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Complexation of Soy Protein Microgels and Isoflavones: Implication in Modulating Bioavailability

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Abstract: The soy protein aggregate particles-isoflavones complexes (SPIGs) were prepared by thermally induced protein-polyphenol co-assembly and the *in vitro* bioavailability of isoflavones with different soy protein matrices was evaluated. The SPIG formed by heating SPI-isoflavone dispersion at 120°C in a narrow pH range, which was defined as spherical particles with uniform size, exhibited better colloidal stability and high isoflavone binding capacity, which was higher than that of native SPI and unheated mixture. The results also showed that different pH had no significant effect on fluorescence quenching by protein-isoflavone interaction. The bioavailability of isoflavones was significantly elevated when it was encapsulated in the particles. The overall bioavailability of isoflavones maybe depends on the biotransformation of isoflavones and the production of bioactive metabolites.

Keywords: Soybean Protein Isolates, Particles, Isoflavones, Interaction, Bioconversion

Introduction

There have been many *in vitro* and clinical trials on the pharmacological properties of isoflavones and isoflavone-rich soy protein, such as antioxidative, anti-inflammatory, neuroprotective, and anticarcinogenic effects, as well as protective effects against bone loss, hormone-dependent and independent cancers, cardiovascular diseases and autoimmune diseases (Liu *et al*., 2017; Chatterjee *et al*., 2018). Unfortunately, data from intervention and clinical trials to evaluate the possible beneficial effects of isoflavone-rich protein and soy foods on a variety of health outcomes remain to be inconsistent and controversial (Amaral *et al*., 2017; Applegate *et al*., 2018; Hu *et al*., 2020). The variability highlights the gap in our understanding of soy isoflavone uptake, metabolism, distribution, and overall bioavailability. Some studies have highlighted the importance of soy isoflavone metabolism and the critical role of human intestinal flora in the bioavailability of these compounds and their metabolites (Chen *et al*., 2022). In addition, the bioavailability of isoflavones is also affected by different factors such as the food matrix and the interaction with other food components. This inconsistency is probably attributed to the lack of standardization of the sources of isoflavones in clinical trials, especially the neglect of protein-isoflavone association when dealing with soy matrixes with different protein content and denaturation states (Malaypally and Ismail, 2010).

Owing to their polyphenolic nature, isoflavones can non-covalently interact with the soy protein matrix, thus probably altering their bioaccessibility, bioavailability, and specific biological activities in the intestinal tract systemically. Malaypally and Ismail confirmed that the binding of soy isoflavones to soy protein greatly affected its extractability and thermal stability. Currently used solvent extraction techniques considerably underestimate isoflavone content in complex soy systems with high protein content and proteolysis and enhance the extractability of isoflavones, especially in samples containing denatured protein (Malaypally and Ismail, 2010; Jang *et al*., 2020). The binding of polyphenols to proteins may have multiple consequences, but the biological implications of these interactions remain to be unclear (Li *et al*., 2021, Dumitrascu *et al*., 2020, Jovcevski *et al*., 2020). In addition, as far as we know although the interaction of soy protein and isoflavones is very important to explain the variability in the overall bioavailability of isoflavones, there were few studies conducted in this field.

This study aims to prepare the soluble SPI aggregates-isoflavone complexes by thermally inducing protein-polyphenol co-assembly strategy and further evaluates the *in vitro* bioconversion of isoflavones trapped in the

