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yan liu

yanliu02046@163.com

College of Tea (Pu'er), West Yunnan University of Applied Sciences

Ruijun Li

College of Tea (Pu'er), West Yunnan University of Applied Sciences

yangyang qian

College of Tea (Pu'er), West Yunnan University of Applied Sciences

Wenshu Peng

College of Tea (Pu'er), West Yunnan University of Applied Sciences

Linchun Mao

College of Tea (Pu'er), West Yunnan University of Applied Sciences

Lifen Shi

Honghe University

Liang Tao

Yunnan Agricultural University

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Improvement of functional and antioxidant properties of Sacha Inchi protein through glycosylation with chitosan oligosaccharide

Yan Liu^{1#}, Ruijun Li^{1#}, Yangyang Qian¹, Wenshu Peng⁴, Linchun Mao¹, Lifen Shi^{3*}, Liang

Tao^{2*}

¹ College of Tea (Pu'er), West Yunnan University of Applied Sciences, Pu'er, 665000, China

² College of Food Science and Technology, Yunnan Agricultural University, Kunming, 650201, China

³ School of Biological and Food Engineering, Honghe University, Mengzi 661100, China

⁴ Puer Institute of Pu-Erh Tea, Pu'er 665000, China

These authors contributed equally to this work.

* Corresponding author.

E-mail address: 2017043@ynau.edu.cn

1 **Abstract:**

2 Sacha inchi (*Plukenetia volubilis* L.) protein (SIP) is a promising plant-based resource, yet its
3 industrial application is severely hindered by poor solubility and limited functional properties. In this
4 study, we employed a green and efficient glycosylation strategy to prepare SIP-chitosan
5 oligosaccharide (COS) conjugates via the Maillard reaction. Conditions were optimized using response
6 surface methodology (1:1.2 SIP:COS ratio, protein concentration of 15%, 70 °C, pH7). Structural
7 analysis (SDS-PAGE, FT-IR, CD, and SEM) confirmed successful covalent bonding and a transition
8 toward more flexible protein conformations. Crucially, glycosylation significantly improved
9 functionality: solubility at the isoelectric point (pH 4.0) surged from 5.20% to 35.41% (a 5.8-fold
10 increase), with an average 106.5% enhancement across pH 2.0–12.0. Emulsifying, foaming, and
11 antioxidant activities were also markedly elevated. These results demonstrate that SIP-COS
12 glycosylation is a green and effective strategy to overcome the solubility bottleneck of sacha inchi
13 protein, broadening its application in functional food systems.

14 **Keywords:** Sacha inchi protein; Chitosan oligosaccharide; Maillard reaction; Structural
15 characterization; Physicochemical functional properties.

Introduction

Sacha Inchi (*Plukenetia volubilis* L.), also known as Inca peanut or mountain peanut, is a perennial woody liana native to the Amazon rainforest(Kodahl and Sørensen, 2021).Its seeds are notable for their exceptionally high lipid content (35%–60%) and protein content (25%–30%). "In 2013, the Ministry of Health of China officially approved Sacha Inchi oil as a 'new food ingredient,' which significantly accelerated its industrial application in the oil and fat sector.". However, the byproduct of oil extraction, Sacha Inchi Press-Cake (SIPC), still contains over 50% protein. This protein is rich in essential amino acids, including cysteine, tyrosine, threonine, and tryptophan(Ruiz et al., 2013) Despite being a high-quality plant protein source, SIPC is currently treated primarily as waste or low-value animal feed. Therefore, the high-value utilization of this resource has become a critical focus in food science research.

Previous studies have demonstrated that Sacha Inchi Protein (SIP) possesses significant bioactive properties, including antioxidant(Chirinos et al., 2020), antihypertensive, enzyme inhibitory, and anti-inflammatory activities(Suwanangul et al., 2021), highlighting its potential in functional foods and nutraceuticals. However, native SIP exhibits notable limitations during food processing: its high surface hydrophobicity causes easy aggregation and precipitation near the isoelectric point, resulting in poor solubility, emulsification, and foaming properties. These drawbacks severely restrict its application in functional food and beverage formulations. Therefore, optimizing the structure and functionality of Sacha Inchi peptides through rational modification techniques is crucial for expanding their application scope and enhancing the added value of SIPC.

In recent years, Maillard-driven glycosylation has emerged as a green and efficient protein modification strategy, garnering increasing attention in the food industry(Hou et al., 2017). The covalent conjugation of proteins and carbohydrates via the Maillard reaction can effectively enhance protein solubility emulsifying properties and antioxidant activities(Xiao et al., 2021). For instance, explored the effects of various saccharides on oat protein conjugates, systematically revealing the correlation between saccharide type, degree of grafting, and functional properties such as solubility, emulsifying activity, and water-holding capacity(Xu et al., 2023).

Chitosan oligosaccharide (COS), as natural water-soluble polysaccharides, offer excellent biocompatibility, safety, and non-toxicity, making them ideal substrates for glycosylation. Glycosylation with Chitosan oligosaccharide (COS) has been shown to alter the secondary structure and enhance the functional properties of perilla seed meal protein(Li et al., 2024). Additionally, Cong et al. demonstrated that COS-modified silver carp (*Hypophthalmichthys molitrix*) protein not only improved solubility and emulsification but also significantly delayed oxidative denaturation(Cong et al., 2023). Although glycosylation offers substantial advantages, research on the covalent binding of Sacha Inchi protein with COS and its subsequent impact on structural and functional characteristics remains limited.

Beyond being a reactive carbohydrate for the Maillard reaction, chitosan oligosaccharide (COS) is a well-recognized functional ingredient, celebrated for its unique biological activities, including potent antioxidant, antimicrobial, and immunomodulatory properties(Wang et al., 2025; Xie et al., 2024). Unlike traditional polysaccharides, the low molecular weight and high degree of deacetylation of COS endow it with superior bioavailability and the ability to scavenge free radicals directly. Therefore, the conjugation of COS with sacha inchi protein (SIP) serves a dual purpose: it not only acts as a physical modifier to overcome the solubility and emulsification bottlenecks of the protein but also functions as a "bioactive booster." By covalently grafting COS onto the SIP backbone, the resulting conjugates are

expected to inherit and potentially synergize the antioxidant merits of both components, transforming a simple plant protein into a multi-functional bioactive ingredient with enhanced health-promoting potentials.

