolysis. This phenomenon can be attributed to



(A)



(B)

Fig. 2. FTIR spectra (A) and the secondary structure percentage (α helix, β turn, β sheet and random coil) of different protein samples (B).

the enzymatic hydrolysis of SPI and zein, resulting in the formation of a greater content of shorter polypeptides. Namely, the content of the ordered structure in protein mixture became lower. Recently, a similar phenomenon has also been observed in black bean protein (Xu, Han, Chen, Li, & Jin, 2018) and rice bran protein (Singh et al., 2021). Random coil structures can endow the modified proteins with more flexible conformations, which are conducive to the improvement in protein solubility.

3.4. Free amino groups and total free thiol groups

The changes in free amino groups and free thiol groups are illustrated in Fig. 3. As for free amino group, limited enzymatic hydrolysis for 15–30 min resulted in a significantly increased content of free amino groups ($P < 0.05$), compared with untreated proteins. This fact was mainly due to the release and exposure of peptides and free amino acids by enzymatic hydrolysis, which leads to a significant increase of free amino groups in the hydrolyzed samples (Wang, Cheng, Wang, & Yang, 2022). Limited enzymatic hydrolysis is able to induce the structural alterations of proteins, but extensive hydrolysis may provoke a remarkable reduction in molecular weight, leading to the impaired functionality (Vogelsang-O'Dwyer, Sahin, Arendt, & Zannini, 2022).

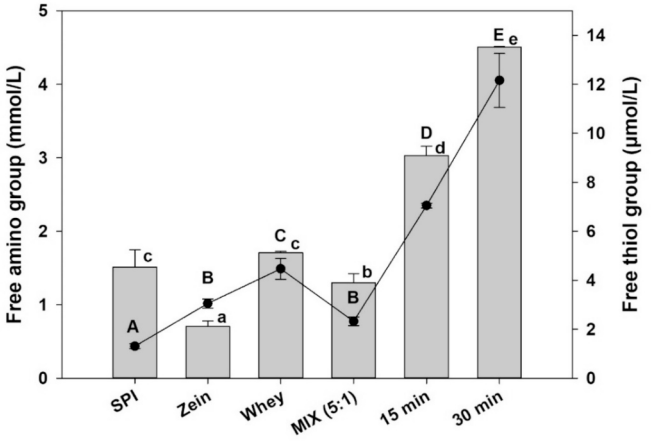


Fig. 3. The content of free amino groups and total thiol group. Different lowercase superscripts (a-e) for free amino group and capital letters (A-E) for thiol group mean significant difference ($P < 0.05$).

Similarly, limited enzymatic hydrolysis can lead to a remarkable increase of total free thiol groups in the hydrolyzed samples ($P < 0.05$). The enhanced content of thiol groups was attributed to the unfolding of protein and breakage of disulfide bonds by enzymatic hydrolysis (Ali, Rawel, & Hellwig, 2023), which may contribute to the improved functionality, *e.g.* foaming capacity.

3.5. In vitro digestibility

Fig. 4 illustrates the digestibility of SPI, zein, protein mixture and the hydrolyzed protein samples. Notably, WPI exhibited the highest digestibility (90.8%), while limited enzymatic hydrolysis led to a significant increase in *in vitro* digestibility in protein mixture ($P < 0.05$). Compared with the unhydrolyzed protein mixture, the digestibility was remarkably improved by 12.9% after 15-min enzymatic treatment ($P < 0.05$). After 30-min enzymatic hydrolysis, the *in vitro* digestibility was increased by 23.9% ($P < 0.05$), which was close to WPI. This phenomenon can be explained by the fact that the modified structure of proteins as a result of limited hydrolysis makes them more susceptible to degradation by digestive enzymes. Recently, a similar study has revealed that the *in vitro* digestibility of soybean meal was significantly increased through enzymatic treatment (Ketnawa & Ogawa, 2021).

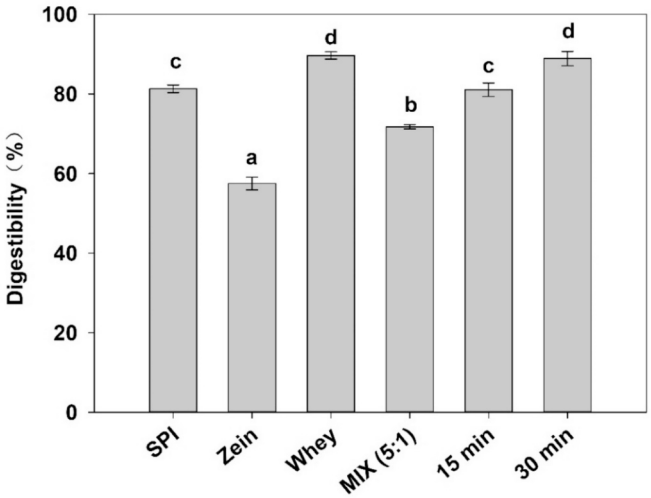


Fig. 4. *In vitro* digestibility of different protein samples. Different lowercase superscripts (a-d) mean significant difference ($P < 0.05$) between different protein samples.

