Preparation of modified Jiuzao glutelin isolate with carboxymethyl chitosan by ultrasound-stirring assisted Maillard reaction and its protective effect of loading resveratrol/quercetin in nano-emulsion

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ABSTRACT

Jiuzao glutelin isolate (JGI) was reported to possess interface and functional properties. To enhance the stability and properties of JGI, conjugation between JGI and carboxymethyl chitosan (CTS) through ultrasound-stirring assisted Maillard reaction (UTSA-MR) was investigated and optimized. The changes of molecular distribution, secondary structure, morphology, and amino acid composition of JGI were detected after conjugation with CTS. The solubility, foaming property and stability, viscosity, and thermal stability of four conjugates (CTS-JGI, with weight ratios of 0.5:1, 1:1, 2:1, and 4:1) were significantly increased compared to native JGI. Under the optimal glycation, the conjugate (CTS/JGI, 2:1, w/w; CTS-JGI-2) exhibited the best emulsifying ability and stability against NaCl solution, in vitro antioxidant activity, and cholesterol-lowering ability. CTS-JGI-2 stabilized oil-in-water nano-emulsion improved resveratrol (RES) and quercetin (QUE) encapsulation efficiency (80.96% for RES and 93.13% for QUE) and stability during the simulated digestion process (73.23% for RES and 77.94% for QUE) due to the connection through hydrogen bonds, pi-anion, pi-sigma, and donors between CTS-JGI and RES/QUE. Taken together, the modification of JGI by conjugating with CTS through UTSA-MR could be an excellent method to improve the functional properties of JGI. CTS-JGI-2 is a potential conjugate with functions that can be used to encapsulate functional substances in the stabilized nano-emulsion.

1. Introduction

Baijiu is the Chinese national liquor and is popular with people of different ages. Jiuzao is the solid by-product after baijiu distillation [16]. In recent years, baijiu production has shown an increasing trend, and more content of Jiuzao has been produced. The raw materials of baijiu distillation are mainly sorghum, accompanied by rice, wheat, corn, barley, and glutinous rice [41]. These raw materials have a high protein content. Some proteins in the raw materials are the primary nutrients for microorganisms in the fermentation process [18]. However, the proteins not utilized by microorganisms remain in Jiuzao after the distillation process due to their high boiling points. Currently, the kafirin and glutelin in Jiuzao have been studied in the literatures [18,55]. Although the content of kafirin is higher than glutelin in sorghum, glutelin possesses higher essential amino acids that the body needs [6]. In addition, our previous study has extracted and purified Jiuzao glutelin isolate (JGI) and identified its structure by nano ultra-performance liquid chromatography-mass spectrum/mass spectrum [18]. High interfacial properties (such as foamability, foam stability, and water and oil retention properties) and functional properties (such
as free radical scavenging ability and ACE inhibitory activity) of JGI were found. Therefore, JGI has higher research and utilization values.

Proteins are a class of substances that has a variety of functions. Animal and plant proteins are the two most common protein sources [11]. Compared with animal protein, plant protein is mainly obtained from crops and is derived from by-products of food processing [48]. Compared with the acquisition of animal protein, there is a wider variety of plant protein. Using by-products can also reduce the pollution they cause to the environment. Plant protein with sensory quality similar to specific animal protein is mainly processed from raw plant materials, which not only meets the people’s pursuit of taste but also provides the nutritional requirement of reducing saturated fatty acid and cholesterol intake. Plant protein is an active substance that plays a vital role in sustaining normal metabolism and is essential in the body [2]. Plant protein can be utilized as a carrier for active ingredients [40]. However, when proteins are influenced by external conditions (such as pH changes, temperature, acidity, alkalinity, ion concentration, and others), their original functional properties are also altered [1]. Therefore, it is imperative to improve the stability of proteins.

Maillard reaction occurs between amino groups of proteins and peptides with carbonyl groups of polysaccharides [24]. Although the degree of Maillard reaction is difficult to control, relevant studies have demonstrated that protein stability and functional properties can be significantly improved by combining with polysaccharides at the optimal Maillard reaction degree. Klinchongkon conjugated whey protein with subcritical-water hydrolyzed pectin and found that the conjugates exhibited better emulsifying properties than whey protein [20]. Wen prepared soy protein isolate-lentinan conjugates through Maillard reaction by slit divergent ultrasonic-assisted wet heating method and found better functional properties of conjugates [45]. As a new environmental protection technology, ultrasound has been widely used in the food industry. The high temperature and pressure caused by the ultrasonic process provide the conditions for the Maillard reaction. Ultrasound has been applied to accelerate the Maillard reaction, resulting in less hazardous ingredient formation and increasing the conjugate functions. Zhang utilized the ultrasonic-assisted wet-heating method to produce whey protein-flaxseed gum product and found better physicochemical properties and encapsulation efficiency of astaxanthin [53]. Jiang also prepared pea protein-inulin conjugates with higher surface load and thicker interfacial layers via ultrasound Maillard reaction to improve the sensory characteristics and emulsifying ability [15]. Yang used energy-divergent type ultrasound to promote the glycosylation of protein-hydrolysate from grass carp to improve the flavor of the product [52].

Chitosan is the product of chitin deacetylation. Chitosan has a variety of physiological functions such as biodegradability, biocompatibility, non-toxicity, antibacterial, anti-cancer, lipid-lowering, and immune enhancement. Chitosan is used in various industries, including the food industry, drug sustained-release materials, gene transduction carriers, biological medical, and daily chemical industries [27,43]. Chitosan has many unique properties such as biodegradability, cell affinity, and biological effects [30]. In Xu’s and Du’s studies, chitosan was conjugated with soybean protein isolate (SPI) to improve the SPI’s emulsifying activity and stability [9,50].

Rserveratrol (RES, a non-flavonoid polyphenol compound) and quercetin (QUE, a flavonol compound) are proved to possess functional properties, such as antioxidant, anti-inflammatory, anti-cancer, and cardiovascular protection. However, their applications in the food and pharmaceutical industries are limited due to their instability, low water solubility, and poor oral bioavailability. Therefore, many delivery strategies, for instance, liposomes [5], liquid self-micro emulsifying drugs [13], and polymeric micelles [10] have been studied to overcome these limitations. Emulsions have a wide range of applications in the food field, including homogeneous milk, cream, and seasonings [4]. Nano-emulsion is a new type of emulsion with the advantages of using less emulsifier, having no surface-active additives, and being stable [18]. Nano-emulsion can be used to encapsulate and protect the release of hydrophobic natural products in the delivery system, considering the enhancement of water solubility, thermal stability, gastrointestinal stability, and bioavailability because of its versatile technical functions and biological properties [7].