The objective of this study was to prepare Sacha Inchi protein – Chitoooligosaccharides (SIP-COS) conjugates via a wet Maillard reaction. The preparation process was optimized using a Box-Behnken response surface methodology. The microstructural evolution of the conjugates was thoroughly analyzed using SDS-PAGE, Fourier transform infrared spectroscopy (FT-IR), Ultraviolet-Visible spectroscopy (UV-Vis), Circular Dichroism (CD), and Scanning Electron Microscopy (SEM). By optimizing the process parameters through single-factor experiments and response surface design, this study aimed to evaluate the structural and functional properties of the glycosylated proteins, thereby providing a theoretical foundation for the high-value utilization of Sacha Inchi protein resources and the development of functional plant-based protein ingredients.

Materials and Methods

Preparation of Sacha inchi protein

Sacha inchi protein was extracted using alkali solution and acid isolation methods. Deionized water was added at a solid-to-liquid ratio of 1:20 (w/v), and the pH was adjusted to 9.0 using 1 mol/L NaOH. After stirring at room temperature for 2 hours, the slurry was centrifuged at 3500 rpm for 20 minutes. The precipitate was then extracted once more using the same solid-to-liquid ratio, and the two supernatants were combined. The combined supernatant was adjusted to pH 4.4 with 1 mol/L HCl. Following centrifugation at 3500 rpm for 20 minutes, the precipitate was resuspended in deionized water and adjusted to pH 7.0. The Sacha inchi protein (SIP) was obtained by vacuum freeze-drying (FD-1A-50 vacuum freeze-dryer, Nanjing Xian Ou Instrument Manufacturing Co., Ltd.) and stored at –20 °C until further analysis.

Preparation of SIP-COS Glycosylation Products

SIP-COS conjugates were prepared by reacting SIP and COS in an aqueous system at pH 8.5. After overnight hydration at 4°C, the mixture was heated at 60°C for 1 hour, cooled ([Kodahl and Sørensen, 2021](#)), and dialyzed using a 3500 Da cutoff membrane for 24 hours. The lyophilized supernatant was then used for subsequent characterization. The effects of temperature (40–80°C), protein-to-carbohydrate ratio (3:1 to 1:3), pH (6.0–10.0), and protein concentration (5%–25%) on the grafting degree and browning index were initially evaluated through single-factor experiments. For process optimization, a Box-Behnken Design (BBD) incorporating four factors at three levels was applied. The grafting degree (Y) served as the objective function to optimize pH (A), protein concentration (B), protein-to-carbohydrate ratio (C), and temperature (D), with factor levels detailed in **Table S1**.

Grafting degree (DG)

The degree of glycosylation was determined using the o-phthalaldehyde (OPA) method with some modifications([Wang et al., 2024](#)). Briefly, 80 mg of OPA was dissolved in 2 mL of ethanol. Then, 3.81 g of sodium tetraborate, 0.1 g of sodium dodecyl sulfate (SDS), and 88 mg of DL-dithiothreitol (DTT) were completely dissolved in 75 mL of deionized water. The OPA reagent was prepared by mixing these two solutions, and the total volume was adjusted to 100 mL with deionized water. Subsequently, 200 µL of a 2.5 mg/mL sample solution was thoroughly mixed with 4 mL of the OPA reagent and incubated in the dark for 10 minutes at room temperature. The absorbance of each sample was

measured at 340 nm using a UV–Vis spectrophotometer (TU-1810, Beijing Puxi Instruments Co., Ltd.). Deionized water was used as the blank control. The degree of glycosylation (DG, %) was calculated using equation :

$$DG(\%) = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 represents the solution's absorbance prior to the glycosylation process and A_t represents the solution's absorbance following the reaction.

Browning degree

For the measurement of browning extent, SIP-COS samples were diluted in 0.1% SDS to achieve a protein concentration of 0.2%. Browning was quantified by measuring the absorbance at 420 nm (Zhong et al., 2019).

Structural characteristics analysis

SDS-PAGE analysis

SDS–PAGE was performed using a 4% stacking gel and a 15% separating gel. The proteins were denatured in a metal bath at 95 °C for 10 minutes after mixing the sample solution (5 mg/mL) with the loading buffer at a volume ratio of 1:5. Next, 10 µL of the sample supernatant and 5 µL of protein standard (10–180 kDa) were loaded into the gel wells. Electrophoresis was conducted at 50 V for the stacking gel in Tris-HCl buffer and at 100 V for the separating gel. After electrophoresis, the gel was destained in a solution containing 10% glacial acetic acid and 5% ethanol, then stained for one hour with Coomassie Brilliant Blue R-250. Finally, gel images were captured using a Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad, USA).

Ultraviolet-visible (UV–Vis) absorption spectroscopy

The UV absorption spectra of the samples were determined using a previously described method with slight modifications (Xie et al., 2015). The samples were dissolved in deionized water to prepare a 0.1 mg/mL solution and analyzed using a UV-visible spectrophotometer (UV3600, Shimadzu, Japan) with a scanning wavelength range of 200–600 nm.

Fourier transform infrared spectroscopy (FTIR)

The samples were measured in the range of 400–4000 cm^{-1} using an FTIR-4700 spectrometer (Jasco, Japan). All the samples were freeze-dried, mixed with KBr, and then pressed into small wafers for the FTIR measurements.

Circular dichroism spectra measurements

Circular dichroism (CD) spectra were determined by the spectropolarimeter (JASCO-810, Tokyo, Japan). Proteins were dissolved in 0.1 M phosphate buffer (pH 7.4) to obtain the 0.2 mg/mL protein solution. The solution was assayed in 1mm path length cell at 25 °C between 190 and 250 nm. The spectra were recorded and each scan was acquired three times. To obtain the proportion of secondary structures, the data was analyzed by the CD Pro software package.

Scanning electron microscope (SEM) analysis

The SEM (S-3400 N, Hitachi Ltd., Japan) was used to observe the microstructure of the protein powder sample at an acceleration voltage of 10 kV, with magnifications of 200×, 1000×, and 2000× to examine the surface micro-morphological features.

Determination of functional properties

Protein solubility

SIP/SIP-COS were dissolved in buffer solutions with pH values ranging from 2.0 to 9.0 at a concentration of 1 mg/mL. The pH of the solutions was then adjusted to between 2.0 and 12.0 using 0.1 M HCl or NaOH. After stirring at room temperature for 20 minutes, the samples were centrifuged for 20 minutes at 10,000 rpm and 4 °C, the protein content of the supernatant was determined.