Overall, the *in vitro* digestibility of protein mixture after limited hydrolysis was higher. Given that 30-min hydrolysis rendered a close digestibility to WPI, the samples hydrolyzed for 45-min and 60-min were not selected for the subsequent study. In agreement with several previous reports, limited enzymatic hydrolysis using proteases has also been reported to elevate the *in vitro* digestibility of peanut protein (Chen et al., 2021) and soybean protein (Song et al., 2018).

3.6. Functional properties

3.6.1. Solubility

As one of the most important functional properties of proteins, solubility can reflect the degree of denaturation and aggregation of the internal structure of protein (Klost & Drusch, 2019). In this study, the solubility of six protein samples was measured and displayed in Fig. 5A. The results demonstrated that the solubility of SPI, zein and untreated protein mixture was much lower than that of WPI. In contrast, after limited hydrolysis by papain, the solubility of protein mixture was significantly improved ($P < 0.05$), with an increase of approximately 70% after 30-min of hydrolysis treatment. Even though there is a certain gap in comparison with WPI, the improvement effect of solubility in protein mixture by limited hydrolysis was still obvious. This fact is attributed not only to the reduction in the molecular weight of proteins by limited hydrolysis, but also the increase of soluble peptides as well as the corresponding increase in ionizable amino and carboxyl groups (Mokni Ghribi et al., 2015).

3.6.2. Foaming capacity and foam stability

Proteins are composed of both hydrophobic and hydrophilic amino acids. They have a typical amphiphilic structure with good interfacial activity, which is prone to form foams at the air/water interface after external stirring (Jin, Wang, Tang, Regenstein, & Wang, 2020). Foaming capacity and foam stability are important functional properties of proteins during food processing. As displayed in Fig. 5B, prior to enzymatic hydrolysis treatment, SPI, zein and protein mixture exhibited relatively low foaming capacity and stability, compared with WPI. It was worth noting that zein was unable to properly form foams, probably due to its high hydrophobicity and folded structure. However, the foaming capacity of protein mixture after limited enzymatic hydrolysis was significantly improved ($P < 0.05$). After 15-min hydrolysis, the foaming capacity of protein mixture was increased by 84% (comparable to the level of WPI), followed by an insignificant decline after 30 min post-hydrolysis ($P > 0.05$). The reason for the improved foaming capacity in the initial stage of enzymatic hydrolysis is that the formation of foam requires the wrapping of peptide chains around air bubbles on the air–water interface, and the aqueous film can be formed on the aqueous surface (Liang et al., 2020). With the aid of limited enzymatic hydrolysis, proteins are degraded to generate more small peptides, which further participate in the formation of aqueous films so that the foaming capacity is improved (Fig. 5B). However, as the enzymatic hydrolysis progressed, the peptide chain became shorter and were unable to form strong aqueous films, which ultimately led to the weakening of foaming capacity or formation of fewer foams.

