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Correlation between surface hydrophobicity changes and surface activity changes of soybean protein isolates caused by structural changes

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Abstract

The present work aimed to examine the association between the changes in the surface hydrophobicity and surface activity of soybean protein isolate (SPI) following structural alterations. To this end, the effects of heating, pH modification, ultrasonication, surfactant (SDS, sodium dodecyl sulphate) treatment, alkaline protease hydrolysis, peracetic acid treatment, and acylation on the surface activity and surface hydrophobicity of SPI were investigated. The results demonstrated that the changes in the surface hydrophobicity of SPI could accurately reflect the changes in its surface activity. A strong correlation between surface hydrophobicity and surface activity alterations was noted after the SPI molecules underwent structural modifications. The higher the surface hydrophobicity of the treated SPI, the greater its surface activity. Given that the surface hydrophobicity of SPI reflects the actual distribution of hydrophobic residues on its surface, this parameter can serve as a key indicator for evaluating and predicting changes in the surface activity of SPI.

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Introduction

Soybean protein is increasingly being used as a versatile raw material in various industries — including the food, agriculture, and biotechnology sectors — owing to its functional attributes, nutritional content, and low cost. The excellent surface activity of soybean protein is an important attribute, contributing to the development of soybean protein products, as it facilitates interfacial adsorption and thus enables the maintenance of phase stability while preventing phase separation. However, the inherent surface activity of most globular proteins is typically low due to the scarcity of hydrophobic groups on their molecular surface. Owing to the formation of secondary bonds, primarily hydrophobic interactions, proteins tend to orient their hydrophilic regions outwards in their natural states, shielding hydrophobic segments within the molecular core to minimise contact with surrounding water molecules. This allows the proteins to remain stable in an aqueous environment. Given that hydrophobic interactions drive the aggregation of non-polar groups in aqueous environments, they promote hydrogen bonding between the polar groups of proteins and water molecules, while reducing the area of contact between non-polar groups and water. Accordingly, they stabilise protein molecules in aqueous environments (Cardamone and Puri, 1992; Chandler, 2005). Consequently, proteins in their native states often exhibit suboptimal surface characteristics (Lam and Nickerson, 2013; Tang *et al*., 2021). The surface activity of soybean protein can be modulated by altering its secondary and tertiary structures, facilitating the exposure of hydrophobic groups on the surface of the protein, and adjusting the balance between hydrophilic and hydrophobic moieties (Yan *et al*., 2021a; Liu *et al*., 2022). Therefore, numerous studies have attempted to elucidate the relationship between structural modifications and changes in protein surface activity using diverse methodologies. However, changes in the spatial structure of a protein do not always correspond to changes in its surface activity.

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Soybean protein isolate (SPI) is a mixture of various protein components with different structures and physicochemical properties, and its conformational characteristics are complex and unstable. As a result, it is challenging to understand the correlation between the structure and surface activity of SPI (Wang *et al*., 2014; Jiang *et al*., 2015). In light of these complexities, identifying a pertinent factor that not only influences SPI function, but also correlates significantly with parameters governing surface activity, could enable us to simultaneously evaluate and predict the diverse functional properties of the protein.

Typically, the surface activity of conventional proteins primarily depends on the hydrophilic-lipophilic balance (HLB) of their molecular surface. Although protein molecules are amphiphilic and contain both hydrophilic and hydrophobic groups, most water-soluble spherical proteins do not exhibit optimal surface activity due to the lack of sufficient hydrophobic groups on their molecular surface (Lam and Nickerson, 2013; Tang *et al*., 2021). In early studies on protein hydrophobicity, the hydrophobicity of the protein was calculated based on the total hydrophobicity of the protein's amino acid side chains. However, this value failed to represent the true "effective hydrophobicity" on the surface of the protein molecule (Kato *et al*., 1981). Today, it is understood that only the number of hydrophobic groups distributed on the surface of a protein molecule determines its surface activity.

The fluorescent molecule 8-aniline-1-naphthalene sulfonate (ANS) only emits fluorescence in non-polar environments. Therefore, the real surface hydrophobicity of a protein molecule after conformational changes can be evaluated *via* the fluorescence-based measurement of the binding between the non-polar aniline-1-naphthalene group of ANS and the hydrophobic groups on the protein surface (Matulis *et al*., 1999). Kato and Nakai (1980) used this fluorescent probe to effectively measure the surface hydrophobicity changes in protein molecules after conformational alterations, and discovered a significant correlation between the changes in the surface hydrophobicity of protein molecules and their surface activity. Numerous studies have explored the association between surface hydrophobicity and surface activity in proteins, and a good relationship between the two parameters has been identified (Nakai *et al*., 1980; Voutsinas *et al*., 1983). Determining changes in surface hydrophobicity is simpler and often more accurate than evaluating complex structural changes. Hence, surface hydrophobicity appears to be a good measure of changes in protein surface activity (Nir *et al*., 1994).

After conformational changes, both the hydrophilic and hydrophobic moieties present on the protein surface can be altered. This leads to changes in the HLB of the protein molecules, and endows them with new surface activities, including wettability, solubilisation, emulsification, and foaming properties. Among them, emulsification activity (measured based on the emulsifying activity index [EAI]), emulsification stability (measured based on the emulsion stability index [ESI]), foaming properties (measured based on foaming capacity [FC]), and foaming stability (FS) are the most important and commonly used parameters. Importantly, these parameters are closely related to the real surface hydrophobicity of protein molecules.

Therefore, in the present work, ANS was employed as a fluorescent probe to measure the real surface hydrophobicity changes in protein molecules following conformational modification. The correlation between changes in the surface hydrophobicity and surface activity of SPI induced by structural changes due to sonication, heating, pH modification, sodium dodecyl sulphate (SDS) treatment, alkaline protease hydrolysis, peracetic acid treatment, and acylation, was analysed. The aim of the present work was to verify whether surface hydrophobicity can serve as a key indicator of protein surface activity in order to guide the development of protein-based surfactants in the future.

Materials and methods

Materials and instruments

Materials

SPI was obtained from Shandong Fuwangjia Biotechnology Co., Ltd.; o-phthalaldehyde (OPA) (Sigma), bovine serum protein, β-mercaptoethanol, and Coomassie brilliant blue (G250) were obtained from Sinopharm Chemical Reagents Ltd.; ANS was obtained from Aladdin Biotechnology Co., Ltd.; alcalase (alkaline protease), glycine, and lysine were obtained from Shanghai Yuanye Biological Co., Ltd.; and soybean oil was obtained from Yihai Kerry Grain and Oil Co., Ltd. Other chemicals and solvents were of analytical grade, and purchased from Guangzhou Xilong Chemical Co. Ltd.

Instruments

The following detection and analytical instruments were used in the present work: centrifuge (Avanti J-26 XPI, USA), freeze dryer (Christ Alpha 1-4LDplus, Germany), UV-Vis spectrophotometer (Cary-60, USA), fluorescence spectrophotometer (Shimadzu F-320, Japan), high-speed shear mixing emulsifier (100LX, China), magnetic stirrer (DF-101S, China), and pH meter (PHS-25CW, China).

Methods

Preparation of samples

SPI solution

SPI was dissolved in 0.2 mol/L, pH 7.5 phosphoric acid buffer at a 3% (w/v) ratio. The mixture was stirred at 25°C for 90 min, and then centrifuged at 4,000 *g* for 20 min to remove any insoluble substances. The supernatant was used for subsequent experiments.