Emulsifying properties

The samples SIP and SIP-COS were dissolved in phosphate buffer solution (10 mM, pH 7.0) to obtain a final protein concentration of 1 mg/mL. For emulsion formation, corn oil and protein solution (1:3, v/v) were homogenized at 24,000 rpm for 1 minute. After homogenization, at 0 and 30 minutes, 50 µL of the emulsion was immediately taken from the bottom of the beaker and diluted 1:100 with 0.1% SDS solution. The absorbance of the diluted emulsion was recorded at 500 nm. The emulsifying activity index (EAI) and emulsifying stability index (ESI) were calculated using the following equations:

$$ESI(min) = \frac{A_0}{A_0 - A_{30}} \times 30$$

$$EAI (m^2 / g) = \frac{2 \times T \times A_0 \times N}{C \times \Phi \times 1000}$$

Where N is the dilution factor (100), C is the protein concentration (g/mL), Φ is the oil volume fraction (0.25), and A_0 and A_{30} are the absorbance values of the emulsion at 0 minutes and 30 minutes, respectively.

Measurement of foaming capacity (FC) and foam stability (FS)

50 mL of SIP aqueous dispersion (2%, w/v) was prepared, and the pH of the dispersion was adjusted to 8. The dispersion was whipped at 16,000 rpm using a high-speed homogenizer (IKA-T18, ULTRA-TURRAX, GE) for 2 minutes. The foam volume was recorded immediately (0 minutes) and after 30 minutes. FC and FS were calculated using the following equations:

Where V_0 (mL), V_1 (mL), and V (mL) represent the foam volumes at 0 minutes, 30 minutes, and the initial time, respectively.

$$FC = (V_0/V) \times 100$$

$$FS = (V_1/V_0) \times 100$$

Where V_0 (mL), V_1 (mL), and V (mL) represent the foam volumes at 0 minutes, 30 minutes, and the initial volume, respectively.

Antioxidant Activity

DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of vitamin C, SIP, and SIP-COS was determined using a previously reported method with slight modifications (Fan et al., 2023). Samples with varying concentrations (0.5, 1, 1.5, 2, and 2.5 mg/mL) were prepared. Solutions of SIP, SIP-COS conjugates, and vitamin C at these concentrations were mixed with 2 mL of 0.2 mmol/L DPPH ethanol solution and 1 mL of phosphate buffer (0.2 mol/L, pH 7.0). All reactions were conducted in the dark for 30 minutes, and absorbance was measured at 518 nm. The DPPH radical scavenging activity was calculated using

the following formula:

$$\text{DPPH radical scavenging (\%)} = [1 - (A_i - A_j) / A_0] \times 100$$

Where A_i , A_j , and A_0 represent the absorbance of the sample, blank, and control, respectively.

Determination of Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity was determined using a previously described method with slight modifications (Lv et al., 2023). Different mass concentrations (0.5, 1, 1.5, 2, and 2.5 mg/mL) of vitamin C, SIP, and SIP-COS were each mixed with 0.5 mL of salicylic acid ethanol solution (10 mmol/L) and 0.5 mL of ferrous sulfate solution (10 mmol/L). All reactions were carried out in the dark for 30 minutes, and the absorbance was measured at 510 nm. The hydroxyl radical scavenging activity was calculated using the following formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (A_i - A_j) / A_0] \times 100$$

Where A_i , A_j , and A_0 represent the absorbance of the sample, blank, and control, respectively.

Statistical Analysis

Results are expressed as mean $\pm$ standard deviation (SD) for all experiments conducted in triplicate. Statistical analysis was performed using SPSS software (version 18.0; Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for statistical comparisons, with a significance level set at $P < 0.05$.

Results and Discussion

Single-Factor Experiment Analysis

Glycation occurs through a condensation reaction between the carbonyl group of reducing sugars and free amino groups in proteins, forming a Schiff base. The degree of grafting (DG) is commonly used to characterize the extent of glycosylation reactions. The effects of different pH values (6–9) on the grafting degree and browning degree of glycosylation products are shown in Figure 1A. As the pH increases, the grafting degree initially rises and then declines. At pH 9, the maximum grafting degree of 39.04% was achieved in the SIP-COS reaction ($P < 0.05$). This is because glycosylation is an alkali-catalyzed reaction. When the solution pH reaches a slightly alkaline state, it promotes the glycosylation reaction, leading to an increased grafting degree. However, excessive alkalinity can cause deamination, decarboxylation, and peptide bond cleavage in the polypeptide, resulting in protein denaturation and a reduction in the number of sugar-peptide bonds, which adversely affects the grafting reaction. The browning degree, indicated by the absorbance at 420 nm, was 0.122 at pH 9. Based on these results, an optimal pH of 9 was selected for further optimization to achieve a suitable grafting degree while minimizing the formation of harmful compounds.

As shown in Figure 1B, protein concentration (5%–25%) significantly influenced both the grafting degree and browning intensity of the glycosylation product. With increasing protein concentration, the grafting degree and browning intensity of SIP-COS initially increased and then decreased. Within the 5%–10% protein concentration range, the grafting degree significantly rose from $(14.5 \pm 0.3)\%$ to a peak value of $(22.3 \pm 0.5)\%$ ($P < 0.05$), consistent with findings reported by Li et al. (Li et al., 2013), indicating that higher substrate concentration enhances glycosylation efficiency. The browning intensity increased from (0.10 ± 0.01) to a maximum of (0.42 ± 0.02) within the 5%–15% range, reflecting the synergistic relationship between the Maillard browning reaction and glycosylation. When the peptide concentration exceeded 10%, the grafting degree gradually declined. The browning intensity also decreased beyond 15%, falling to (0.30 ± 0.02) at 25%. This phenomenon is attributed to

increased steric hindrance among peptide molecules at high concentrations, which hinders the binding of sugar molecules to active sites(Cao et al., 2023). Additionally, excess peptides binding to Maillard reaction intermediates reduce the formation of colored substances(LU et al., 2024). To balance grafting efficiency and browning degree, a protein concentration of 15% was selected for further optimization.

The effect of the SIP:COS ratio on the grafting degree and browning intensity of SIP-COS is illustrated in Figure 1C. Both the glycosylation grafting degree of SIP-COS and the browning intensity initially increased and then decreased as the SIP:COS ratio varied. Within the SIP:COS ratio range of 3:1 to 1:3, the grafting degree significantly increased from $(1.2 \pm 0.1)\%$ to $(28.1 \pm 0.4)\%$ ($P < 0.05$), while the browning intensity rose from (0.10 ± 0.01) to a peak value of (2.15 ± 0.03) . These findings are consistent with the conclusion reported (Qiu et al., 2017), which states that glycosylation efficiency improves with an increasing proportion of sugar substrate. When the protein-to-carbohydrate ratio was further increased to 1:4, the grafting degree decreased to $(11.2 \pm 0.3)\%$, and the browning intensity declined to (1.30 ± 0.02) . This phenomenon can be attributed to steric hindrance caused by an excessively high carbohydrate concentration, which inhibits effective binding between protein and carbohydrate molecules. To balance grafting efficiency and browning intensity, SIP:COS ratio of 1:2 was selected for subsequent experiments.