Carboxymethyl chitosan (CTS) has a better water solubility than chitosan. Currently, no study attempts to prepare Maillard products using JGI and CTS as raw materials to explore the improvement of their physicochemical properties and utilization in the delivery systems for RES and QUE. The purpose of this study was to conjugate JGI with CTS via the ultrasound-stirring assisted Maillard reaction (UTSA-MR) to improve the physicochemical, interfacial, and functional properties as well as to explore its encapsulation effect on RES and QUE in oil-in-water nano-emulsions. The main contents include 1) optimization of UTSA-MR conditions; 2) determination of interfacial properties and JGI structure changes of Maillard products; 3) measurement of Maillard products stabilization on oil-in-water nano-emulsions; 4) investigation of functional properties of Maillard products (in vitro antioxidant and cholesterol-lowering effects); 5) exploration of RES and QUE encapsulation ability of conjugate stabilized nano-emulsion. The above research aims to broaden the application of JGI in food emulsions and functional foods industry, improve the utilization of JGI, and reduce the environmental pollution caused by Jiuzaoo’s untimely treatment.

2. Materials and methods

2.1. Materials and reagents

Jiuzaoo of strong-flavor type (after three times of distillation; main material composition, sorghum, wheat, corn, rice, sticky rice, and millet; auxiliary material composition, rice husk) was provided by Bandaqing Liquor Co., Ltd. (Zibo, China). The identified JGI (Table S1) was obtained according to our previous method [18]. CTS, RES, and QUE (purity > 98%) were purchased from Macklin Biochemical Co., Ltd (Shanghai, China). Other materials and reagents were exhibited in the Supplementary Material.

2.2. Preparation of carboxymethyl chitosan-Jiuzao glutelin isolate (CTS-JGI) conjugates by ultrasound-stirring assisted Maillard reaction (UTSA-MR)

CTS-JGI conjugates were prepared via the UTSA-MR in a wet-heating system following a method [26] with some modifications. First, CTS and JGI were dissolved in ultrapure water to obtain a concentration of 10 mg/mL solutions. The solutions were stirred for 3 h at 25 °C. The dissolved CTS and JGI solutions were mixed to obtain a series ratio of 0.5:1, 1:1, 2:1, and 4:1 (CTS/JGI, w/w), which were expressed as CTS-JGI-0.5, CTS-JGI-1, CTS-JGI-2, and CTS-JGI-4. The mixture solutions were stored at 4 °C overnight to obtain complete hydration. Next, the mixture was transferred to glass bottles and capped to prevent water evaporation during the reaction. The pH of the CTS-JGI solutions was adjusted to 7.0 and 11.0. The sealed samples were incubated and stirred in a magnetic stirrer (IKA, RCT basic, Baden-Wuerttemberg, Germany) at 90 °C along with the treatment of ultrasound (400 W, 10 s on and 10 s off at a frequency of 25 kHz) using an ultrasound cell disruptor (Shanghai Huxi Industrial Co., Ltd) with a φ6 horn. The samples were collected at different times (0, 15, 30, 60, 90, 120, and 180 min). The collected samples were immediately cooled in an ice-water bath to stop reaction and stored at 4 °C for further experiments. JGI treated without UTS was used as a control.

2.3. Determination of the pH changes, browning index (BI), Astaxanthin grafting degree (GD), and surface disulfide bond content

The methods of the pH changes, BI, Astaxanthin grafting degree (GD), and surface disulfide bond content was exhibited in the Supplementary material.
2.4. Structure properties

2.4.1. Molecular distribution analysis by high-performance size-exclusion chromatography (HPSEC)

CTS-JGIs with the best Maillard reaction degree obtained in each CTS/JGI ratio were used for the subsequent investigation.

HPSEC was performed to investigate the molecular size distributions of CTS-JGIs. The analysis was conducted using an Agilent HPLC instrument (model 1260, Santa Clara, CA, USA) equipped with a TSKgel SW2000 column (separation range 15–150 kDa, TOSOH, Tokyo, Japan). 50 μL of CTS-JGI (0.67 mg/mL) solution was injected by an autosampler. The same mobile phase and elution procedure in a previous study [18] was used to analyze the molecular distribution. The chromatograms were recorded at 214 and 280 nm.

2.4.2. Ultraviolet (UV)-visible spectroscopy

The sample solutions (0.1 mg/mL) were prepared with ultrapure water. The UV–visible spectra of CTS-JGIs were recorded from 240 to 320 nm at room temperature (25 °C) using a UV–vis spectrophotometer UV-2700 (Shimadzu, Kyoto, Japan) to measure the structure changes of CTS-JGIs.

2.4.3. Fourier transform infrared (FT-IR) analysis

The samples were obtained after being freeze-dried using a lyophilizer (Christ, Alpha 1–4 LSC basic, Osterode, German). The FT-IR spectrum was recorded using a vector 33 IR spectrophotometer (Bruck, Ettingen, Germany) at a wavenumber of 4000–800 cm⁻¹.

2.4.4. Circular dichroism (CD) analysis

2.5 mg/mL of the sample solutions were prepared and filled in a precision quartz cell of 2.0-mm. The CD spectrum of CTS-JGI was recorded using a J-815CD Spectrometer (JASCO, Tokyo, Japan) at a wavelength of 195 to 300 nm. The secondary structure of CTS-JGI was analyzed by the Dicropot software.

2.4.5. Determination of surface hydrophobicity (H₀)

The samples (0.01, 0.05, 0.1, 0.15, and 0.2 mg/mL) were prepared with 10 mM pH 7.0 PBS solution. The method of H₀ measurement was shown in the Supplementary Material.

2.4.6. Scanning electron microscopy

The samples adhered to a conductive paste. After gold spraying, the apparent morphology of the conjugates was recorded at 3.0 kV using a scanning electron microscope (SEM) (FEI Quanta 250 FEG, FEI Inc., QR, USA).