Heating

The SPI samples (50 mL, 3% w/v) were heat-treated in a water bath at 20, 25, 40, 55, 70, 85, and 100°C for 60 min. Then, they were cooled in an ice water bath for 15 min before testing.

pH modification

The pH of the SPI samples (50 mL, 3% w/v) was adjusted to 2, 4, 6, 7, 8, 10, and 12 using 2 mol/L HCl or 2 mol/L NaOH. The samples were maintained at these pH values for 4 h at 4°C. Then, the pH of each solution was adjusted to 7, and the volume was increased to 100 mL using 2 mol/L NaCl.

SDS treatment

The SPI samples (50 mL, 3% w/v) were treated with SDS solutions with concentrations of 0, 0.1, 0.2, 0.4, 1, 1.4, and 1.8% for 12 h at 4°C in the dark. The samples were finally freeze-dried for subsequent detection.

Ultrasonication

The SPI samples (50 mL, 3% w/v) were treated with a 500 W power ultrasonic device for 0, 5, 10, 20, 30, 40, and 80 min before testing.

Enzymatic hydrolysis

The SPI samples (50 mL, 3% w/w) were treated with alcalase, and the pH was adjusted to 7, 7, 7, 8, 8, and 8, respectively, using 2 mol/L HCl or 2 mol/L NaOH. The samples were stirred for 30, 30, 60, 120, 240, and 360 min, respectively. After the hydrolysis reaction was completed, the treated SPI samples were heated in a boiling water bath for 10 min to inactivate the enzyme. After cooling to room temperature, the mixture was centrifuged at 10,000 *g* for 20 min to remove insoluble materials. The supernatant was freeze-dried for subsequent detection.

Peracetic acid

The SPI samples (50 ml, 3% w/v) were treated and adjusted with peracetic acid solution to a fixed volume of 100 mL, and to peracetic acid concentrations of 0, 0.1, 0.5, 0.75, 1.5, 3, or 5%, respectively, for 12 h at 4°C in the dark, and were finally freeze-dried for subsequent detection.

Acylation

The SPI samples (50 ml, 3% w/v) were treated and adjusted with succinic anhydride to a fixed volume of 100 mL, and to succinic anhydride concentrations of 0, 0.1, 0.5, 1, 5, 10, or 15%, respectively, and stirred for 4 h at 50°C. After 48 h of flowing water dialysis at room temperature, the treated SPI samples were freeze-dried for subsequent detection.

Determination of protein concentration

The method developed by Buroker-Kilgore and Wang (1993) was used to measure the spectral changes, with slight modifications. First, 0.1 g of Coomassie brilliant blue (G250) was dissolved in 50 mL of 90% ethanol and 100 mL of 85% (v/v) phosphoric acid. The volume of the mixture was adjusted to 1,000 mL with distilled water, and stored in a brown bottle protected from light. The protein samples were diluted ten-fold, and mixed with Coomassie brilliant blue at a 1:1 (v/v) ratio, and the absorbance of the solutions was measured at 595 nm. Bovine serum protein was used as the control.

Determination of degree of hydrolysis (DH)

A modified version of the method developed by Adler-Nissen (1976) was employed for the spectrophotometric analysis. During alkaline enzyme hydrolysis, the pH of the mixture was kept constant by adding 1 mol/L NaOH throughout the process. The DH was calculated using the PH-STAT method as shown in Eq. 1:

$$DH(\%) = \frac{N_b V}{\alpha M_P h_{hot}} \times 100 \quad \text{(Eq. 1)}$$

where, N_b = molar concentration of NaOH; V = volume of NaOH consumed by the reaction; α = average degree of dissociation of the α-amino group; M_p = substrate protein content; and h_{hot} = total number of hydrolysable peptide bonds in the protein.

Determination of disulphide bond cleavage rate

The determination was carried out using a method similar to that described by Li *et al*. (2019a). A 0.1% DTNB solution, a 0.1% NTSB solution, and a 2 mg/mL glutathione standard solution were prepared for subsequent experiments. The oxidised SPI (0.4 mL) and DTNB solution (0.4 mL) were first mixed at room temperature (25°C) for about 20 min. Then, the mixture was diluted with phosphate buffer (pH 8.0) to 10 mL for spectroscopic analysis. Spectral absorbance was measured using a UV-Vis spectrophotometer (Cary-60, USA) at 412 nm. A mixture containing 0.4 mL of SPI oxide and 0.4 mL of NTSB solution was prepared in the same manner to measure the total disulphide and sulfhydryl content. A standard curve was generated using different concentrations of glutathione, as shown in Eq. 2:

$$y = 0.0278x - 0.0025 \ (R^2 = 0.9977) \quad \text{(Eq. 2)}$$

where, y = absorbance at 412 nm, and x = mass concentration of reduced glutathione (mg/mL). The thiol concentration in oxidised SPI was calculated using Eq. 3:

$$\text{Thiol concentration (mol/L)} = \frac{0.0278x - 0.0025}{307.32} \quad \text{(Eq. 3)}$$

where, molecular mass of reduced glutathione = 307.32 g/mol. The disulphide bond cleavage rate of oxidised SPI was calculated using Eq. 4.

$$\partial = \frac{x_1 - x_2}{x_1} \quad \text{(Eq. 4)}$$

where, ∂ = disulphide bond cleavage rate; x_1 = disulphide bond content before modification; and x_2 = disulphide bond content after modification.

Determination of degree of acylation (DA)

First, 1 mL of ninhydrin solution was added to the protein sample, and the mixture was heated in a boiling water bath for 16 min before being cooled in a water bath to 20°C. Then, 5 mL of KIO_3 was added to the treated samples, and the optical density of the mixture was measured at 570 nm. The ninhydrin solution was used as a control to measure the degree of reaction between the free amino groups and the ninhydrin solution (Pan *et al*., 2020). The DA was calculated using Eq. 5:

$$DA\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{(Eq. 5)}$$

where, A_0 and A_1 = absorbance values of the samples before and after succinylation, respectively.

Intrinsic fluorescence emission spectroscopy

Analysis was carried out using a method similar to that employed by Li *et al.* (2019a). The samples were centrifuged at 4,000 *g* for 20 min, and the supernatant was fixed at 0.1 mL to 10 mL. The intrinsic fluorescence spectra of the above samples were measured using a fluorescence spectrophotometer. The excitation wavelength was set at 290 nm, the slit widths were all 5 nm, and the scanning range was 300 - 420 nm.

Determination of surface hydrophobicity

A slightly modified version of the method originally developed by Hayakawa and Nakai (1985) was adopted to determine the surface hydrophobicity. The surface hydrophobicity of SPI, before and after treatment, was determined using ANS as a fluorescent probe. First, 20 μL of ANS (8 mm/L) were added to samples of different concentrations. The fluorescence spectra of these samples were obtained using a fluorescence spectrophotometer. The slope of the initial phase of the curve (H_0) was considered the surface hydrophobicity index of the samples.

EAI and ESI

The EAI and ESI were determined based on the method developed by Pearce and Kinsella (1978). First, 15 mL of soybean oil was mixed with 15 mL of the treated samples, and sheared at 10,000 rpm for 1 min at room temperature. Then, 100 μL of the protein emulsion was added to 10 mL of 0.1% SDS solution, and mixed well. Subsequently, the absorbance values were measured at 0 and 30 min at 500 nm, using 0.1% SDS solution (without any of the protein emulsion) as the control. The EAI and ESI were calculated using Eqs. 6 and 7, respectively:

$$\text{EAI } (m^2/g) = 2T \times \frac{A_0 \times N}{C \times \varphi \times 10000} \quad \text{(Eq. 6)}$$

$$\text{ESI (min)} = \frac{A_0}{A_0 - A_{30}} \times 30 \quad \text{(Eq. 7)}$$

where, A_0 and A_{30} = absorbance values of the samples at 500 nm at 0 and 30 min, respectively; C = concentration of the protein sample (g/mL); Φ = volume fraction of oil in the experimental emulsion (0.5); T = 2.303; and N = dilution factor.