The effects of different temperatures (30°C – 80°C) on the grafting degree and browning degree of SIP-COS are shown in Figure 1D. When the temperature reached 70°C , the grafting degree of SIP-COS peaked at 50.1% ($P < 0.05$). As the temperature increased further, the grafting degree began to decline, and the browning degree also decreased to 0.017 ± 0.001 . Consistent with the findings of Zuo et al.(Bu et al., 2021), elevating the temperature moderately promotes the reaction by accelerating molecular motion, increasing substrate collision frequency, and boosting enzyme activity. However, excessively high temperatures ($>60^{\circ}\text{C}$) induce degradation of Maillard intermediate products and cause partial denaturation of peptide chains. This reduces the number of active sites on reactants, consequently inhibiting grafting efficiency and diminishing the formation of colored(Liu et al., 2025b).

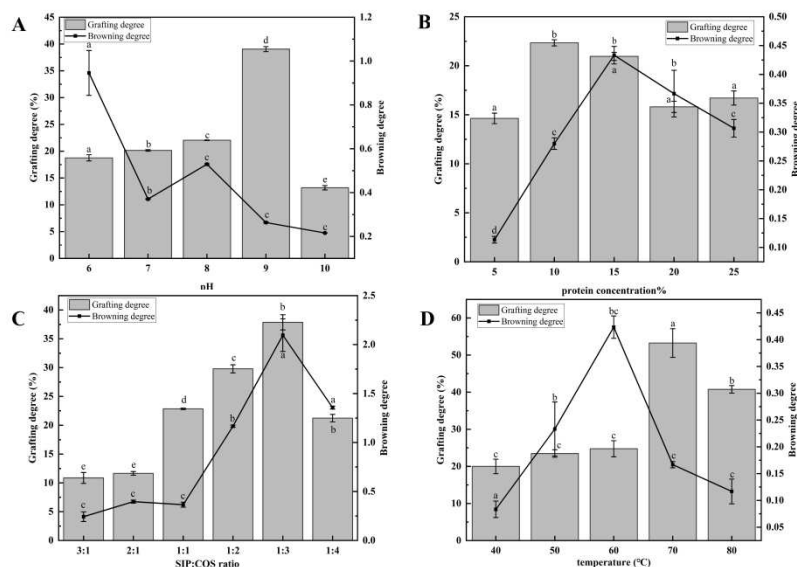


Figure.1. Effects of pH (A), protein concentration (B), SIP:COS ratio (C), and temperature (D) on the grafting degree and browning index of SIP-COS conjugates.

Response Surface Analysis

The preliminary single-factor experiments indicated that the optimal processing conditions for synthesizing glycosylated SIP-COS were pH 9.0, a protein concentration of 15%, SIP:COS ratio of 1:2, and a temperature of 60 °C. To further optimize these parameters, the effects of four independent variables—pH (A), protein concentration (B), protein-to-carbohydrate ratio (C), and temperature (D)—were investigated. A Box-Behnken design (BBD) was employed to plan and conduct 29 experimental runs, as summarized in Table S2.

The above results were subjected to multivariate analysis using Design Expert 12 software. The binary polynomial regression equation relating the grafting degree (Y) to the independent variables pH (A), protein concentration (B), SIP:COS ratio (C), and temperature (D) was obtained as follows: Grafting degree % (Y) = $40.60 + 0.5A + 0.9083B + 1.15C + 0.2275D - 0.25AC + 0.25AD - 0.25BC - 0.475BD - 0.65CD - 5.45A^2 - 5.58B^2 - 4.17C^2 - 5.21D^2$. According to Table 3, the R^2 of the regression model was 0.9786, indicating that the measured grafting degree values fit well with the predicted values. The adjusted R^2 was 0.9893, signifying that the model accounts for 98.93% of the variance in the response values. The model exhibited a highly significant level ($P < 0.001$), confirming its adequacy in simulating and predicting the effects of the selected parameters on the grafting degree.

ANOVA and the Influence of Factors Based on the Analysis of Variance (ANOVA) results (Table S3), the linear terms pH (A) and protein concentration (B) had significant effects, while the linear term SIP:COS ratio (C) and all quadratic terms (A_2 , B_2 , C_2 , D_2) exhibited highly significant impacts ($P < 0.01$). The significant interaction term CD indicates that the combined effect of the protein-to-carbohydrate ratio and temperature on the response is complex and not purely linear. According to the F-values, which serve as indicators of factor influence, the hierarchical order of factors affecting the grafting degree of SIP-COS was: SIP:COS ratio (C) > protein concentration (B) > pH (A) > reaction temperature (D).

Three-dimensional (3D) response surface plots were generated using Design-Expert 13 (Figure 2). The steepness of the response surface indicates the sensitivity of the grafting degree to variations in processing conditions. The analysis confirmed that the interactions between the variables significantly influenced the grafting degree. The observed hierarchy of factor influence was consistent consistently with the ANOVA results.

The software predicted the optimal processing conditions to as follows pH 7.06, protein concentration 14.34%, SIP:COS ratio of 1:2.93, and reaction temperature of 70.43°C, with a predicted grafting degree of 40.6%. Considering practical feasibility, the adjusted optimal conditions were set to pH 7.0, protein concentration 15%, SIP:COS ratio 1:3, and reaction temperature 70°C. Under these conditions, the experimental grafting degree was $40.6 \pm 0.2\%$, demonstrating excellent agreement with the predicted value. These results validate the reliability and feasibility of the response surface based optimization.