2.4.7. Amino acid composition analysis

The amino acid compositions of the samples were analyzed by an automatic amino-acid analyzer (HITACHI, L-8900, Tokyo, Japan). First, the samples were hydrolyzed by 5.7 M HCl at 110 °C for 12 h. Then, 1 mL of hydrolysate was evaporated in rotation. Finally, the hydrolysate was redissolved in 0.02 M HCl and filtered through a 0.22 μm membrane.

2.5. Physicochemical properties

The solubility, foaming property and stability, emulsification activity index (EAI), emulsification stability index (ESI), viscosity, and thermal stability methods were exhibited in the Supplementary Material.

2.6. Physical stability of oil-in-water nano-emulsions during 28 days of storage

2.6.1. Preparation of oil-in-water nano-emulsions

The soybean oil and CTS-JGI conjugates (5 mg/mL) were mixed equably at a ratio of 1:100 (v/v). The mixed solution was homogenized for 3 min at 15,000 rpm in an ice bath to obtain the coarse emulsion. The oil-in-water nano-emulsion was obtained by homogenizing the coarse emulsion circularly using a high-pressure homogenizer (APV 2000, Beijing, China) at 750 bars for 6 min. The prepared oil-in-water nano-emulsions were used for subsequent analysis.

2.6.2. Particle size, polydispersity index (PDI), and zeta-potential measurement

The particle size, PDI, and zeta-potential of the emulsions were directly measured using a Malvern Mastersizer 3000 (Malvern Panalytical Inc., Malvern City, UK) at 1, 7, 14, 21, and 28 days.

2.6.3. Backscattering intensity (BSI) and Turbiscan scan index (TSI) measurement

The BSI and TSI of conjugates were evaluated via acceleration testing at 40 °C using a multiple light scattering spectroscopy (Turbiscan Tower, Formulation, Toulouse, France). The prepared emulsions were transferred to 20 mL glass tubes. The glass tubes were scanned from the bottom to the top for 3 d, measuring BSI and TSI to inspect the destabilization mechanism.

2.6.4. Stability of emulsions against NaCl

NaCl solution (5 M) was added to the emulsions to achieve the final concentrations of 50 and 150 mM in the emulsions. The particle size and PDI of the emulsions were directly measured at 1, 7, 14, 21, and 28 days.

2.7. Functional properties

2.7.1. In vitro antioxidant activity

The CTS-JGI solutions were prepared with 0.125, 0.25, 0.625, 0.9375, and 1.25 mg/mL concentrations. ABTS, DPPH, hydroxyl radical scavenging, and ferrous reducing abilities were evaluated according to the instruction manufacturers.

2.7.2. Cholesterol-lowering activities

The cholesterol-binding capacity (CBC) of JGI and CTS-JGI-2 were measured according to Zhu’s method [56] with some modifications. The specific method was shown in the Supplementary Material.

The micellar cholesterol inhibition (MCI) of the samples was measured according to the method described by [46] with minor modifications. The specific method was stated in the Supplementary Material.

The specific method of bile acid-binding capacity (BAC) of JGI and CTS-JGI measurement was shown in the Supplementary Material.

α-amylase inhibitory activity (AIA) of the samples was determined according to a DNS assay kit instruction [23]. The specific method was exhibited in the Supplementary Material.

2.8. Effect of RES/QUE in CTS-JGI-2 stabilized oil-in-water nano-emulsion (RES/QUE-CTS-JGI-2-O/W-NE)

2.8.1. Construction of RES/QUE-CTS-JGI-2-O/W-NE

Weighted RES and QUE were dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 100 μM. Then, the prepared solution was mixed with CTS-JGI-2 solution (10 mg/mL, pH 8.5) at room temperature for 4 h to integrate fully. Afterward, the O/W-NE were prepared with the same method in Section 2.6.1. Native JGI formed oil-in-water nano-emulsion loaded with RES and QUE (RES/QUE-JGI-O/W-NE) was used as the control.

2.8.2. Encapsulation efficiency (EE) and loading capacity (LC)

The EE and LC of RES and QUE in the emulsions were measured by the method of [7] with slight modification. The RES/QUE-CTS-JGI-2-O/W-PE was diluted 50-fold with DMSO and centrifuged at 4,000 g for 15 min. Finally, the absorbance was measured at 320 nm and 360 nm, respectively. The EE and LC were calculated according to the following equations and compared to RES/QUE-JGI-O/W-NE:
\[ EE(\%) = \left( \frac{A - B}{A} \right) \times 100\% \] (1)

where A is the initial content (mg/mL) of RES and QUE, and B is the content of free RES and QUE content in the supernatant.

\[ LC(\%) = \left( \frac{A - B}{W} \right) \times 100\% \] (2)

where W is the weight (mg) of RES/QUE-CTS-JGI-2-O/W-NE or RES/QUE-JGI-O/W-NE.

2.8.3. In vitro simulated digestion
20 mL of RES/QUE-CTS-JGI-2-O/W-NE was prepared for the in vitro simulated digestion assay. The method was determined according to our previous study [16] with slight modification; that is, the digestion of gastric and intestinal stages were 2 h. After digestion, 1 mL of the digesta was centrifuged for 20 min at 4 °C and 5,000 g. The supernatant was collected and diluted by DMSO (20-fold). The content of RES and QUE was measured by the method in Section 2.9.2. The release rate (RR) and bioavailability (BAY) were measured by the following equations and compared with RES/QUE-JGI-O/W-NE:

\[ RR(\%) = \left( \frac{\text{Content of RES and QUE in the supernatant}}{\text{Total RES and QUE content in the digesta}} \right) \times 100\% \] (3)

\[ BAY(\%) = \left( \frac{\text{Content of RES and QUE in the emulsion}}{\text{Total RES and QUE content before digestion}} \right) \times 100\% \] (4)

2.8.4. SEM observation of RES- and QUE-loaded CTS-JGI-2 powder
To investigate the connection of RES and QUE with CTS-JGI-2, SEM was used to observe the microstructure with the same protocol in Section 2.4.6.