FC and FS

The FC and FS were determined as described by Li *et al*. (2019b). First, 10 mL of the treated samples were diluted with distilled water to a volume of 50 mL before being homogenised at room temperature using a high-speed shear disperser at 10,000 rpm for 1 min. The total volume of the homogenate was recorded at 0 and 30 min after homogenisation. The FC and FS of the protein were calculated using Eqs. 8 and 9, respectively:

$$\text{FC } (\%) = \frac{V_0}{50} \times 100\% \quad \text{(Eq. 8)}$$

$$\text{FS } (\%) = \frac{V_{30}}{V_0} \times 100\% \quad \text{(Eq. 9)}$$

where, V_0 = volume of the foam at 0 min (mL), and V_{30} = volume of the foam at 30 min (mL).

Determination of surface tension and critical micelle concentration (CMC) values

In order to determine the relationship between the CMC and surface tension of SPI, an automated surface tension meter was employed. First, samples of different SPI concentrations were prepared. Each sample was analysed thrice, and the average value of three measurements was taken. Subsequently, a graph of concentration (X-axis) *versus* surface tension (Y-axis) was generated. The inflection point in the relationship between surface tension and concentration, after which there was minimal change in the surface tension, was considered the CMC value of the sample.

Statistical analysis

All analytical and graph preparation procedures were carried out using Origin software (version 8.0, Origin Lab, USA). The correlation between functional properties and surface hydrophobicity was determined based on Pearson correlation coefficient analysis and Principal Component Analysis (PCA), performed using SPSS (version 22.0, USA).

Results and discussion

Heating

Intrinsic fluorescence is commonly used to detect hydrophobic amino acids (*e.g.*, tryptophan [Trp]), and thus estimate changes in the spatial structure of a protein. As shown in Figure 1a, the fluorescence intensity of SPI increased as the reaction temperature increased from 25 to 70°C, with λ_{max} values of 342, 343, 346, and 350 nm, respectively. This could have been due to a heat-induced alteration in the normal structure of SPI molecules (α-helix, β-fold, *etc*.), resulting in the unfolding of the tertiary structure, and the exposure of hydrophobic fragments on the surface of the SPI molecules (Ulrichs *et al*., 2015). As a result, the intrinsic fluorescence peak was enhanced, and a red shift occurred. Interestingly, the fluorescence intensity decreased as the treatment temperature increased from 70 to 100°C (λ_{max} of 350, 348, and 346 nm). This indicated that when the heating temperature was too high, the secondary structure of SPI became highly disordered. Excessive unfolding of the spatial structure could lead to the rearrangement and aggregation of SPI molecules, allowing the hydrophobic groups to be buried again. As a result, the fluorescence intensity decreased, and a blue shift occurred (Wang *et al*., 2012).

Collectively, the findings showed that the surface hydrophobicity of SPI increased with an increasing reaction temperature, and peaked at 70°C, before decreasing as the temperature increased (Figure 1b). In effect, the results indicated that heat treatment loosened and expanded the structure of SPI molecules, exposing more hydrophobic regions (favourable for ANS probe binding), and increasing hydrophobicity. However, when the temperature was too high, the relaxed globular proteins aggregated again, and the previously exposed hydrophobic groups were re-buried within the molecule. Hence, there was a decrease in the hydrophobic groups present on the surface of the SPI molecules, which reduced surface hydrophobicity (Wang *et al*., 2014).

As shown in Figure 1c, the emulsification and foaming capacity of SPI showed a similar trend as that of its surface hydrophobicity. Both emulsification and foaming capacity increased with increasing treatment temperature up to 70°C, and then

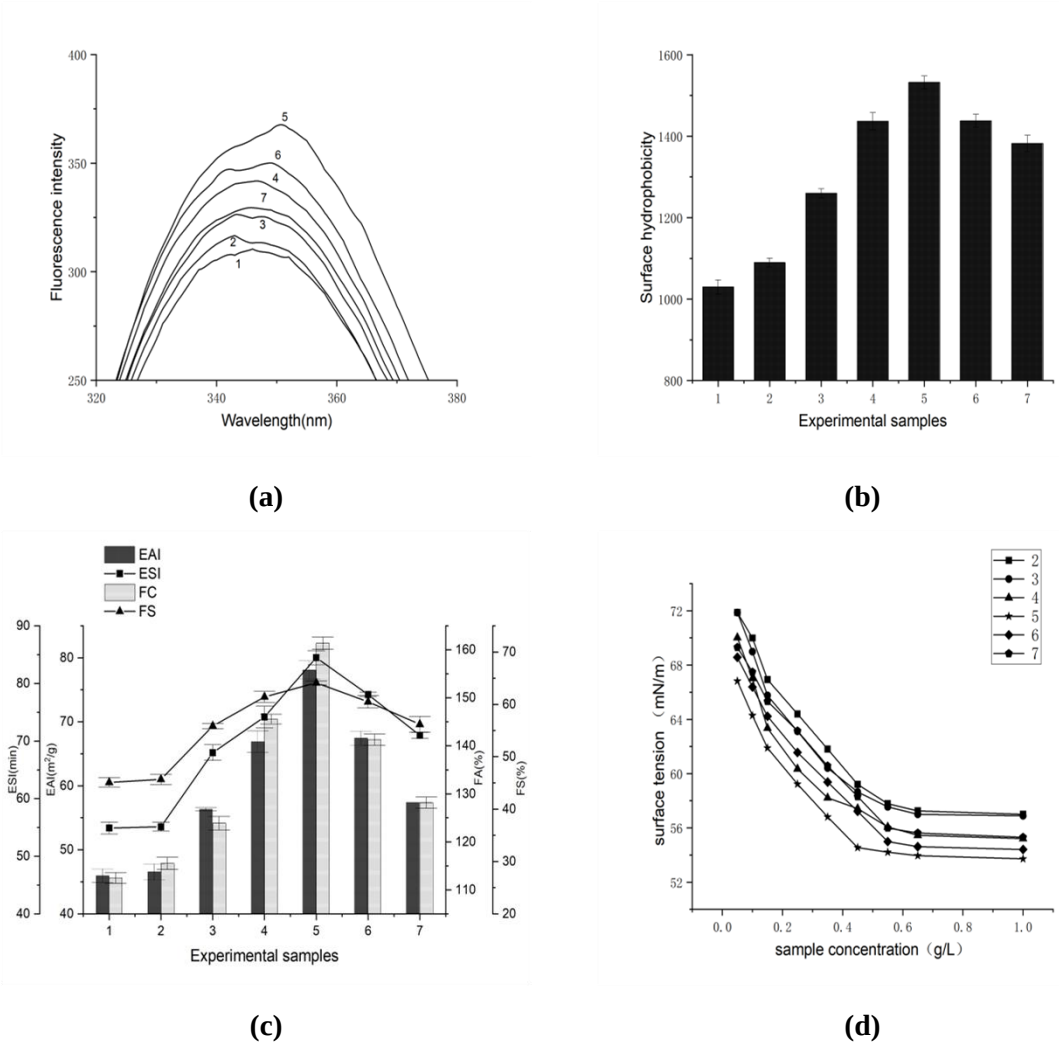

Figure 1. Intrinsic fluorescence emission spectra **(a)**, surface hydrophobicity **(b)**, surface activities **(c)**, and surface tension **(d)** of SPI after heating. 1 - 7 in **(a)**, **(b)**, **(c)**, and **(d)** correspond to heating temperatures of 20, 25, 40, 55, 70, 85, and 100°C, respectively.

decreased thereafter. As the temperature increased, the secondary structure was disrupted, loosening the SPI molecules, and exposing internal hydrophobic groups. As a result, the SPI molecules achieved better interfacial ability, and increased emulsification and foaming capacity. In contrast, when the temperature was too high, the protein likely underwent re-agglomeration, and the aggregates impeded the increase in its surface activity (Ren *et al*., 2009).