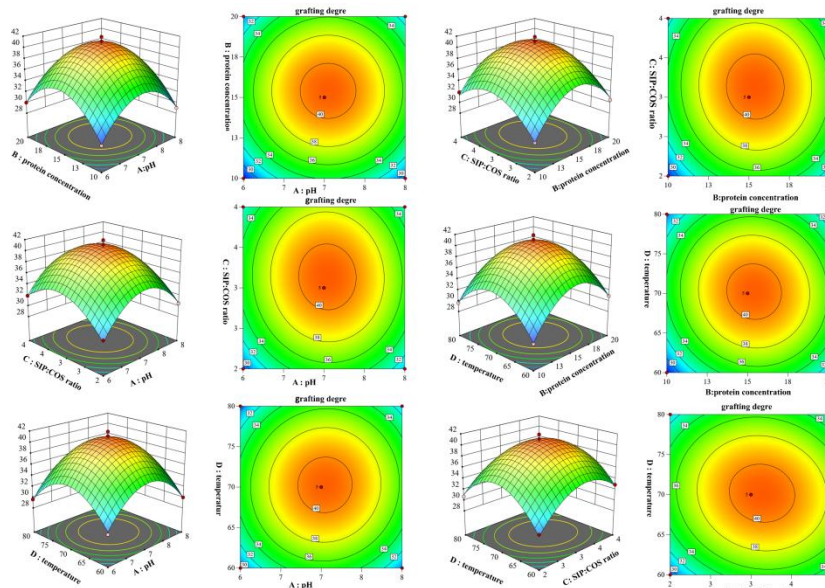


Figure 2. Effect of factor interactions on grafting degree

Structural Analysis

Analysis of Amino Acid Composition

According to reports, protein-polysaccharide graft reactions result from Maillard reactions between the ϵ -amino groups of proteins and the reducing-end carbonyl groups of polysaccharides (Li et al., 2014). Table 1 illustrates the changes in the amino acid composition of SIP-COS following the glycosylation of SIP. The total amino acid content decreased from 537.19 mg/g to 292.88 mg/g, representing a reduction of 45.48%. This significant depletion of amino acids confirms the occurrence of the Maillard reaction, consistent with established trends in plant protein glycosylation research (Lan et al., 2010; Wen et al., 2024). Most individual amino acids exhibited a significant decrease ($P < 0.05$). Notably, the preferential consumption of specific amino acids during the glycosylation process can be fundamentally elucidated by their distinct side-chain chemistries, particularly concerning nucleophilicity and steric hindrance. The exceptionally high depletion rates of Gly (-83.41%) and Ala (-73.63%) are primarily attributed to their minimal steric hindrance. The extraordinarily small side chains of these residues (-H for Gly and -CH₃ for Ala) create an open spatial microenvironment. This lack of steric bulk not only highly exposes adjacent reactive sites (such as N-terminal α -amino groups) but also enhances the local structural flexibility of the peptide chain, which dramatically facilitates collision frequencies and subsequent condensation with the relatively bulky COS molecules.

Conversely, the substantial consumption of Arg (-53.88%) and Lys (-48.38%) is thermodynamically and kinetically driven by their strong nucleophilicity. The highly basic guanidino group of Arg and the ϵ -amino group of Lys serve as the primary nucleophiles that attack the electrophilic carbonyl carbon at the reducing end of COS to form the crucial Schiff base intermediate.

Furthermore, the significant reduction in Pro (-67.13%), which possesses a unique cyclic secondary amine, suggests its high surface exposure (often located in flexible β -turns or loop regions of the protein) and its potential to form reactive iminium intermediates during the Maillard cascade. Finally, the moderate consumption of Asp (29.50%) and Glu (33.70%) might be associated with their side-chain carboxyl groups, which can act as local acid-base catalysts to accelerate the initial steps of the Maillard reaction (Chevalier et al., 2001).

Table 1 Amino acid composition of SIP and SIP-COS

Amino acids	SIP	SWIP-COS	Reduction(%)
Asp	52.24 ± 1.20	36.83 ± 0.90	29.50
Thr	15.98 ± 0.50	8.78 ± 0.30	45.09
Ser	26.95 ± 0.80	14.68 ± 0.30	45.54
Glu	85.0 ± 2.00	56.37 ± 1.50	33.70
Gly	70.43± 1.80	12.39 ± 0.40	83.41
Ala	35.33 ± 1.00	9.32 ± 0.30	73.63
Cys	16.07 ± 0.50	17.35 ± 0.60	-8.00
Val	21.91 ± 0.70	13.86 ± 0.50	36.77
Met	6.12 ± 0.20	5.32 ± 0.20	13.15
Ile	9.69± 0.60	13.60 ± 0.50	15.70
Leu	26.61± 0.80	13.97 ± 0.50	47.52
Tyr	17.58± 0.50	9.80 ± 0.30	44.27
Phe	21.55± 0.70	25.00 ± 0.80	16.01
His	9.52 ± 0.30	7.45 ± 0.25	21.79
Lys	19.35± 0.60	9.99 ± 0.30	48.38
Arg	57.79± 1.50	26.65 ± 0.80	53.88
Pro	35.05± 1.00	11.52 ± 0.40	67.13
amino acid content	537.19	292.88	-45.48

315

316 SDS-PAGE Profiles

317 SDS-PAGE is an efficient and reliable method to confirm the formation of covalent bonds
318 between SIP and chitosan oligosaccharide, as well as to determine changes in protein content and
319 molecular weight following glycosylation. As shown in Figure 3A, the molecular weights of the
320 subunit bands in the SIP and control groups (SIP heated at 80 °C) primarily ranged from 15 to 25 kDa,
321 with a few bands appearing near 35 kDa. Compared to SIP, the bands in SIP-COS shifted progressively
322 upward and exhibited reduced intensity. This indicates that the amino groups (-NH₂) on the lysine
323 residues of SIP underwent covalent cross-linking with chitooligosaccharides via glycosylation,
324 resulting in a decrease in free amino groups and an increase in relative molecular mass. This finding
325 provides evidence for the occurrence of glycosylation. Consistent with our results, glycosylation has
326 been shown to increase the molecular weight of hemp protein(Ding et al., 2023). Furthermore, higher
327 molecular weight bands were observed at the top of the separation gel, confirming that chitosan
328 oligosaccharide cross-linked with SIP via glycosylation or formed strong bonds that could not be
329 cleaved by SDS. Similar phenomena have been observed in the coconut protein-pectin complex(Li et
330 al., 2022) .

331 Ultraviolet-visible Absorption Spectroscopy (UV-Vis)

332 As shown in Figure 3B, the UV spectrum of SIP differs significantly from that of SIP-COS. SIP
333 exhibits characteristic absorption peaks at 195 nm and 275 nm. After glycosylation with chitosan
334 oligosaccharides, the characteristic absorption peak shifts from 195 nm to 192 nm. This shift may be
335 attributed to the influence of glycosylation on the protein chain conformation or the chromophore
336 environment, causing the lone electron pairs in the glycosylated product to move toward shorter
337 wavelengths. This results in a π - π^* transition of the carbonyl group and a blue shift in the absorption
338 band(Blindauer et al., 2009). Additionally, the red shift of the UV absorption peak from 273 nm to 275
339 nm may result from a weak $n \rightarrow \pi^*$ electron transition of the carbohydrate carbonyl group, accompanied
340 by a significant decrease in absorbance. The differences in the UV spectra between SIP and SIP-COS
341 provide evidence of glycosylation reactions, forming unique substances distinct from the original

protein structure.