2.8.5. Prediction of the potential binding mechanism of RES and QUE with CTS-JGI-2
Molecular docking software AutoDock 4.2 (Scripps, CA, USA) was used to predict the docking sites of RES and QUE with CTS-JGI. Discovery Studio 2019 (BIOVIA, CA, USA) and PyMOL 2.5 (Schrodinger, DE, USA) were used to produce the docking graphs. The detail method and JGI macromolecule were the same as in our previous study [17,18]. The CTS structure unit (CTSU) was obtained from http://www.chemspider.com/Default.aspx with a ChemSpider ID of 64780. RES and QUE were prepared using Chem3D Professional 16.0 (CambridgeSoft, MA, USA).

\[ Fig. 1. (A) pH changes during the reaction. (B) BI rate changes during the reaction. Different letters showed a significant difference at p < 0.05 in the UTS treated groups. \]
2.9. Statistical analysis

All measurements were performed on at least four freshly prepared samples and reported as mean and standard deviation. The results were analyzed using SPSS software (SPSS Inc., Chicago, IL, USA) for one-way ANOVA analysis. The level of significance was determined at $p < 0.05$.

3. Results and discussion

3.1. Degree of Maillard reaction

Maillard reaction can improve the properties of proteins. However, overreaction also produces harmful substances, such as carcinogens.
Therefore, to control the degree of reaction, the overall reaction time was controlled at 180 min to optimize the experiment to avoid excessive reaction producing harmful substances in this study.

### 3.1.1. pH changes

pH changes are shown in Fig. 1A. There was no noticeable change in pH when reacted in the neutral condition. pH showed an upward trend in CTS-JGI-0.5 and CTS-JGI-1 conjugates during the first 1 h. This result is due to the opening of the JGI spatial structure after the reaction started, and the exposed amount of alkaline amino acids was more than the amount involved in the reaction ascribed to the low ratio of CTS. This increase did not occur in CTS-JGI-2 and CTS-JGI-4 conjugates, which proved that a high proportion of CTS could react rapidly with these alkaline amino acids. However, in the pHe 11.0 condition, the pH value exhibited a significant (p < 0.05) decrease in the four conjugates. The pH changes in UTSA-MR are lower than untreated ones, indicating the reaction degree treated by UTS is higher than the traditional method. During the reaction, many alkaline amino acids were consumed when reacted with the carbonyl of CTS to produce hydroxymethyl furfural or furfural [19]. The pH of the solution cannot maintain in a high alkaline condition, so it dropped rapidly after the reaction started. As the reaction progressed and approached completion, the trend of pH decline slowed down. The above results proved that the Maillard reaction occurred between CTS and JGI.

### 3.1.2. BI and λ294

A color change accompanies with the Maillard reaction. Yellow and yellow/brown products are formed in the middle and final stages of the Maillard reaction, respectively [36]. Therefore, measuring the color change can reflect the progress of the reaction. The characteristic absorbance at 420 nm and 294 nm was measured (Fig. 1B and Fig. 2A). It could be seen that the absorbance at 420 nm and 294 nm in the pHe 7.0 condition along with 294 nm in the pH 11.0 condition presented an upward trend when reacting. This result indicates that as the reaction proceeds, the content of colored substances gradually increases. However, a different trend appeared in the absorbance at 420 nm in alkaline conditions. The absorbance increased rapidly in the first 15 min and decreased subsequently. This phenomenon is caused by the decreased pH during the reaction process, which reduces the solubility of trace tannins and anthocyanins in JGI [18], causing different colors of tannins and anthocyanins to exhibit under different acid-base conditions [29]. The degree of color lightening caused by the decrease in solubility of tannins and anthocyanins is higher than the content of brown substances produced in the reaction. Thus, the absorbance value at 420 nm in the subsequent reaction decreases. The absorbance value under alkaline conditions is significantly higher than that under neutral conditions. One reason is that JGI has a high solubility under alkaline condition, which contributes to complete reaction. Thus, higher content of brown substances is formed. The other reason is that tannins and anthocyanins interfere with color.

### 3.1.3. GD

The GD during the reaction was measured to investigate the degree of the Maillard reaction. The result is shown in Fig. 2B. Under neutral and alkaline conditions, GD shows an upward trend. With the increase of the CTS proportion, the GD value increased, indicating that the GD value was positively correlated with the amount of CTS added. Moreover, the GD between CTS and JGI is higher at pH 7.0 than at pH 11.0.

### 3.1.4. Surface disulfide bond content

In addition, the content of surface disulfide bonds can reflect the changes in protein structure during the reaction process, which can indirectly reflect the degree of Maillard reaction [52]. The result is presented in Fig. 2C. As the reaction progressed, the surface disulfide bond content increased at both pH values. However, the reaction times to reach the maximum value were different among the four ratio reactions. Besides, comparing the reactions at pH 7.0 and pH 11.0, the surface disulfide bond content is significantly higher in the neutral environment than in the alkaline environment. This phenomenon indicates that the Maillard reaction proceeds to a higher degree in a neutral environment.

Based on the above four indicators, the BI, intermediate products, GD, and surface disulfide bond changes in UTSA-treated groups are higher than in untreated traditional reaction groups. This result proves that UTSA could accelerate the reaction rate by supporting more free amino groups of JGI and accelerating their contact with carbonyl radicals of CTS. Same results were also reported in Zhang and Jiang’s studies [18,53]. In addition, four proportions of CTS-JGI conjugates under the condition of the highest degree of UTSA-MR were selected for subsequent experiments. The optimal conditions are CTS-JGI-0.5 reacted for 120 min at pH 7.0 (with a total change percent of 69.27 % in four indexes), CTS-JGI-1, CTS-JGI-2, and CTS-JGI-4 reacted for 180 min at pH 7.0 (with total change percent of 81.38 %, 116.48 %, and 144.54 %, respectively, in four indexes). This result proves that as the proportion of CTS elevates, the degree of reaction increases significantly.