The surface tension of SPI decreased after the heat treatment, and the CMC also showed a decrease. This indicated that heat treatment could reduce the surface tension of SPI, and enhance its ability to form micelles at the air interface, thus increasing its surface activity, and changing its properties to further improve its application potential.

pH modification

Although the fluorescence intensity of SPI was higher at pH 2 than at pH 4, it gradually increased from pH 4 to 12 (λ_{max} of 345, 339, 342, 343, 346, and 348 nm) because the isoelectric point of SPI is pH 3.5 - 4. Under its natural state, the pH of SPI dissolved in water was found to be around 7.5, as shown in Figure 2a.

Near the isoelectric point, the spatial structure of a protein becomes more compact, and chromospheres such as Trp get buried inside the protein. Hence, proteins may undergo structural de-folding, and a subsequent rearrangement of de-folded proteins (to form a tighter structure) may occur as the surface charge of the protein changes from negative to positive due to a shift in pH (Chen *et al*., 2016). In

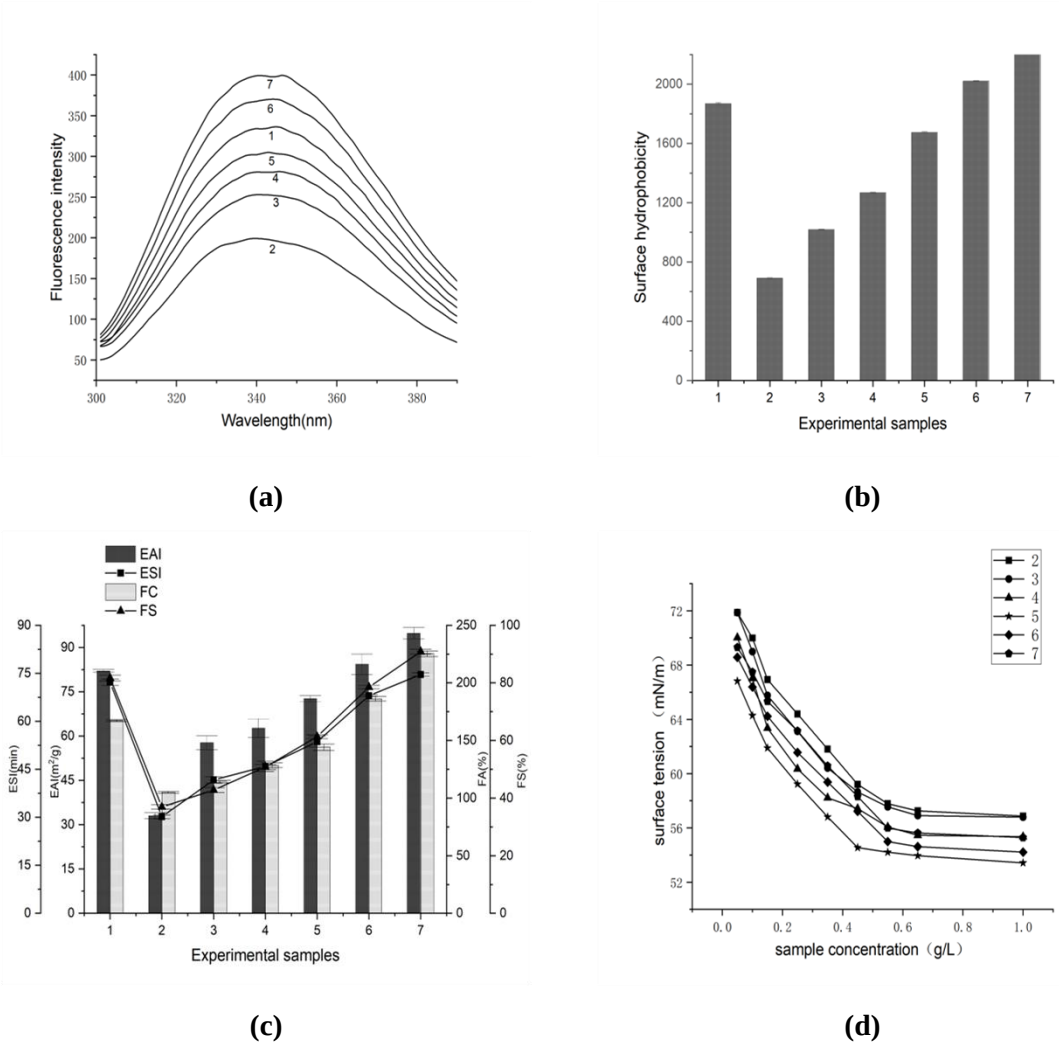

Figure 2. Intrinsic fluorescence emission spectra **(a)**, surface hydrophobicity **(b)**, surface activities **(c)**, and surface tension **(d)** of SPI after different pH-shifting. 1 - 7 in **(a)**, **(b)**, **(c)**, and **(d)** correspond to treatment conditions of pH 2, 4, 6, 7, 8, 10, and 12, respectively.

the present work, the fluorescence intensity increased as the pH became further from the isoelectric point. At pH 2, 10, and 12, the internal structure of the SPI molecules was disrupted, leading to a gradual unfolding of the protein structure, and the exposure of internal hydrophobic groups, enhancing fluorescence intensity.

Figure 2b shows that the hydrophobicity of SPI was the lowest near its isoelectric point, and then increased with an increase in pH. Changes in pH alter protein structure, causing an increase in electrostatic repulsion to affect the unfolding and stretching of the protein's spatial structure (Xu *et al*., 2021). This increase was more pronounced under alkaline conditions than under acidic conditions due to the greater shift from the isoelectric point, resulting in stronger intramolecular electrostatic repulsion, more extensive unfolding, and higher protein hydrophobicity.

The pH-related changes in the emulsification and foaming capacity of SPI were also broadly similar to the changes observed in surface hydrophobicity, as shown in Figure 2c. This was mainly because the interfacial properties of SPI treated with different pH conditions were better than those of natural SPI. The stretching of the spatial structure exposed the non-polar groups of treated SPI molecules, resulting in increased protein surface activity (Tan *et al.*, 2021). Weaker intermolecular interactions caused by electrostatic repulsion under acidic or alkaline conditions create a more flexible protein conformation. After pH modification, the protein structure swells and becomes more flexible, which improves its emulsification properties. In

addition, in the present work, the pH treatment also significantly improved the emulsion stability of the proteins. The ability of the proteins to stabilise emulsions was significantly higher after alkali treatment than after acid treatment, which may be due to the solubility of the acid-treated protein and its surface hydrophobicity (Yan *et al.*, 2021a). At the same time, the exposure of hydrophobic groups and the increased molecular flexibility contributed to the foaming activity and stability of SPI (Li *et al*., 2019a; Luis and María, 2015).