complexes, thus better understanding the effect of soy protein-isoflavone interaction on the bioaccessibility of isoflavones. The colloidal stability and morphological properties of SPIG prepared with different combinations of temperature and pH were characterized by Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM), and TEM. The interaction of isoflavones with soy protein isolate and its main components, *β*-conglycinin, and glycinin were accessed by fluorescence quenching. Bioaccessibility is defined as the fraction of ingested nutrients that are absorbed and utilized during gastrointestinal digestion (Wu *et al*., 2012). The overall bioavailability of isoflavones depends on the biotransformation of isoflavones in the intestinal tract and the production of bioactive metabolites, which largely depends on the nature of intestinal flora. The bioconversion from the naturally occurring isoflavone glucosides into aglycones forms catalyzed by the cytosolic and bacterial *β*-glucosidases of the small intestine is considered to be a key and limited step to promote their absorption and bioavailability (Guo *et al*., 2012). However, it is difficult to study the *in vivo* changes of these components through the digestive tract. Therefore, *in vitro* conversion of isoflavones enriched the soy protein aggregate particles during the fermentation in the presence of probiotic *Lactobacillus* bacteria from the human intestinal tract was used as an alternative approach to *in vivo* studies, to assess the bioaccessibility of isoflavones with different protein matrices.

Materials and Methods

Experimental Materials

Soy Protein Isolate (SPI) lyophilized powder with a protein content of 89.66±0.16% was prepared by acidic precipitation technology as described in the previous study (Guo *et al*., 2012). Soy isoflavones product with a purity of 98% (w/w) was purchased from Shanghai Fusheng biological technology Co., Ltd. Soy isoflavones standards were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Pancreatin from porcine pancreas 8× USP specifications and sodium deoxycholate were purchased from Sigma Chemical Co., Ltd. Pepsin (DH232-1; activity, 1:3000) were purchased from Gen-View Scientific Inc (Wilmington, DE). *Lactobacillus casei* LC-L134-1-M and *Lactobacillus plantarum* LP-L134-1-P were obtained from China General Microbiological Culture Collection Center, then domesticated and cultivated in our lab. All chemicals used are analytical grade or higher.

Preparation of Soy Protein Aggregate Particles Enriched with Soy Isoflavones

The SPI dispersions (2%, w/v) were prepared by dissolving lyophilized SPI in Millipore water (18.2 MΩ·cm), stirred at room temperature for 2 h, and hydrated overnight at 4°C. Insoluble proteins were removed by centrifugation at 10000 g for 20 min. The pH of prepared SPI dispersions was adjusted to different pH with 1 M HCl. Isoflavone dispersion was prepared by dispersed isoflavone mixture (80% W/W) in 5 mm acetate buffer solution (pH = 4.5) to give a concentration of 0.04 mg/mL, then passed through a homogenizer (Microfuidizer M-110 EH Processor Co., Lampertheim, USA) at 500 bar pressure for two times to obtain a homogenous dispersion. An excess amount of soy isoflavones (0.5 mL) was mixed with 40 mL SPI dispersions (2.0%, w/v) by homogenizing at 8000 rpm for 5 min using an Ultra-Turrax T25 (IKA-Werke GMBH & CO., Germany). The resulting mixture was heated at 90°C for 30 min in a water bath (TW12; Julabo, Seelbach, Germany) and at 120°C for 30 min in an autoclave (YX280D, Huatai Co. Ltd., Hefei, China), respectively. The obtained mixtures were taken out and cooled down in an ice bath to room temperature and then freeze-dried.

Determination of Total Isoflavones and Bound Isoflavones in Soybean

SPIG was suspended at 2.0% w/v in distilled water and stirred at room temperature for 2 h. For solvent extraction of overall isoflavones, 5 mL methanol and 1 mL 0.1 M HCl (pH 2.0) were added into 2 mL dispersion and then stirred for 5 min. After centrifuging at15,000g for 20 min, the supernatant was filtered through a 0.45 *μ*m filter (Millipore, Billerica, MA, USA) before HPLC determination (the content of overall isoflavones). For enzyme-assisted extraction of total and binding isoflavones (Malaypally and Ismail, 2010; Murphy *et al.*, 2002), sample mixtures were incubated firstly with papain (2400 U/g sample, pH 6.5) at 50°C for 2 h and then added 5 mL acetonitrile and 1 mL 0.1 M HCl to terminate enzyme activity. The resulting supernatant was centrifuged and filtered for HPLC determination (the content of total isoflavones). The binding mass of isoflavones with the protein matrix was calculated as follows:

$$B = T - O$$

where, *B* represents the mass of bound isoflavones, *T* represents the content of total isoflavones, and *O* represents the content of overall isoflavones.