Fourier transform infrared spectroscopy (FTIR)

Typically, FTIR is employed to identify changes in protein secondary structure and intermolecular interactions. As shown in Figure 3C, following glycosylation of SIP and COS, their FTIR spectra exhibited significant peak shifts and intensity alterations across multiple key amide bands. The characteristic absorption peak of SIP-COS shifted from 1654 cm^{-1} in non-glycosylated SIP to 1651 cm^{-1} . This peak primarily originates from the stretching vibration of C=O bonds in protein chains and minor contributions from the planar bending vibration of N-H bonds. In their study of protein-polysaccharide grafts, the shift of the amide I band to lower wavenumbers is a hallmark of the Maillard reaction. Glycosylation consumes free amino groups, and the introduced sugar chains induce partial unfolding of the peptide chain through steric hindrance effects. This facilitates the formation of more stable hydrogen bond networks between the carbonyl groups (C=O) and the newly introduced active groups, thereby reducing their vibration frequencies (Xu et al., 2019). This promotes the formation of a more stable hydrogen bond network between the carbonyl (C=O) group and the newly introduced active groups, thereby reducing its vibrational frequency. In the amide III band region, the absorption peak of the glycosylated product blue-shifted from 1255 cm^{-1} to 1363 cm^{-1} . This shift originates from the coupling of N-H bending and C-N stretching vibrations, which is similar to what has been observed for (He et al., 2021). In the amide A and B band regions, the non-glycosylated SIP exhibits characteristic absorption peaks at 2970 cm^{-1} (amide B band) and 3246 cm^{-1} (amide A band). After the reaction, the amide B band red-shifted to 2929 cm^{-1} , while the amide A band blue-shifted to 3336 cm^{-1} . The amide A band ($\sim 3300\text{ cm}^{-1}$) is primarily attributed to N-H stretching vibrations and readily overlaps with O-H stretching vibrations. Reported in their study on the binding of soybean isolate protein to carboxymethyl cellulose that the peak position in this region shifted to higher wavenumbers (blue shift) and broadened after glycosylation (Su et al., 2010). This was attributed to the introduction of numerous hydroxyl (-OH) groups from the sugar molecules, causing the O-H stretching vibration signal to dominate the $3300\text{--}3500\text{ cm}^{-1}$ region. It also reflected the formation of a complex new hydrogen bond network between the hydroxyl groups of the sugar chains and the amino groups of the protein chains. The strong absorption peak at 3336 cm^{-1} in this study directly confirms the successful grafting of COS and the introduction of numerous hydrophilic hydroxyl groups. SIP-COS exhibits a strong absorption peak at 1087 cm^{-1} that is absent in the original SIP spectrum (original peak position at 1070 cm^{-1}). This region corresponds to the characteristic fingerprint zone for C-O-C and C-OH stretching vibrations in carbohydrates. Research by (Nooshkam and Madadlou, 2016) indicates that the emergence or enhancement of absorption peaks in this region provides conclusive evidence of successful covalent anchoring of the sugar chain to the protein backbone.

Circular Dichroism Spectra (CD)

The position and intensity of absorption peaks in circular dichroism (CD) spectroscopy can reflect the secondary structures of proteins or polypeptides. Figure 3D shows the CD spectra of Sacha Inchi protein (SIP) and its glycosylated conjugate SIP-COS, both of which exhibited negative absorption peaks in the range of 190–200 nm. Specifically, unmodified SIP presented a strong negative peak at 198 nm, which is a typical characteristic of α -helices; in contrast, the peak shape of the glycosylated peptide was significantly attenuated and broadened, indicating that glycosylation directly altered the characteristic absorption signal of α -helices. The secondary structure of native SIP consisted of 8.18% α -helices, 61.88% β -sheets, 3.99% β -turns and 19.96% random coils. After glycosylation, the contents

of β -sheets and β -turns in SIP-COS decreased to 61.72% and 0.56%, respectively, while those of α -helices and random coils increased significantly to 15.35% and 22.13% ($P < 0.05$). This variation in secondary structures was consistent with the results reported by (He et al., 2021). The conversion of β -sheets and β -turns into α -helices and disordered structures such as random coils indicated that the introduction of sugar chains facilitated the deformation and unfolding of SIP-COS during glycosylation, which was closely associated with the formation of a novel hydrogen bond network. In addition, the increased α -helix content suggested that SIP cross-linked with COS to form macromolecular aggregates, and the protein chains in the protein contracted to a more compact and ordered state. The elevated random coil content implied that the introduction of sugar chains might induce conformational unfolding and functional group exposure of protein chains at the binding sites, thereby converting partial ordered structures into disordered ones (Sebii et al., 2021). The α -helix/ β -sheet ratio is indicative of the molecular flexibility of proteins, where a higher ratio corresponds to lower molecular flexibility; the enhancement of molecular flexibility is conducive to the improvement of protein functional properties, which also provides a structural basis for the subsequent improvement of the functional properties of SIP-COS.

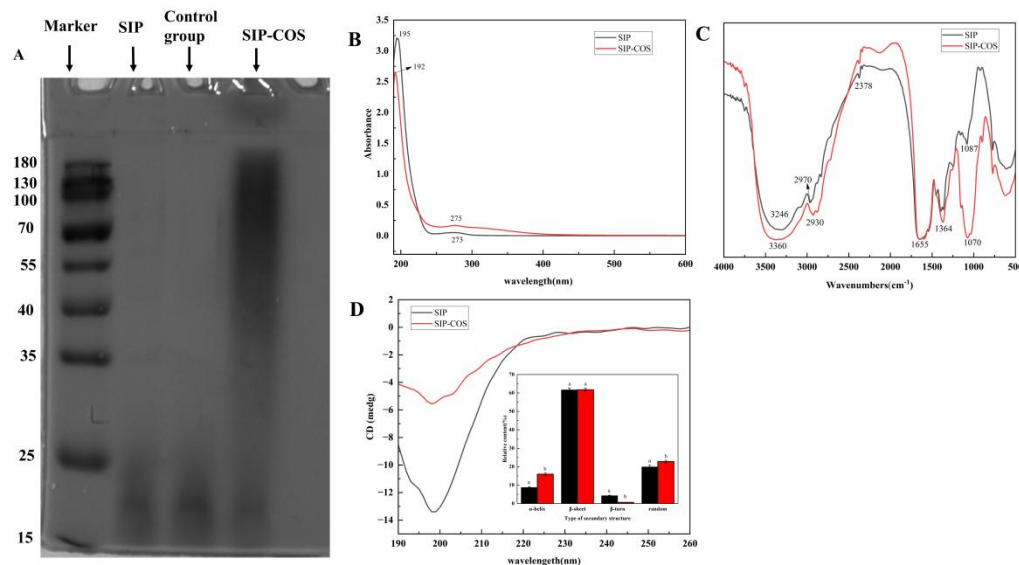


Figure 3. Structural characterization of SIP and SIP-COS complex: (A) SDS-PAGE; (B) UV-Vis spectra; (C) FT-IR spectra; (D) CD spectra and secondary structure contents.