Both acid and alkali treatments led to a very significant improvement in the surface tension of SPI. This was because the tertiary conformation of SPI changed, and the proteins partially unfolded during treatment. The hydrophobic fragments and groups originally buried in the interior of the molecule were exposed to the surface, reducing the surface tension. When the protein modification became excessive, the molecular structure was completely opened, the spatial structure was loosened, and the molecules became rearranged due to hydrophobic and intermolecular interactions. Subsequently, the hydrophobic fragments became regrouped to generate a hydrophobic core that was completely surrounded by hydrophilic fragments.

Ultrasonication

As shown in Figure 3a, the fluorescence intensity of the samples increased continuously (λ_{max} of 343, 345, 346, and 347 nm) as the duration of ultrasonic treatment increased from 0 to 20 min. This may be related to the cavitation and shear stress caused by ultrasound, which changed the three-dimensional structure of proteins. This resulted in the exposure of hydrophobic groups within the protein molecules, and also caused the swelling of the protein molecules. Therefore, the fluorescence intensity was enhanced after ultrasonication, and a red-shift was observed (Liu *et al*., 2022). When the samples were sonicated for 40 to 80 min, the fluorescence intensity decreased, and a blue-shift was detected. Excessive ultrasound treatment can cause the previously exposed hydrophobic groups of proteins to re-agglomerate, and form a more stable spatial structure, leading to a decrease in the exposed hydrophobic groups on the protein surface, and thereby reducing surface hydrophobicity (Huang *et al.*, 2019).

Figure 3b shows that appropriate ultrasonic treatment could induce a certain degree of unfolding in SPI molecules, thus increasing the number of hydrophobic moieties exposed on the surface of the SPI molecules. In contrast, excessive ultrasonic treatment could cause partial protein denaturation and repolymerisation *via* hydrophobic bonds and other interactions, thus reducing the hydrophobicity of the protein's surface (Sui *et al.*, 2017).

Ultrasound treatment for 20 min could improve the EAI and ESI of SPI. This duration of treatment also provided the highest EAI and ESI values. In contrast, prolonged ultrasound treatment could reduce the EAI and ESI of SPI, as shown in Figure 3c. This may be because ultrasound promotes protein unfolding, making protein molecules more flexible, promoting adsorption, and creating a more stable structure at the water-oil interface. As a result, the EAI and ESI increased (Yan *et al*., 2021b; Liu *et al*., 2022). However, excessive ultrasonic treatment caused excessive disruption of the natural structure of SPI, leading to the re-aggregation of exposed hydrophobic groups, and a reduction in EAI and ESI, in line with previous findings regarding intrinsic fluorescence (Li *et al*., 2019a; Yan *et al*., 2021b).

In the present work, the foaming and emulsifying properties of ultrasonically treated SPI showed a similar trend to that of its surface hydrophobicity. This may be because ultrasound pre-treatment can induce stretching of the protein molecule, bringing out the hidden hydrophobic moieties to the surface of the protein molecule. This increases adsorption and diffusion at the air-water interface, thus improving the foaming capacity of proteins (Dai *et al.*, 2022). However, we found that excessive ultrasonic treatment for more than 20 min caused a rapid decrease in the foaming capacity and foam stability of SPI. This may be because the rigid structure of SPI loosened with prolonged sonication, and SPI could only maintain its optimal foaming ability and stability when the flexible and rigid structures were present at the right ratio.

Collectively, our findings indicated that due to ultrasonic treatment, protein molecules could undergo conformational changes that loosened their molecular structures. The hydrophilic and hydrophobic groups within the molecules were rearranged until an inflection point, which represents the CMC. These structural changes contributed to the surface activity of SPI, but excessive treatment induced protein aggregation, the refolding of protein subunits, and a decrease in the surface activity of SPI.

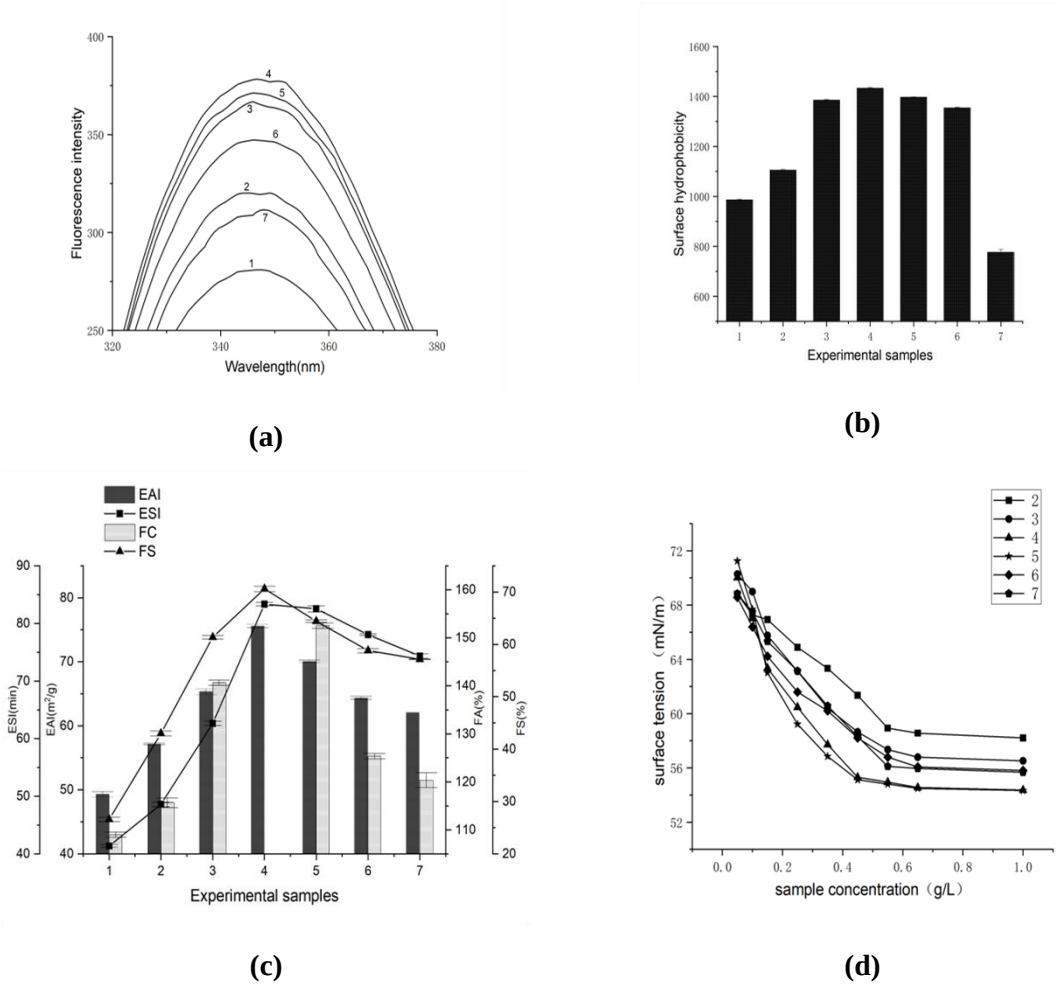

Figure 3. Intrinsic fluorescence emission spectra **(a)**, surface hydrophobicity **(b)**, surface activities **(c)**, and surface tension **(d)** of SPI after different ultrasonication. 1 - 7 in **(a)**, **(b)**, **(c)**, and **(d)** correspond to ultrasound durations of 0, 5, 10, 20, 30, 40, and 80 min, respectively.