### 3.2. CTS-JGI structure properties

#### 3.2.1. HPSEC analysis

To accurately analyze the changes in the molecular weight distribution of JGI after the reaction, HPSEC is used instead of SDS-PAGE to analyze whether new molecular weight peptides are generated during the reaction. The elution profiles of the CTS-JGI conjugates at 214 nm and 280 nm are shown in Fig. 3A and B. Large molecules are usually eluted before small molecules in HPSEC. Compared with the native JGI molecular distribution stated in the previous study [18]. The prominent peak of JGI at 10 min resolved into two peaks when conjugated with high CTS ratios (CTS-JGI-2 and CTS-JGI-4). This result was due to sufficient CTS-JGI reaction in CTS-JGI-2 and CTS-JGI-4 to unwind the GD, and surface disulfide bond changes in UTSA-treated groups are higher than in untreated traditional reaction groups. This result proves that UTSA could accelerate the reaction rate by supporting more free amino groups of JGI and accelerating their contact with carbonyl radicals of CTS. Same results were also reported in Zhang and Jiang’s studies [18,53]. In addition, four proportions of CTS-JGI conjugates under the condition of the highest degree of UTSA-MR were selected for subsequent experiments. The optimal conditions are CTS-JGI-0.5 reacted for 120 min at pH 7.0 (with a total change percent of 69.27 % in four indexes), CTS-JGI-1, CTS-JGI-2, and CTS-JGI-4 reacted for 180 min at pH 7.0 (with total change percent of 81.38 %, 116.48 %, and 144.54 %, respectively, in four indexes). This result proves that as the proportion of CTS elevates, the degree of reaction increases significantly.

#### 3.2.2. UV analysis

Proteins have an apparent UV absorption at 280 nm (on account of the presence of conjugated double bonds in tryptophan and tyrosine residues), so the UV absorption of CTS-JGI at 280 nm was determined. The result is shown in Fig. 3C. Blue shifts of the absorbance in CTS-JGI appeared compared to native JGI studied in the previous study [18], which may be ascribed to the change in solution polarity, pH, and the decrease in the degree of JGI conjugation as the reaction proceeds. A slight red shift appears in the UV absorption as the proportion of CTS increases. This red shift is caused by the increase in the proportion of CTS, which makes it react fully with JGI and significantly changes the spatial structure of JGI. In addition, the formation of organic compounds such as ketones and aldehydes during the reaction increases the polarity of the solution, which also contributes to the red shift phenomenon. The same phenomenon was also reported in Chen’s study [7].

#### 3.2.3. FT-IR analysis

FT-IR is an effective method to explore the structures of proteins and polysaccharides. As shown in Fig. 3D, CTS possesses noticeable peaks at 2989.123 cm⁻¹ and 2901.378 cm⁻¹, an -NH angular deformation peak at 1592.913 cm⁻¹, and a C=O–O= peak at 1066.442 cm⁻¹ [9]. JGI exhibits amino acid characteristic bands at 2924.87 cm⁻¹ (C–H stretching), 1651.50 cm⁻¹ (α-helix in amide I), 1532.52 cm⁻¹, 1517.70 cm⁻¹, 1457.12 cm⁻¹, 1377.09 cm⁻¹, and 1321.36 cm⁻¹, indicating the fish scales peptides and xylene were cross-linked [8].
cm⁻¹, 1453.07 cm⁻¹ (N–H bond vibrations), and 1233.26 cm⁻¹ (β-strand) [18]. After conjugation, the characteristic bands of CTS at 2989.123 cm⁻¹, 2901.378 cm⁻¹, 1592.913 cm⁻¹ and JGI at 1532.52 cm⁻¹, 1517.70 cm⁻¹, and 1453.07 cm⁻¹ disappeared. This result indicates that CTS-JGI electrostatic interaction occurred in four combinations of conjugates. Red shifts appeared at 2925.996 cm⁻¹, 1655.569 cm⁻¹, 1484.437 cm⁻¹, 1405.370 cm⁻¹, 1247.718 cm⁻¹, 1194.685 cm⁻¹, and 1064.996 cm⁻¹. These shifts are associated with the consumption of amino groups and the progress of the UTSA-MR [37]. New peaks were exhibited at 1323.892 cm⁻¹, which indicated new substances were formed. These changes demonstrate that the conjugation occurred between CTS and JGI under the effect of UTSA-MR. As the proportion of CTS increases, the characteristic peaks of CTS-JGI are getting closer to CTS, which is caused by the masking of the characteristic peaks of JGI after the increase of CTS proportion.

3.2.4. CD analysis
CD is a relatively simple and effective technique for studying the secondary structure of proteins [7]. The secondary structure distribution of CTS-JGI conjugates is predicted by CD and presented in Table S2. A significant decrease of α-helix and increase of β-sheet and random in the heated JGI and CTS-JGI conjugates compared to the native JGI in the previous study with 54.11 % α-helix, 18.60 % β-sheet, and 27.29 % other structures [18]. These changes in the JGI secondary structures indicate a significant change in JGI spatial structure after heating and glycosylation. The reaction of the polysaccharide carbonyl group with the protein amino group is usually in the α-helix region and its surrounding structures. The decrease in the α-helix ratio shows that the helical region is the central region of the reaction. Besides, ultrasound can also influence the secondary structure ratios considering its effect on exposing the interior amino acids to the surface. The same result also appeared in Li’s study that α-helix of peanut protein decreased after conjugating with gum Arabic and dextran through ultrasonic treatment [22].

3.2.5. Surface hydrophobicity (H₀)
The surface hydrophobicity of proteins refers to folding proteins in aqueous media that tends to bury hydrophobic residues inside the molecule [21]. The balance of hydrophobic and hydrophilic interactions plays a vital role in protein structure and function. The H₀ values of CTS-JGI conjugates are shown in Table S3. The H₀ values of CTS-JGI-0.5, CTS-JGI-1, and CTS-JGI-2 were significantly (p < 0.05) higher than the heated JGI, which was mainly caused by the hydrophilic peptides’ exposure to the surface of JGI and the addition of polysaccharides tends to compete with ANS, thereby inhibiting the binding of ANS to protein groups. However, the lowest H₀ appeared in CTS-JGI-4 due to the steric hindrance of CTS [54]. Meanwhile, as the proportion of CTS increases, the surface hydrophobicity tends to decrease in the four conjugates. This phenomenon is due to the binding of hydrophilic linear polysaccharides in CTS increasing the hydrophilicity of the conjugate molecule, thereby reducing the exposure of buried hydrophobic groups within the molecule. A study showed that the surface hydrophobicity of proteins was related to α-helix [44]. The link between α-helix and H₀ is again demonstrated by the reduction of the helix structure and H₀ value after the reaction in this experiment. This reduction is because glycosylation loosens the structure of JGI, allowing it to cross-link with CTS.