Scanning electron microscopy (SEM)

The micro-morphological evolution of SIP and its glycosylated derivative, SIP-COS, was characterized using scanning electron microscopy (SEM) (Fig. 4). As illustrated, the native SIP (a1–a3) primarily exhibited compact spherical granules with heterogeneous particle sizes (5–50 μm). These granules displayed a rough surface and a distinct lack of porous structures, reflecting the highly folded and aggregated state of the native protein. In contrast, the morphology of the glycosylated product, SIP-COS, underwent a dramatic transformation (b1–b3). The tight granular structure evolved into a sheet-like network characterized by interconnected three-dimensional channels. This structural remodeling provides robust evidence that the covalent conjugation of chitosan oligosaccharides (COS) with the peptide chains induced the unfolding and organized re-aggregation of the protein molecules (Fu and Zhao, 2017). The formation of this porous architecture is primarily attributed to the steric hindrance exerted by the attached COS chains, which interferes with the dense packing of peptide chains and alters intermolecular interactions (such as hydrophobic interactions and hydrogen bonding).

This highly developed, open network structure increases the contact area between the protein and the aqueous phase, thereby providing a structural foundation for the significant improvement in functional properties, including solubility and emulsification(Chen et al., 2025).

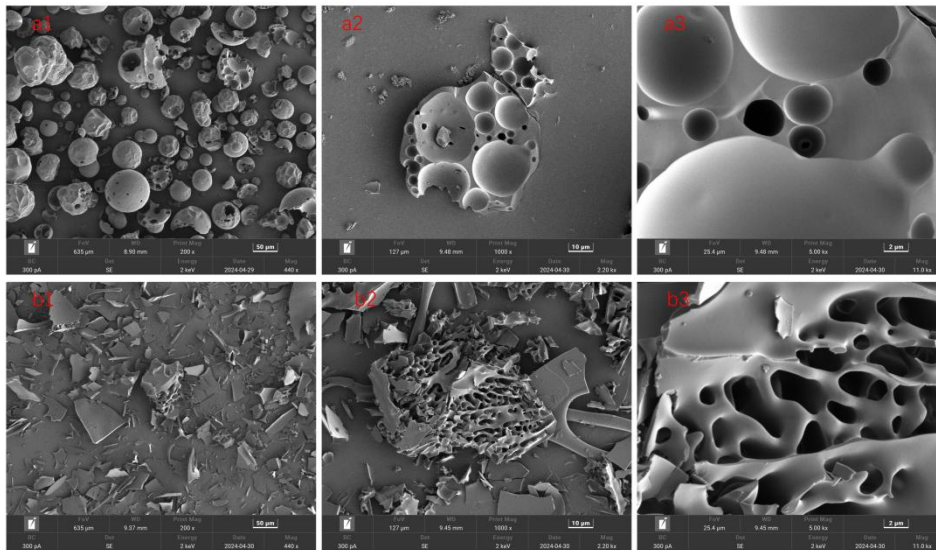


Figure 4. SEM images of SIP and SIP-COS: (a1 - a3) SIP at magnifications of 200×, 1000×, and 5000 × magnification; (b1-b3) SIP-COS at magnifications of 200×, 1000×, and 5000 × magnification

Solubility

Figure 5A illustrates the solubility of SIP and SIP-COS as a function of pH (ranging from 2.0 to 12.0). The results indicate that the solubility of both samples followed a characteristic U-shaped trend, reaching a minimum near pH 4.0. This behavior aligns with the typical isoelectric point (pI) of sachinchi protein, where the net molecular charge approaches zero, leading to minimized electrostatic repulsion and subsequent severe protein aggregation.

Notably, the glycosylation with chitosan oligosaccharide (COS) significantly enhanced the solubility of SIP across the entire pH range. Specifically, at pH 4.0 (the pI), the solubility of native SIP was remarkably low at 5.20%, whereas that of the SIP-COS conjugates surged to 35.41%, representing a dramatic 5.8-fold increase. Throughout the tested pH values, the conjugates demonstrated an average solubility improvement rate of 106.5%. The introduction of numerous hydrophilic hydroxyl groups from COS effectively increases the surface hydrophilicity of the protein. Furthermore, the grafted polysaccharide chains form a robust hydration layer on the protein surface, creating a strong steric hindrance effect.

emulsifying properties

Emulsification is closely related to the structural changes in sachinchi protein itself and has a certain relationship with other functional properties. EAI refers to the ability of protein to produce emulsion; ESI refers to the ability of protein to stabilize lotion for a certain time. In this study, the emulsifying ability (EAI) and emulsifying stability (ESI) of SIP and SIP-COS conjugates were measured, as shown in Fig 5B. In this study, the emulsifying activity index (EAI) and emulsifying stability index (ESI) of SIP and the SIP-COS conjugate were determined, with the results presented in Fig. 5B. Compared to native SIP, the SIP-COS complex exhibited significant improvements in both EAI and ESI ($P < 0.05$). Specifically, the EAI of SIP was $15.29 \pm 0.28 \text{ m}^2/\text{g}$, which increased approximately 2.3-fold to $34.73 \pm 0.37 \text{ m}^2/\text{g}$ for the SIP-COS conjugate. Similarly, the ESI increased

significantly from 52.06 ± 0.45 min to 109.82 ± 0.97 min. These results indicate that the incorporation of chitooligosaccharides (COS) remarkably enhanced the interfacial properties of sacha Inchi protein.