SDS treatment

Figure 4a shows that the fluorescence intensity of SPI increased as the SDS concentration increased from 0 to 1%. However, it subsequently decreased as the SDS concentration increased from 1 to 10%. As an anionic surfactant, SDS disrupts hydrogen bonds and hydrophobic interactions when it interacts with proteins, thus altering the protein conformation (Krainer *et al*., 2020). The large negative charge of SDS greatly exceeds that of most native proteins. Thus, after SDS binds to proteins, it masks the charge differences between proteins, and confers all proteins with a similar density of negative charge (Mahanta *et al*., 2010). When the SDS concentration is too high, the surfactants interact with each other to produce micelles that encapsulate protein molecules. Therefore, the fluorescence intensity associated with hydrophobicity decreases, and the λmax shifts to a shorter wavelength.

As shown in Figure 4b, the surface hydrophobicity of SPI also increased as the SDS concentration increased from 0 to 1%. However, the surface hydrophobicity of SPI gradually decreased as the SDS concentration increased from 1 to 10%. A previous study examining structural changes in proteins after SDS treatment revealed that when the SDS concentration was less than 1%, SDS unfolded SPI molecules. Hence, the hydrophobic groups previously buried inside the molecule were exposed, and the surface hydrophobicity of SPI increased (Huang and Sun, 2000). In contrast, when the concentration of SDS was too high, the surfactant interacted with the proteins, and formed micelles surrounding the protein molecules. As a result, the

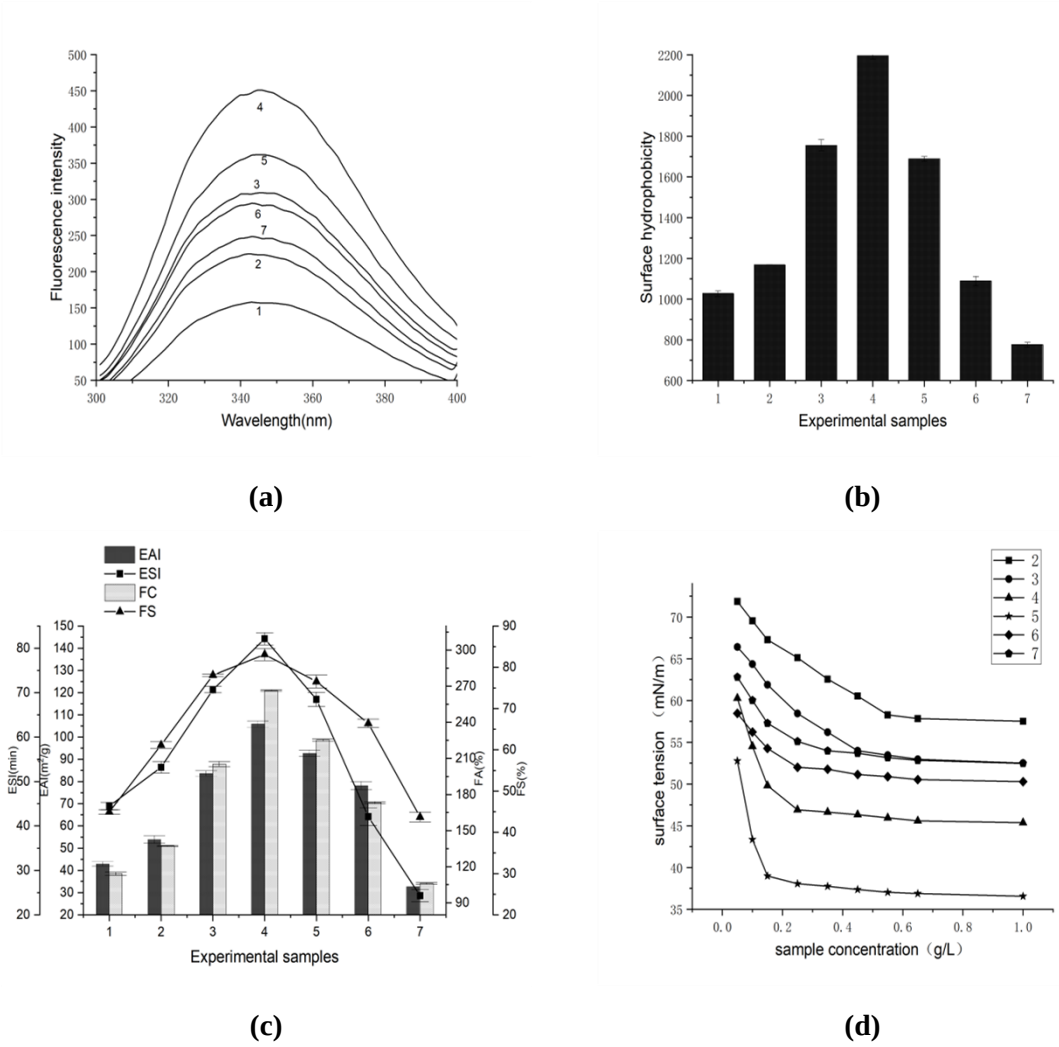

Figure 4. Intrinsic fluorescence emission spectra **(a)**, surface hydrophobicity **(b)**, surface activities **(c)**, and surface tension **(d)** of SPI after different SDS treatments. 1 - 7 in **(a)**, **(b)**, **(c)**, and **(d)** correspond to SDS treatment concentrations of 0, 0.1, 0.5, 1, 5, 10, and 15%, respectively.

hydrophobic groups on the SPI surface were covered by negatively charged micelles. ANS was unable to bind to the hydrophobic regions due to micelle coverage, reducing the readout of surface hydrophobicity (Maikokera and Kwaambwa, 2007).

As shown in Figure 4c, the changes in the emulsification and foaming properties of SPI after SDS treatment were similar to those observed with surface hydrophobicity. The stretching of the natural molecular structure of SPI had a positive effect on emulsification and foaming capacity. After molecular stretching, the emulsifying properties were enhanced. However, when the protein molecules were encapsulated by micelles and the hydrophobic moieties were not effectively exposed, the surface properties deteriorated.

As shown in Figure 4d, following SDS treatment, protein molecules underwent conformational changes, which made their molecular structure less compact. The hydrophilic and hydrophobic groups within the molecules were rearranged, generating an inflection point corresponding to the CMC. Such structural changes contributed to the surface activity of SPI. However, excessive treatment led to protein aggregation, the refolding of protein subunits, and a decrease in the surface activity of SPI.