3.2.6. SEM observation
The morphology of the JGI and CTS-JGI conjugates is shown in Fig. 3E-H. JGI exhibited a smoother and stiffer form. In contrast, the conjugates after the UTSA-MR showed a cross-networking structure. This result is ascribed to the changes in JGI structure after glycosylation with CTS. The Maillard reaction at high temperatures is beneficial to drawing polysaccharides and proteins, thereby improving the efficiency
of the grafting reaction. This improvement of the reaction may be due to the high temperature that unfolds the protein structure and accelerates molecular movement, promoting the covalent interaction of the protein with the polysaccharides.

3.2.7. Amino acid composition

An amino acid analyzer was used to analyze the amino acid composition of JGI to explore the changes in the amino acid composition of JGI during the reaction. The result is exhibited in Table 1. The total amount of amino acids in the conjugate is significantly reduced compared to native JGI, indicating that the reaction between CTS and JGI consumes the amino acids of JGI. Besides, the proportion of hydrophilic amino acids in the conjugate decreased while the proportion of hydrophobic amino acids increased. Pro, Ala, Val, Ile, Leu, Phe, Ser, Tyr, Lys, His, and Arg content significantly decreased, demonstrating that these amino acids are potential binding sites with CTS. A content increase of Met, Gly, and Cys appeared, which contributed to the unfolding of JGI structure, exposing these internal amino acids. Moreover, Thr, Asp, and Glu had no significant content change. One reason is that they are not involved in the reaction. Another deduction is that they are consumed in the same amount exposed from the internal structure during the reaction. Maillard reaction does not increase the total amount of essential amino acids. Only the content of Met significantly (p < 0.01) increased.

3.3. CTS-JGI physicochemical properties after UTSA-MR

3.3.1. Solubility analysis

The solubility of the conjugates is shown in Fig. 3i. Four conjugates have high solubility. This increase in solubility is consistent with the fact that the Maillard reaction can improve the solubility of substances. This result may ascribe to the isoelectric point of CTS as opposite to JGI. The Maillard reaction can improve the solubility of substances. This phenomenon is due to the CTS at a high proportion is not combined with JGI, and the isoelectric point of this part of the CTS does not change and cannot be dissolved.

3.3.2. Foaming property and stability

The foaming property and stability are shown in Fig. 3j. The foaming property of CTS-JGI-1 and CTS-JGI-2 is the highest and significantly higher than native JG measured in our laboratory [18]. The foaming stability in the four conjugates was higher than native JG, in which CTS-JGI-2 exhibited the highest stability. This result indicates that the UTSA-MR can enhance the conjugate’s foaming property and stability under proper ratios between polysaccharides and proteins. This improvement in foamaibility and stability can be attributed to the increase in cross-linker solubility and random structure content [45].

3.3.3. Emulsification activity index and emulsification stability index

The formation of protein-polysaccharide conjugates combines protein adsorption properties at the oil-water interface and the solvation properties of polysaccharides in aqueous media. Therefore, glycation could enhance the emulsifying property of the conjugates [49]. The EAI and ESI of CTS-JGI conjugates are shown in Table 2. As expected, the conjugates EAI and ESI were significantly (p < 0.05) increased compared with heated JGI. These results can explain that the UTSA-MR promote the stretching of protein molecules, enhance the attachment of more proteins to the polysaccharide and reduce the tension, thus improving the emulsion performance [45]. Therefore, CTS is an ideal polysaccharide to improve the emulsifying properties of JGI through protein–polysaccharide graft reactions. A study reports that the random structure in protein contributes to better emulsion properties [51]. This result is proved by the increased random ratio in CTS-JGI conjugates.

3.3.4. Viscosity

The viscosity of CTS-JGI conjugates is exhibited in Table 3a. The viscosity of the CTS-JGI conjugates was increased significantly (p < 0.05) compared to native JGI in the previous study [18], which was ascribed to the combination with CTS and the increase of the conjugate solubility. Pirestani’s study also reported that the Maillard reaction could improve the viscosity of canola protein isolate with gum Arabic conjugate owing to the significant effect of gum Arabic addition and formation of a new macromolecule after covalent conjugation [28]. However, the viscosity of the four CTS-JGI conjugates did not show regularity, and there was no significant difference in viscosity.

3.3.5. Thermal stability

It has been studied that the Maillard reaction can improve the thermal stability of proteins and their mixtures [26]. DSC can detect changes in the heat flow of the sample during temperature changes, which can provide beneficial information about the thermal stability of the sample [54]. The thermal stability of JGI and CTS-JGI conjugates was presented in Fig. 3i-3j. Compared with JGI, the fastest weight loss rate was at 280 °C, significantly higher than JGI at 248 °C. This result indicates that the UTSA-MR could enhance the thermal stability of CTS-JGI conjugates. Meanwhile, ΔH values in the conjugates significantly

| Table 1 | Amino acid composition of JGI and CTS-JGI conjugate. |
|---------|---------------------------------------------------|
| Amino acids | Content (mg/g) | JGI ESI |
| **Hydrophile** | | |
| Asp | 17.72 ± 1.26 | 18.60 ± 1.44 |
| Ser | 12.95 ± 1.18 | 10.12 ± 0.74* |
| Gly | 48.23 ± 1.99 | 43.24 ± 2.14 |
| Cys | 11.38 ± 0.95 | 29.37 ± 1.98* |
| Tyr | 0.97 ± 0.034 | 13.08 ± 0.78* |
| Lys | 25.63 ± 1.41 | 13.78 ± 1.11* |
| His | 9.26 ± 0.69 | 4.61 ± 0.20 |
| Arg | 8.48 ± 0.79 | 4.61 ± 0.31 |
| **Total** | 157.62 ± 6.98 | 145.48 ± 3.30* |

| **Hydrophobicity** | | |
| Thr | 10.79 ± 0.44 | 9.38 ± 0.58 |
| Pro | 23.63 ± 1.72 | 17.93 ± 1.21 |
| Ala | 22.64 ± 1.56 | 16.47 ± 1.10 |
| Val | 17.92 ± 1.23 | 11.58 ± 0.21** |
| Met | 3.14 ± 0.15 | 19.75 ± 1.54 |
| Ile | 13.61 ± 0.81 | 9.67 ± 0.63* |
| Leu | 33.97 ± 2.15 | 26.04 ± 1.45* |
| Phe | 27.78 ± 1.52 | 15.01 ± 1.02** |
| **Total** | 153.38 ± 10.25 | 125.83 ± 7.74*** |