Enzymatic hydrolysis can also lead to impaired foam stability

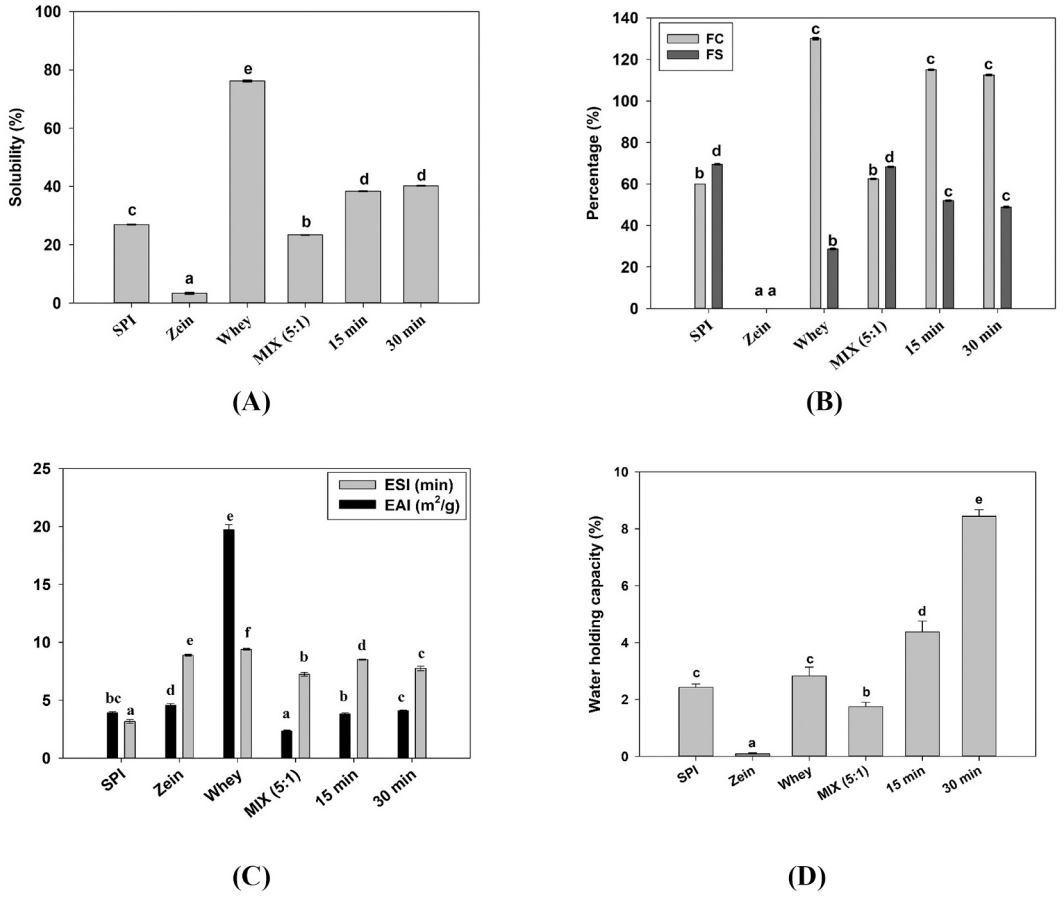


Fig. 5. (A) Solubility of different protein samples. Different lowercase superscripts (a-e) mean significant difference ($P < 0.05$) between different protein samples. (B) Foaming capacity and foam stability of different protein samples. Different lowercase superscripts (a-d) mean significant difference ($P < 0.05$) between different protein samples. (C) Emulsifying activity and emulsifying stability of different protein samples. Different lowercase superscripts (a-f) mean significant difference ($P < 0.05$) between different protein samples. (D) Water holding capacity of different protein samples. Different lowercase superscripts (a-f) mean significant difference ($P < 0.05$) between different protein samples.

(Fig. 5B). The reason may be that small peptides generated from enzymatic hydrolysis gave rise to weak and fragile aqueous films, which reduced the resistance of interfacial films to gravity and thus reduced foam stability (Song et al., 2021). In addition, due to the fragmentation of peptides after limited enzymatic hydrolysis, the formation of a large number of peptides increased the charges of proteins. The electrostatic effect generated by static charges could hinder the adsorption of proteins on the air bubble surface, which in turn reduced foam stability (Song et al., 2021). This phenomenon is in good agreement with the fact that the foam stability of protein hydrolysates decreased with a decline in the hydrophobic surface of proteins rather than the release of hydrophobic amino acids (García Arteaga, Apéstegui Guardia, Muranyi, Eisner, & Schweiggert-Weisz, 2020). Even though the foaming stability of the hydrolyzed proteins was reduced, the values were still higher than that of WPI.

3.6.3. Emulsifying activity and stability

The emulsifying activity of proteins can be determined using emulsifying activity index (EAI) and emulsifying stability index (ESI) (Zang, Yue, Wang, Shao, & Yu, 2019). As shown in Fig. 5C, WPI contains both hydrophilic and lipophilic groups. SPI and zein exhibited relatively low EAI and ESI values. The EAI and ESI of protein mixture treated by limited enzymatic hydrolysis were remarkably improved ($P < 0.05$). As enzymatic hydrolysis progressed, the molecular rigidity of proteins can be reduced as a result of conversion into smaller peptides, which facilitates increased molecular flexibility and more orderly arrangement at the oil-water interface (Wang et al., 2022; Wang, Cheng, et al., 2022). In addition, with the extension of enzymatic hydrolysis time, the hydrophobic residues embedded in the protein inner core are gradually exposed to interact with the oil/water interface, thereby reducing the interfacial tension and improving the emulsifying activity and emulsifying stability (Akharume et al., 2021). According to the data in Fig. 5C, the emulsifying stability of protein mixture after 30-min hydrolysis treatment was improved by 63.7% ($P < 0.05$), compared with the untreated protein mixture. The emulsifying activity was suggested to be closely related to the exposure of hydrophobic groups of proteins, so that proteins with high hydrophobicity had better emulsifying activity (Yin et al., 2008).