Enzymatic hydrolysis

As shown in Figure 5a, when the DH value of SPI ranged from 0 to 10.84%, the fluorescence intensity of the molecule increased. The fluorescence

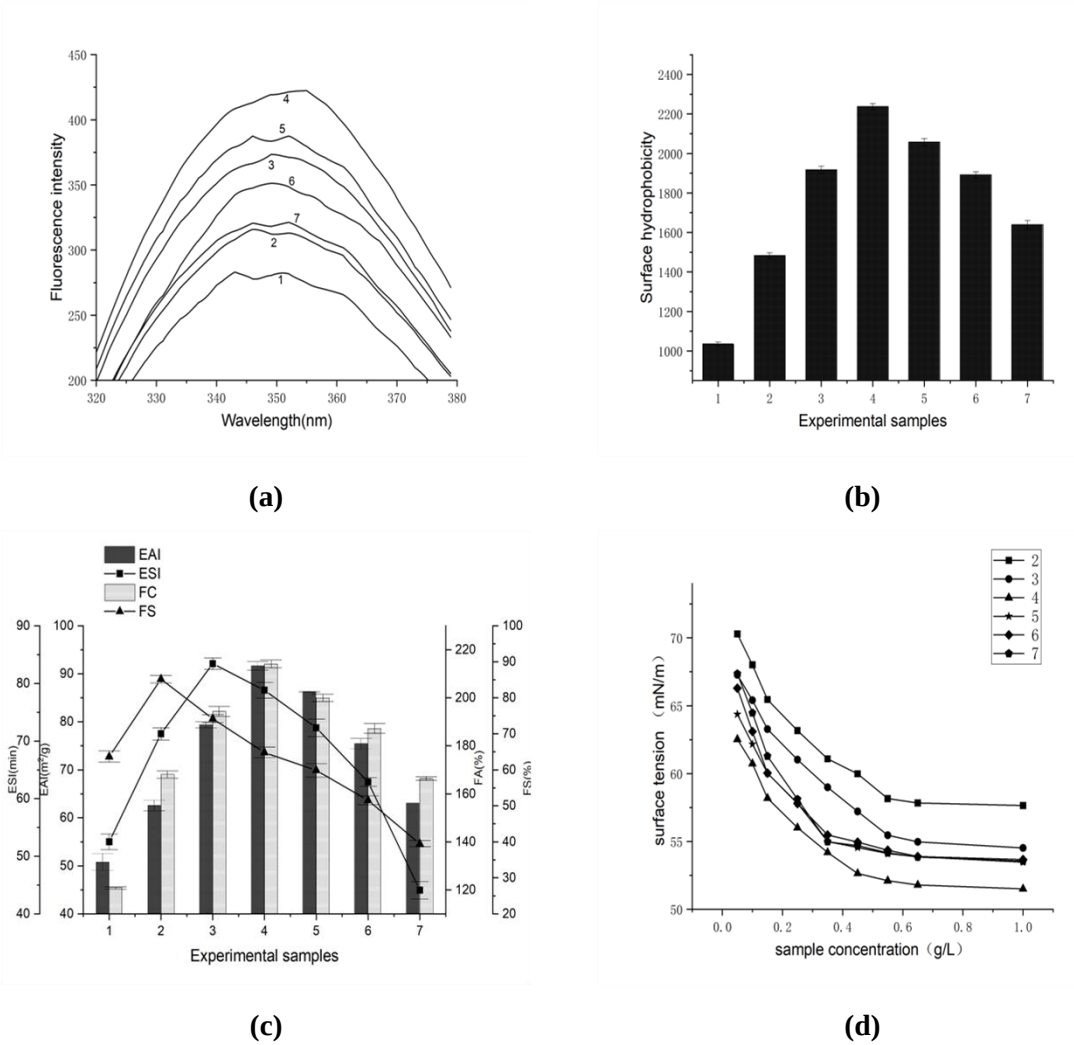

Figure 5. Intrinsic fluorescence emission spectra **(a)**, surface hydrophobicity **(b)**, surface activities **(c)**, and surface tension **(d)** of SPI after different enzymatic. 1 - 7 in **(a)**, **(b)**, **(c)**, and **(d)** correspond to DH values of 0, 2.24, 6.38, 10.84, 13.25, 16.32, and 19.83%, respectively.

intensity peaked when the DH value was 10.84%, and a red-shift phenomenon was detected at this stage. After the DH value of SPI reached 10.84%, the fluorescence intensity of SPI decreased with an increase in the DH value, and a blue-shift phenomenon was detected at this stage. An appropriate duration of enzymatic hydrolysis can make the natural structure of proteins less compact, allowing the overall structure of SPI molecules to become looser and more diffuse. As a result, more hydrophobic groups from the interior of these molecules can get exposed on the protein surface. Therefore, the fluorescence intensity of SPI changes significantly before and after enzymatic hydrolysis, showing a red-shift or a blue-shift depending on the DH. Excessive enzymatic hydrolysis causes numerous peptide fragments to reform and reorganise. Additionally, some exposed hydrophobic groups are reabsorbed into the interior of the SPI molecules due to hydrophobic interactions, thus decreasing the fluorescence intensity of SPI (Zhang *et al.*, 2015).

Figure 5b demonstrates that the surface hydrophobicity of SPI also changed in line with its intrinsic fluorescence intensity after enzymatic hydrolysis. When the hydrophilic portion of the SPI molecule was gradually hydrolysed by alcalase during the early stage of enzymatic hydrolysis, the hydrophobic groups within SPI gradually became exposed. As a result, the number of hydrophobic regions and sites suitable for ANS probe binding increased. However, following excessive hydrolysis, SPI molecules of different sizes underwent rearrangement *via* hydrophobic interactions. Hence,

the hydrophobic regions suitable for ANS binding were buried within the core of the SPI molecules again. Therefore, the surface hydrophobicity decreased (Ren and Li, 2022).

As shown in Figure 5c, the emulsification activity and foam stability of SPI increased gradually with the increase in the DH until the DH reached 10.84%. An appropriate level of enzymatic hydrolysis can enable the exposure of hydrophobic groups on the surface of SPI molecules, making the molecules soft and elastic, and improving their emulsifying activity and foam stability (Zhang *et al*., 2015). However, excessive enzymatic hydrolysis induces the reassembly of SPI fragments hydrolysed by enzymes, and the exposed hydrophobic groups are once again buried in the interior of the protein. This leads to a decline in surface activity. The molecular fragments of SPI become smaller with an increase in the DH, and the presence of small molecular fragments is not conducive to emulsification and foam stability (Luis and María, 2015).

Figure 5d shows that the surface tension of SPI was reduced after enzymatic treatment, and the CMC also showed a decrease. This indicated that enzymatic treatment could decrease the surface tension of SPI, and enhance its ability to form micelles at the air interface, thereby increasing its surface activity, changing its properties, and enhancing its application prospects.

Peracetic acid treatment

Peracetic acid can oxidise and cleave the S-S bonds in protein molecules through oxidation, and further generate sulfonic acid residues (R-SO_3H), preventing the restoration of broken disulphide bonds. Hence, peracetic acid can be used to cleave disulphide bonds in protein molecules in a controlled manner (Fan *et al*., 2019). The fluorescence intensity of SPI increased with increasing disulphide bond cleavage, and a red-shift appeared within a disulphide bond cleavage rate of 0 to 37.28%. This implied that the reaction of SPI with peracetic acid promoted molecular unfolding, making the protein molecules more flexible and less compact and exposing the hydrophobic groups originally hidden within the protein core (Wang *et al*., 2016). Interestingly, at a disulphide bond cleavage rate of 37.28 to 96.55%, the fluorescence intensity of SPI decreased continuously with increasing disulphide bond cleavage, and a blue-shift was noted. This may be related to the molecular rearrangement of SPI molecules due to excessive disulphide bond cleavage, which causes the surface hydrophobic groups to be buried within the interior of SPI molecules again.

The change in the surface hydrophobicity of SPI followed a similar trend as the change in its intrinsic fluorescence. Both these parameters first showed an upward trend before peaking, and subsequently decreasing with increasing disulphide bond cleavage. The increase in the surface hydrophobicity of SPI may be related to the unfolding of SPI molecules, wherein the oxidation of disulphide bonds made the molecules less compact and eliminated structural constraints. The subsequent decrease in the surface hydrophobicity of SPI may be related to the rearrangement of the molecules in the presence of hydrophobic effects due to excessive disulphide bond cleavage.