Values are means ± standard deviation; significant different between CTS-JGI and JGI was expressed as *, p < 0.05 and ***, p < 0.01, and ****, p < 0.001. Bold amino acids are essential amino acids in human body.

| Table 2 | EAI and ESI of heated JGI and CTS-JGI conjugates. |
|---------|--------------------------------------------------|
| Conjugates | EAI ESI |
| **Heated JGI** | 37.96 ± 3.25* | 225.80 ± 11.21* |
| CTS-JGI-0.5 | 197.00 ± 18.61* | 658.62 ± 16.73* |
| CTS-JGI-1 | 179.98 ± 18.49** | 1124.44 ± 28.00* |
| CTS-JGI-2 | 169.70 ± 4.44* | 866.04 ± 24.46* |
| CTS-JGI-4 | 145.06 ± 4.67** | 1268.04 ± 38.54* |

Values are means ± standard deviation; values with different letters within a column differ significantly (p < 0.05).
may be the reason for the instability of the emulsion. This phenomenon caused by the increase in buoyancy and the decrease in gravity consistent with visual observations (Fig. S4 A). The migration of oil droplets in the emulsions seems to produce stable BSI values, which is differently colored lines in the profile represent the change in BSI of the percentage change in BSI from the initial stage, respectively. The 3.4.2. Backscattering intensity and Turbiscan scan index analysis

The particle size, PDI, and zeta-potential, and graphs of the nano-emulsions were measured. As shown in Fig. 4 A, 5A-B, Table S5, and Fig. 6 A, the particle size remained at 286.2–357.9 nm when the emulsions were formed. The particle size increased with the ratio of CTS due to the viscosity of CTS, causing slight aggregation in the emulsions. Moreover, CTS-JGI-2 exhibited the lowest PDI, indicating stability in the emulsions. The same result existed that CTS-JGI-2 possessed the highest zeta-potential with a value of ~28.05 mV. These results suggest that emulsifying ability of JGI can be positively affected by conjugating with CTS. Due to their improved solubility and viscosity, the CTS-JGI conjugates can be absorbed quickly on the oil-in-water interface.

To investigate the stability of CTS-JGI stabilized oil-in-water nano-emulsions. The particle sizes, PDI, and emulsion states are tracked continuously during 28 days of storage (Fig. 4 A, 5B, and 6A). An increase in particle size can be observed during storage, possibly due to the coagulation of particles in the emulsion. The particle size in the four conjugate-formed emulsions increased by 34.3–53.8 nm during the storage period because of the aggregation, while the change in PDI was not noticeable.

3.4.3. Stability of emulsions against NaCl

The presence of electrolytes in the emulsion makes the system unstable. To determine the ability of the CTS-JGI conjugates to resist ions in the stabilized emulsions. NaCl was added to the emulsion at concentrations of 50 mM and 150 mM. The particle size, PDI changes, and emulsion graphs are shown in Fig. 4B-C, 5C-D, and 6B. The particle size and PDI increased when NaCl was added to the emulsions. This phenomenon is because the addition of ions reduces the electrostatic repulsion between particles, and the system becomes unstable so that particles aggregate to form stable large particles [25]. The emulsion particle size in the 50 mM NaCl groups increased less in the following measurements. The emulsion particle size even became smaller in CTS-JGI-1 and CTS-JGI-2. In the 150 mM groups, the initial particle size of the emulsions increased significantly (p < 0.05), but the change was not apparent in the following 21 days. On the 28th day, both groups significantly (p < 0.05) increased the particle size and PDI, but PDI remained within a stable range during storage. The PDI decreased in the CTS-JGI stabilized oil-in-water emulsions, indicating that CTS-JGI could resist NaCl-induced agglomeration.

The above emulsion stabilization experiments conclude that CTS-
JGI-2 possesses the best stabilization effect on the emulsion. Therefore, it is selected for the following functional experiments.

3.5. Functional properties of CTS-JGI

3.5.1. In vitro antioxidant activities

The hydroxyl radical is a highly reactive oxygen species. The hydroxyl radical scavenging rate is an important index to evaluate the free radical scavenging ability [34]. Therefore, the scavenging activity against hydroxyl radicals is one of the most effective protections against diseases caused by free radical-induced oxidative stress. ABTS and DPPH free radical scavenging abilities are also critical indicators to measure the antioxidant capacity of functional components. The in vitro antioxidant abilities of CTS-JGI-2 are shown in Fig. 6 C-F. The same concentrations of JGI in the previous study were used to evaluate the four assays [18]. CTS-JGI-2 exhibited ABTS, DPPH, and hydroxyl scavenging abilities, and the abilities increased with the concentrations. The DPPH ability of CTS-JGI-2 was the highest among the three radicals. This increase in DPPH ability is due to the Maillard reaction producing new peptides in the low molecular weight region with enhanced DPPH radical scavenging ability. These peptides are hydrophobic and can combine with DPPH radicals, thus showing a stronger scavenging power. In addition, the improvement of the free radical scavenging activity of CTS-JGI-2 is related to the chromogenic groups, deoxyfructose, pyrrolidones, and reducing ketones generated during the reaction [47]. The hydrogen atoms of these antioxidant molecules are combined with the free radicals of the DPPH molecules and stabilize the molecule without chain reactions. This high DPPH radical scavenging ability is precisely consistent with the result of the increased H₂O₂. However, the ferrous reducing ability of CTS-JGI-2 did not significantly increase in the concentrations of 0.125–0.625 mg/mL compared to JGI. The ferrous reducing power increased only at the two high concentrations. Usually, free hydroxyl groups play an essential role in the ferrous force, which can provide hydrogen atoms that destroy the free radical chain and increase the reducing power activity [38]. From this, it can be speculated that CTS-JGI-2’s radical scavenging ability is stronger than ferrous reducing power.