3.6.4. Water holding capacity

The texture and cooking performance of food products can be improved by increasing water holding capacity. Additionally, it has been suggested that the decreased water loss in meat products is closely tied to economic advantages (Nuñez et al., 2021). The results of water holding capacity of different protein samples are illustrated in Fig. 5D. The native protein samples (SPI, zein and mixture) exhibited a relatively low water holding capacity. However, protein samples by limited enzymatic hydrolysis (15 and 30 min) can result in a pronounced rise in water holding capacity. Furthermore, the longer hydrolysis time can generate more low MW peptides with more potency in holding water than high MW peptides. The excellent hydrophilicity of small peptide fractions corresponds to the higher water retention, and they can interact with water through hydrogen bonds (Cumby et al., 2008). However, more studies are still needed to investigate the underlying mechanism.

3.7. DPPH radical scavenging activity

Enzymatic hydrolysis of food-derived proteins can exert an impact on the antioxidant activity of proteins depending on the peptide sequence and exposure of terminal amino acid groups (Moghadam et al., 2020). The DPPH radical scavenging activity of different protein samples was determined and results are shown in Fig. 6. Overall, the untreated protein samples displayed a relatively low antioxidant activity, while DPPH radical scavenging activity of protein mixture was dramatically augmented after limited hydrolysis by producing more soluble peptides to scavenge DPPH radicals. The highest DPPH radical scavenging activity (37.3%) was achieved in protein mixture subjected to 30-min limited hydrolysis, suggesting that the extended hydrolysis time leads to enhanced antioxidant activity. This fact was attributed to the elevated accessibility of hydrogen ions generated during limited hydrolysis as well as the higher free radical scavenging activity compared to the untreated protein (Moghadam et al., 2020). The similar results concerning enzymatic hydrolysis of plant proteins led to enhanced antioxidant activity have also been reported for soy protein (Guan, Diao, Jiang, Han, & Kong, 2018) and walnut protein (Moghadam et al., 2020).

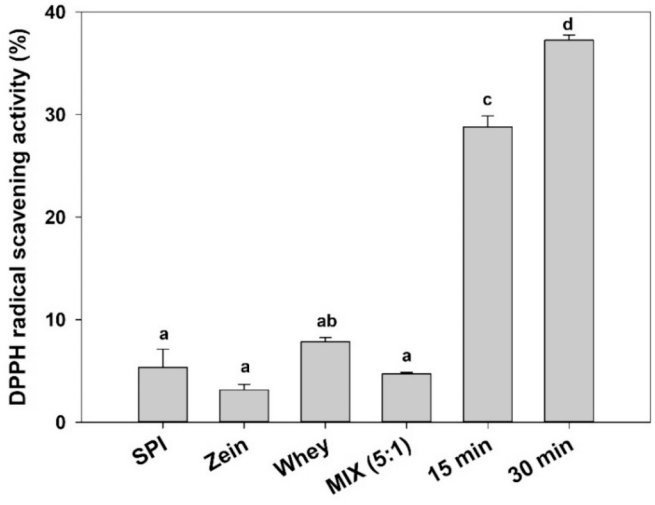


Fig. 6. DPPH radical scavenging activity of different protein samples. Different lowercase superscripts (a-d) mean significant difference ($P < 0.05$) between different protein samples.

4. Conclusion

The SPI-zein mixture (5:1, *w*/w) with the balanced essential amino acid composition was modified by papain-catalyzed limited enzymatic hydrolysis. The digestibility of protein mixture after 30-min hydrolysis was significantly increased, which was close to that of WPI. The SDS-PAGE and structural characterization of protein mixture revealed that proteins were partially degraded to peptides and exhibited a more disordered secondary structure. After limited enzymatic hydrolysis for 15–30 min, the solubility, foaming, emulsifying and water holding capacity of protein mixture were significantly increased. In addition, the antioxidant activity of the hydrolyzed protein mixture was also remarkably elevated. Overall, limited hydrolysis of soy protein-zein mixture led to the significantly elevated digestibility, functionality and antioxidant activity. However, more studies are still necessary to elucidate the structure-function relationship of the hydrolyzed protein mixture. The present study can provide a theoretical basis for the application of limited hydrolysis to modify plant protein mixture to assign the desired digestibility and functionality.

CRediT authorship contribution statement

Dongjing Wu: Investigation, Methodology, Validation, Writing – original draft. **Wei Wu:** Investigation, Validation, Writing – original draft. **Na Zhang:** Writing – review & editing. **Olugbenga P. Soladoye:** Writing – review & editing. **Rotimi E. Aluko:** Writing – review & editing. **Yuhao Zhang:** Resources, Writing – review & editing. **Yu Fu:** Conceptualization, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101550.

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