Foaming and foaming stability

The foaming properties of proteins are characterized by foaming capacity (FC) and foaming stability (FS). As illustrated in Figure 5C, both the FC and FS were significantly enhanced following the glycation reaction. Compared to the native SIP, the FC of SIP-COS increased markedly from 15.24% to $54.54 \pm 3.86\%$. This improvement can be primarily attributed to the structural unfolding of the protein, which led to the exposure of internal hydrophobic groups. This enhanced amphiphilicity allowed more protein molecules to rapidly migrate to and adsorb at the air-water interface, thereby reducing interfacial tension and facilitating the formation of high-quality foam. Furthermore, the FS exhibited a substantial increase, rising from $35.57 \pm 2.14\%$ to $84.12 \pm 3.54\%$. The covalent binding of COS facilitated the formation of a robust viscoelastic film around the bubbles. This steric hindrance, coupled with the increased interfacial thickness, effectively prevented bubble coalescence and retarded liquid drainage, ultimately leading to superior foam stability.

Antioxidant activity

As illustrated in Figures 5D and 5E, the scavenging activities of SIP, SIP-COS, and the positive control (VC) against hydroxyl ($\cdot\text{OH}$) and DPPH radicals exhibited a pronounced dose-dependent relationship. Within the concentration range of 0.5–2.5 mg/mL, the radical scavenging rates increased steadily with sample concentration, demonstrating the robust antioxidant capacity of the modified protein conjugate. The glycation modification significantly bolstered the radical quenching efficiency of the protein. The IC_{50} values for SIP-COS against hydroxyl and DPPH radicals were determined to be 0.53 mg/mL and 0.44 mg/mL, respectively. This enhancement in bioactivity can be attributed to the glycation-induced structural unfolding of the protein, which facilitates the exposure of previously buried amino acid residues with high redox activity, such as sulfur-containing amino acids (Feng et al., 2018). Simultaneously, the covalent attachment of chitosan oligosaccharide (COS) chains, which are abundant in hydroxyl and amino functional groups, exerted a significant synergistic effect with the protein backbone, collectively elevating the free radical scavenging potential of the system.

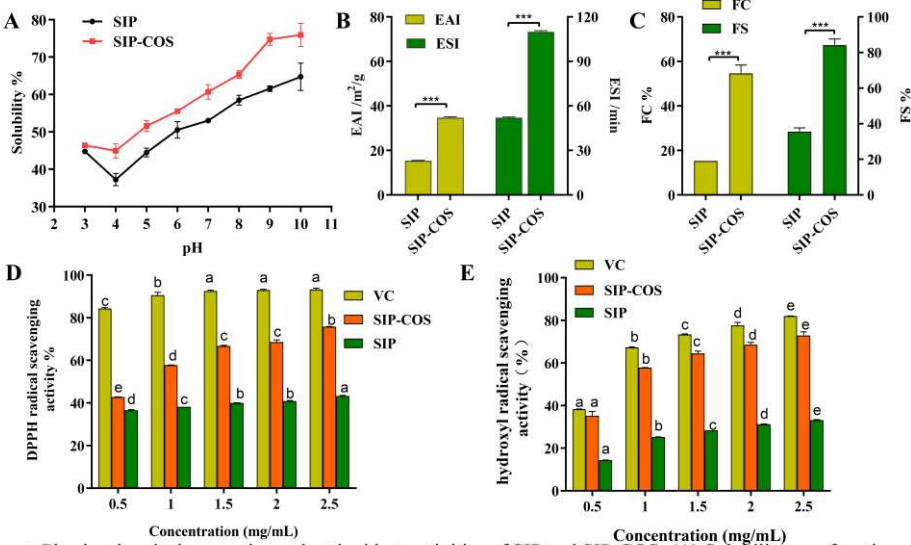


Figure 5. Physicochemical properties and antioxidant activities of SIP and SIP-COS: (A) Solubility as a function of pH; (B) Emulsifying activity index (EAI) and stability index (ESI); (C) Foaming capacity (FC) and stability (FS); (D) DPPH radical scavenging activity; (E) Hydroxyl radical scavenging activity.

Mechanisms Underlying the Enhancement of Functional and Antioxidant Properties

The substantial improvement in solubility at the isoelectric point (pH 4.0) can be attributed to the combined effects of steric hindrance and augmented surface hydrophilicity. Spectroscopic analyses (FT-IR and CD) indicate that the covalent attachment of COS effectively shields the exposed hydrophobic regions of SIP. While native SIP typically aggregates near its pI due to diminished electrostatic repulsion, the introduction of COS chains—which are rich in hydrophilic hydroxyl groups—facilitates the formation of a stable hydration shell. This hydration layer acts as a physical barrier, sterically impeding intermolecular collisions among peptide chains, thereby maintaining dispersion stability despite a neutral net charge.

The enhancement of interfacial properties, including emulsifying (EAI) and foaming (FC) capacities, is inextricably linked to increased molecular flexibility. CD spectroscopy revealed a structural shift from ordered α -helices to disordered random coils. This conformational relaxation enables the SIP-COS conjugates to more rapidly diffuse, unfold, and reorient at oil-water or air-water interfaces. Supported by SEM observations of a morphological transition from dense aggregates to a porous network, this structural architecture promotes the formation of a continuous viscoelastic interfacial film. Within this system, the hydrophobic protein segments anchor into the non-aqueous phase, while the hydrophilic COS tails extend into the aqueous phase. This spatial arrangement generates considerable steric stabilization, which effectively inhibits droplet flocculation and bubble coalescence.

Finally, the elevated antioxidant capacity of the conjugates arises from a functional synergy between the protein backbone and the grafted carbohydrate. The glycation-induced partial unfolding of the tertiary structure exposes previously buried redox-active amino acid residues (e.g., sulfur-containing and aromatic amino acids), thereby increasing their accessibility for free radical quenching. When integrated with the intrinsic radical-scavenging properties of the conjugated COS moiety, this structural modification successfully transforms the native protein into a dual-functional bioactive ingredient with enhanced physicochemical stability.

Author Contributions:

Conceptualization: Y. L., R.J. L., L. T.; Methodology: Y. L., R.J. L.; Validation: W.S.P., Y.Y.Q., L.C.M.; Formal analysis: Y. L.; Investigation: Y. L., R.J. L.; Resources: L. T.; Data Curation: Y. L., R.J. L.; Writing - Original Draft: Y. L., R.J. L.; Writing - Review & Editing: L.C.M., L.F.S., L.T.; Visualization: Y. L., R.J. L.; Supervision: L. T., L.F.S.; Project administration: L.T.; Funding acquisition: L. T.

Data Availability

The data that support the findings of this study are included in the article; further inquiries can be directed to the corresponding author.

Conflict of Interest

The authors declare that they have no competing interests.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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