Quantitative analysis of isoflavone (Murphy *et al.*, 2002) was performed with a reversed-phase Symmetry C18 HPLC column (5 *μ*m, 4.6 × 250 mm) using an HPLC system (Waters, USA) equipped with a Waters 1525 pump and Waters 2487 UV detector (Waters, USA).

Particle Size and Zeta-Potential Measurements

The measurements of size distribution, zeta-potential, and Polydispersity Index (PDI) of protein aggregate particles were performed using a Nano ZS Zetasizer instrument (Malvern Instruments, Worcestershire, UK). Samples were diluted to 0.1 wt % with 20 mm phosphate buffer (pH = 7.0) before loading into the cuvette

(PCS8501). A refractive index of 1.450 was used for dispersion (protein) and 1.331 for the continuous phase (20 mm phosphate buffer, pH = 7.0).

AFM and TEM Observation

AFM images were recorded in tapping mode at a drive frequency of approximately 320 kHz and the scan rate was 1.0 Hz using a MultiMode SPM microscope equipped with a Nanoscope IIIa Controller (Digital Instruments, Veeco, Santa Barbara, CA, USA). Based on TEM (FEI Electronic Optics Co., Ltd, Holland), the negative staining method was used for morphology characterization. Samples were prepared by depositing the protein aggregate particles onto a carbon-coated copper grid for the 60s and the redundant sample was sucked. The samples were dried in a desiccator containing dried silica gel. After that, the samples were negatively stained by phosphotungstic acid (1.0%, w/w) for 60 s and dried for TEM observation.

Fluorescence Spectroscopy Measurements

Quenching constant (K_q), binding constants (K_a), and binding numbers (n) were recorded using an F7000 fluorescence spectrophotometer (Hitachi Co., Japan). Soy isoflavone product with a purity of 98% (W/W) was dissolved in 80% ethanol that was diluted with a 5 mm acetate buffer solution (pH=4.5) at a concentration of 1.5 mg/mL as a stock solution. Protein intrinsic fluorescence was measured at constant SPI concentration (0.04 mg/mL) in the presence of 0 to 4.5 μg/mL isoflavone concentration. Emission spectra were recorded from 300 to 500 nm at an excitation wavelength of 280 nm. Both the excitation and emission slit widths were set to 5 nm.

In Vitro Digestion in the Presence of β--Glycosidase

The release of isoflavones from the SPIG in simulated gastrointestinal fluids was determined (Wu *et al*., 2012; Vitas *et al*., 2020). the pH of the samples (20 mL) was adjusted to 2.0 with 6 M HCl solution and 2.0 mL pepsin was added at a concentration of 5 mg/mL. After continuous stirring at 37ºC for 60 min, the pH of the samples was adjusted to 7.0 using 1 M NaOH, then added a pancreatin from porcine pancreas/bile salt mixture (2.4 mL; 100 mmol L^{-1} sodium deoxycholate, 5 mg mL^{-1} pancreatin and 100 mmol L^{-1} sodium bicarbonate solutions). The preparation was then incubated for a further 120 min at 37°C.