The surface activity of SPI increased with the increase in disulphide bond cleavage. The surface activity of SPI peaked when the disulphide bond cleavage reached 37.28%. When the disulphide bond cleavage increased from 37.28 to 96.55%, the surface activity of SPI did not increase, but instead showed a decrease due to excessive disulphide bond cleavage. Only an appropriate degree of oxidation can ensure optimal protein unfolding, exposing more hydrophobic groups on the surface of SPI molecules and generating soluble aggregates, thus improving the surface activity of SPI. The flexibility and diversity of protein aggregates allow them to accumulate at the air interface and form a thicker adsorption layer, resulting in better emulsification performance and stability (Chen *et al*., 2013). Furthermore, an appropriate amount of disulphide bond cleavage can significantly improve the foaming capacity of oxidised SPI, whereas excessive oxidation can induce the refolding of unfolded polypeptide chains, and result in the formation of large aggregates, thus decreasing the foaming capacity of SPI (Li *et al*., 2019a).

In summary, peracetic acid treatment was found to significantly improve the surface tension of SPI. The results indicated that after peracetic acid treatment, the protein molecules underwent a conformational change that made their molecules less compact. Meanwhile, the hydrophilic and hydrophobic groups within the molecules were rearranged to generate an inflection point representing the CMC. However, excessive peracetic acid treatment can induce protein aggregation and the refolding of protein subunits, release all molecular

structural constraints, loosen the spatial structure of the protein, and cause molecular rearrangement *via* increased hydrophobic and intermolecular interactions. Under these conditions, the hydrophobic fragments regroup to generate a hydrophobic core in the interior of the protein that is completely surrounded by hydrophilic fragments.

Acylation

The fluorescence intensity of SPI increased with the increase in the degree of acylation from 0 to 56.72%. Anionic succinyl groups not only change the charge distribution on the surface of native protein molecules but also alter the molecular structure of the protein. Hence, they cause an expansion of the protein structure, and induce conformational changes, leading to the exposure of buried hydrophobic groups on the protein's surface. This can enhance fluorescence intensity, and cause a red shift in the fluorescence spectrum (Zhao *et al.*, 2017).

The surface hydrophobicity of SPI molecules also increased with the increase in acylation from sample 1 to sample 4. Acylation can lead to the exposure of hydrophobic groups by reducing the positive charge of protein molecules, and enhancing the repulsion between protein subunits. It has been reported that the exposure of internal hydrophobic groups and the reduction in surface charges due to acyl modification promote the binding of ANS to protein molecules (Wang *et al*., 2018).

The changes in the surface activity of SPI induced by acylation were similar to the changes in its intrinsic fluorescence and surface hydrophobicity. On the one hand, acylation affects the native structure of SPI, and stretching protein molecules to enhance their flexibility. This decrease in protein compactness allows more internal hydrophobic groups to be exposed on the protein's surface, increasing the surface activity of SPI. On the other hand, acylation also changes the charge carried by SPI molecules, and enhances their interfacial properties. Thus, acylation significantly improves the emulsification and foaming activities of SPI.

Together, the results indicated that acylation led to a very obvious improvement in the surface tension properties of SPI. This is because the surface structure of SPI molecules is continuously changed during acylation, and the newly introduced moieties greatly improve their adsorption capacity at the air-water interface. Furthermore, the reaction process itself alters the tertiary conformation of the SPI molecules, and partially unfolds them. The restriction on the spatial structure is completely eliminated, allowing the original interior groups to move to the surface of the proteins. This greatly improves surface tension.

Correlations and discussion

After heat treatment, pH modification, ultrasonication, SDS treatment, alkaline protease treatment, peracetic acid treatment, and acylation, the surface hydrophobicity and the surface activity of SPI molecules showed similar changes. Moreover, there was a significant correlation between the surface hydrophobicity and surface activity (including emulsifying activity and stability, and foam activity and stability) of the SPI molecules. The greater the surface hydrophobicity of treated SPI, the better its surface activity. The purpose of the abovementioned treatments was to alter the spatial structure of SPI so as to expose the hydrophobic groups that were originally buried within the interior of the protein on the surface of the SPI molecules. This would ultimately not only change the distribution of hydrophobic groups on the surface of SPI, but also change its hydrophobicity and HLB, thus changing its surface activity, including its emulsifying activity and stability, and foam activity and stability (Wang *et al*., 2012; Liu *et al*., 2022).

The surface activity of a protein is largely determined by the balance of hydrophilic and hydrophobic groups on the protein's surface. Meanwhile, the surface hydrophobicity of a protein reflects the true state of hydrophobic groups on the molecular surface of the protein, and influences its surface activity (Damodaran, 1990). Therefore, the surface hydrophobicity of a protein plays an important role in evaluating and predicting the changes in its surface activity. Our results further demonstrated that surface hydrophobicity could serve as an ideal indicator for evaluating and predicting the surface activity of protein molecules.

PCA is an analytical method used to transform multiple indicators into a few composite indicators in order to derive representative data (Jahirul *et al*., 2021). KMO and Bartlett's Spherical Test are typically employed to verify whether a dataset is suitable for PCA. The KMO and Bartlett's spherical test revealed the following results. The KMO value was 0.797, indicating that the experimental data were suitable for PCA; the *p*-value derived from Bartlett's spherical test was 0.000, which was much lower than

the level of significance. Additionally, a correlation between surface hydrophobicity and surface activity was detected across various sets of data, which made them suitable for PCA.

The indicators related to surface hydrophobicity and surface activity (H0, EAI, ESI, FA, and FC) were subjected to PCA. The eigenvalue of principal component 1 (PC1) was greater than 1 (*i.e.*, 3.381), and PC1 explained 67.62% of the cumulative variance. Hence, this principal component appeared to be well-representative of most of the original data. The table of loading coefficients revealed that H0 (surface hydrophobicity) had the highest loading coefficient on PC1 (0.944), indicating that H0 had the most significant contribution to PC1. In addition, the loading coefficients of EAI, ESI, and FC on PC1 were also high — 0.924, 0.828, and 0.898, respectively — indicating that these indicators also have a strong correlation with PC1. In contrast, the relatively low loading coefficient of FA on PC1 may indicate its weak correlation with PC1. Collectively, the factor loading coefficients indicated that the surface hydrophobicity could effectively reflect the changes in surface activity indexes. Therefore, it can be concluded that the surface hydrophobicity of SPI is closely related to the surface activity of SPI.

Conclusion

The present work demonstrated that the changes in the surface hydrophobicity of SPI were positively correlated with the changes in the surface activity of SPI. The greater the surface hydrophobicity of the treated SPI, the greater its surface activity. The surface activity of SPI could be improved by removing structural constraints *via* alterations in the molecular structure of the protein molecules, allowing more hydrophobic residues to be exposed to the molecular surface. Surface hydrophobicity is one of the most important indexes related to the surface activity of proteins since it directly reflects the actual distribution of hydrophobic residues on the protein's surface. Our correlation analysis confirmed that the changes in the surface hydrophobicity of SPI were positively correlated with the changes in its surface activity after different treatments. Thus, surface hydrophobicity resulting from changes in molecular structure could serve as an ideal indicator for evaluating and predicting the changes in the surface activity of proteins, including SPI.

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