3.5.2. Cholesterol-lowering activities

Many mechanisms explain the cholesterol-lowering functional effect of related components. One of the most accepted explanations is that the ingestion of these components disturbs the enterohepatic circulation, thereby causing the excretion of diseased cholesterol and bile acids. The cholesterol-lowering effect of protein-based components is related to their insolubility. Indigestible proteins or polypeptides rich in hydrophobic amino groups (which can combine with bile acids) are post-
digested or fecal. The cholesterol-lowering abilities of JGI and CTS-JGI-2 were firstly determined. As shown in Fig. 6 G-J, three concentrations of JGI and CTS-JGI-2 exhibited cholesterol-binding capacity (7.46 ± 0.54 – 62.58 ± 3.88 mg/g and 10.12 ± 0.96 – 71.52 ± 4.41 mg/g). The cholesterol-binding capacity of CTS-JGI-2 is significantly higher than JGI. This observation confirms that Maillard reaction conjugates have a comparable potential to bind to cholesterol directly. The inhibiting cholesterol micellar solubility is critical in reducing intestinal absorption of dietary cholesterol. JGI showed MCI values of 4.21 ± 0.21 – 31.03 ± 4.29 %, while higher MCI values appeared in CTS-JGI-2 (6.32 ± 0.54 – 39.48 ± 2.38 %).

Bile acids are biosynthesized from endogenous cholesterol metabolism. If bile acids in the intestinal lumen bind to sequestering agents, such as cholestyramine, enterohepatic circulation will be disrupted, and bile acid reabsorption and endogenous cholesterol levels are reduced [14]. Similar to the above index results, it can be observed that the UTSA-MR remarkably (p < 0.01) increased the bile acid-binding ability in the 5 and 10 mg/mL of CTS-JGI-2 compared to native JGI (increased by 2.14 % and 4.82 %, respectively). Although the inhibition rate was higher than that of cholestyramine resin, the concentration of CTS-JGI-2 was much higher. Thus, the α-amylase inhibitory activity of CTS-JGI-2 does not reach the inhibitory ability of the standard drug.

3.6. Encapsulation efficiency and loading capacity

To estimate the protective effect of CTS-JGI-2 on active substances, RES and QUE were loaded in the CTS-JGI-2-O/W-NE. As illustrated in Fig. 7 A, the EE of RES and QUE loaded in CTS-JGI-2-O/W-NE increased significantly (p < 0.05) than JGI, which was ascribed to the enhancement of loading ability after the UTSA-MR. However, the LC showed no significant different between the two emulsions, which was caused by the high LC leading to lower EE at overload [53]. The EE and LC of QUE were significantly (p < 0.05) higher than RES, proving a better loading ability of QUE in CTS-JGI-2 and JGI. This better loading ability is contributed to the stable binding effect between QUE with CTS-JGI-2 and JGI. The same result was also obtained in Zhang’s study that the Maillard reaction product of whey protein and flaxseed gum also increased the EE and maintained the LC of astaxanthin [53].

3.7. In vitro digestion of emulsions

The release rates of RES and QUE in the emulsions are exhibited in Fig. 7B. It could be seen that the CTS-JGI-2-O/W-NE was resistant to
contributed to the better solubility of JGI in alkaline environment. The increase of RR is digestion rate than JGI-O/W-NE, and it is speculated that the emulsion. RES and QUE in CTS-JGI-2-O/W-NE have a slower digestion process of pepsin is primarily limited by the surface area and erosion rate of the CTS-JGI-2 particles in the emulsion. RES and QUE in CTS-JGI-2-O/W-NE have a slower digestion rate than JGI-O/W-NE, and it is speculated that the emulsion covalently linked to CTS through the UTSA-MR can shield the cleavage site of pepsin by JGI.

When the emulsions were transferred to the intestinal condition, the RR increased significantly in the first 1 h. The increase of RR is contributed to the better solubility of JGI in alkaline environment provided by intestinal fluid, enabling the release of RES and QUE. The RR remained stable at 180–240 min. After the digestion, the total RRs were 57.96 % of RES, 55.69 % of QUE, 40.2 % of RES-JGI-O/W-NE, 38.69 % of QUE-JGI-O/W-NE, 26.77 % of RES-CTS-JGI-2-O/W-NE, and 22.06 % of QUE-CTS-JGI-2-O/W-NE, respectively. As shown in Fig. 7C, the BAY of RES and QUE in JGI-O/W-NE (59.8 % for RES and 61.31 % for QUE) were significantly higher than those in RES-JGI-O/W-NE (73.23 % for RES and 77.94 % for QUE) respectively. Worthy to note that the BAY of QUE in this study was significantly higher than rice bran protein-based nano-emulsion in Chen’s study [7]. Hydrogen bond and pi-anion appeared higher energy than the bindings with JGI (Fig. 8G and I). The binding energy from the outside and cannot spontaneously combine. UTSA-MR provides the energy for the conjugation between JGI and CTSU. More stable combinations between QUE with JGI and CTSU correspond to the higher EE, LC, RR, and BAY of QUE than RES. However, RES and QUE have the same docking site as JGI (Gly-47). Considering that the ratio of RES and QUE is much lower than JGI, there is no competitive docking relationship between the two substances.

Moreover, the potential binding between CTSU and JGI was also predicted. The potential binding sites are shown in Fig. 8K. The binding energy is positive (data not shown), indicating the combination needs energy from the outside and cannot spontaneously combine. UTSA-MR provides the energy for the conjugation between JGI and CTSU, so they can finally combine.

4. Conclusion

In conclusion, JGI was combined with CTS through UTSA-MR to obtain the conjugates with better solubility, thermal stability, emulsifying stability, antioxidant ability, and cholesterol-lowering activity. This improvement might attribute to the JGI structural modification by the reaction with CTS. Furthermore, the O/W-NE stabilized by CTS-JGI-2 was preeminent for entrapment and site-specific release of RES and QUE. The above results highlight that the covalent linkage of JGI and CTS through UTSA-MR can be an ideal method to improve its bioactive
properties and expand its utilization in the food nano-emulsion stabilization and functional substances delivery system. More investigation on the in vivo protection of RES- and QUE-loaded nano-emulsion stabilized by CTS-JGI-2 needs to be carried out in further study.

CRediT authorship contribution statement

Yunsong Jiang: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. Kai Zang: Data analysis. Jinyuan Sun: Funding acquisition, Supervision, Writing – review & editing. Xin-an Zeng: Writing – review & editing. Hehe Li: Resources. Charles Brennan: Writing – review & editing. Mingquan Huang: Writing – review & editing. Ling Xu: Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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