In Vitro Fermentation

Lactobacillus bacteria from the human intestinal tract was used to assess the bioavailability of isoflavones with different protein matrices (Gaya *et al*., 2020). After adding 8.0 wt% of glucose, the sample dispersions were transferred into glass bottles and sterilized at 90-95 °C for 5 min. The prepared soymilk was cooled and stored in a refrigerator (4°C) before use. Domesticated and cultured LAB strains *Lactobacillus casei* LC-L134-1-M and *Lactobacillus plantarum* LP-L134-1-P were isolated from human feces and used as starters for soymilk fermentation in single cultures. The strain was individually propagated twice in 10 mL lactobacilli MRS broth at 37°C for 12 to 15 h. The *Lactobacillus* was inoculated with a single culture (3 mL, 2×10^7 CFU/mL, 2% of soymilk, v/v) and then aliquoted into sterilized 15 mL centrifuge tubes. After tightening the cover, the soymilk tubes were put into an incubator at 37°C for 12 h.

Animal Experiment

The experimental protocol was approved by the Animal Committee of the Guangzhou University of Chinese Medicine (SCXK, Guangdong, NO.00089724). SPF Sprague-Dawley male rats were divided into 3 groups of 8 rats each. Group I received a single oral administration equivalent to 100 mg/kg isoflavones, group II received 100 mg/kg isoflavones mixture and group III received 100 mg/kg SPI microgels enriched isoflavones. Blood samples (0.5 mL each) were collected through the tail vein at 0, 5, 40, 90, 140, and 180 min after administration. The blood samples were centrifuged at 15000g for 15 min and the supernatants were collected as plasma samples in tightly sealed plastic tubes and kept frozen at -20°C until analysis. 50 μL plasma samples were digested by 50 μL papain solution (2400 U/g sample, pH 6.5) at 60°C for 2 h, then added 0.4 mL acetonitrile and vortexed for extraction for the night, centrifuged and filtered for HPLC determination (the content of total isoflavones).

Statistical Analyses

All measurements were carried out in duplicate or triplicate. The analysis of variance (ANOVA) of the data was performed to evaluate significant differences (p<0.05) between the results using the SPSS 13.0 statistical analysis system.

Results and Discussion

The Complexion of Soy Protein Aggregate Particles with Isoflavones

Heat-induced protein unfolding increased both the fraction of accessible binding sites and the binding affinity, which is obviously due to exposure to hydrophobic domains, thus enhancing the hydrophobic interactions between protein and polyphenols. The results showed that the colloidal stable and transparent dispersions of protein-based particles were obtained by heating a 2 wt % solution of SPI at 120°C in a narrow pH range of 6.0–6.4. Severe aggregation and visible precipitation were observed unless the sample was heated at pH = 5.0 (Fig. 1). The morphological structures of the SPI aggregates prepared by heat treatment at 120°C with

different pH were observed by AFM and TEM (Fig.2), confirming the formation of spherical aggregated protein particles with a uniform size of 106±23 nm at pH 6.4 and 280±95 nm at pH 6.0. The particle size was calculated according to the TEM images, which was consistent with the results determined by dynamic light scattering (Table 1). Table 1 summarizes the particle size, PDI, zeta potential, and the overall binding mass of isoflavones of resulted SPI particles. There was a general increasing trend in the particle size, zeta potential, and the mass of overall and binding isoflavones entrapped in the SPI particles with the increase in heating temperature, as well as a decrease in the pH, thus regulating their isoelectric point, IEP. Interestingly, the protein particles prepared at 120°C with a net negative zeta potential of -34.2±0.2 mv (pH = 6.4) and -35.4±0.6 mV (pH = 6.0) is much higher than that of the native SPI solution (-28.6±0.3 mV), which can explain that their remarkable colloidal stability depends on the high surface charge and electrostatic stabilization.

The soy protein aggregates are formed by heating at 120°C, which is a well-defined spherical particle with a diameter of several hundreds of nanometers and relatively low polydispersity (0.122-0.201). They represent an intermediate and self-limited aggregation state of protein between the phase-separated aggregates (Fig. 1), which are formed at pH = 5.0 (near pH = 4.5 of IEP), and small fractal aggregates (≤100 nm) which are formed at pH=7.0. Schmitt *et al*. (2010) suggested that the self-limited aggregation of protein can be explained by an equilibrium between attractive hydrophobic forces arising from protein denaturation by heating and repulsive forces arising from the protein net charger through modulating the pH. Our research work had shown that Soy Protein Isolate (SPI) can form a soluble complex with resveratrol, which can be used as an emulsifier to enhance the oxidative stability of soy protein stabilized emulsion (Wan *et al*., 2013). Soluble soy protein aggregates were prepared by heating the soy protein isolate dispersion at 120°C (hydrothermal treatment) and their internal structure, limited aggregation behavior, and interfacial properties were characterized (Guo *et al*., 2012).

There was a slight increase in the turbidity and particle size with the addition of soy isoflavone mixture into the SPI dispersions. Free isoflavone is almost insoluble in water. However, the transparent dispersions were obtained by reconstituting 2.0wt% heated SPI-isoflavone complex powder in water (Fig. 1). As expected, heat treatment favors the interaction between SPI and isoflavone and results in aggregate particles enriched isoflavone (Table 1). The total content of isoflavone, namely the fraction of solvent extraction solubilized in SPI-isoflavone complex powder, were 5.61-6.08 mg/g (90°C) and 6.42-6.68 mg/g (120°C), which was significantly higher than that of the native (0.72 mg/g) and unheated mixture (1.10-1.24 mg/g). Malaypally and Ismail confirmed the association of the isoflavones with the soy protein, especially in the sample with high protein content and denaturation state, thus significantly affecting the extraction of isoflavones. (Malaypally and Ismail, 2010). The binding content of isoflavone, which means the fraction of enzyme-assisted solvent extraction solubilized in SPI-isoflavone complexes powder was 5.61-6.08 mg/g (90°C) and 6.42-6.68 mg/g (120°C), representing an evident increase of isoflavone compared with the native (0.72 mg/g) and unheated mixture (1.10-1.24 mg/g). The binding properties of soy protein with isoflavones were determined at 25°C using intrinsic fluorescence spectroscopy and expressed based on native and different soy protein aggregate particles formed with the combination of temperature and pH. Intrinsic fluorescence titration relied on the change in the intensity of protein tryptophan fluorescence. The maximum emission wavelength with a red shift was found from 338 nm for native to 355.6 nm for aggregate particles (120°C), suggesting that the increased exposure of major Tryptophan (Trp) residues to a more polar environment, as described early in whey protein isolate (Schmitt *et al*., 2010). With the increase of isoflavone concentration in protein samples, the fluorescence intensity decreased gradually and the obvious red shift was observed. This suggested that there was obvious interaction between SPI and isoflavones in the two systems.

Table 1 summarizes the static quenching rate constant k_q of isoflavone for native and aggregate particles at each pH studied. As expected, the k_q of SPI aggregate-isoflavone complex were 9.42×10^{12} (90°C) and 9.45×10^{12} L M^{-1} s^{-1} (120°C) respectively, which was much higher than that of native SPI, indicating that heat treatment favoring the formation of SPI- isoflavone complex, in addition, this value is also much higher than the maximal dynamic quenching constant (2.0×10^{10} M^{-1} s^{-1}), indicating that the quenching process between SPI and isoflavone is mainly due to static quenching by complex formation. The results also showed that different pH has no significant effect on fluorescence quenching of protein-isoflavone interaction.

In Vitro Digestion

Bioaccessibility can be defined as the fractions of ingested nutrients and nutraceuticals that are absorbed in gastrointestinal digestion. Naturally occurring isoflavones in soy protein products exist in the form of glycosides that are poorly absorbed in the human small intestine. The initial hydrolysis by *β*-glycosidase is necessary to release the free aglycones, which are rapidly absorbed via passive diffusion across the intestinal brush border with high permeability (Wu *et al*., 2012). Isoflavone aglycones are detectable with an initial peak 1 after a meal, indicating that hydrolysis and initial absorption occur readily in the duodenum and proximal jejunum within the first hour of digestive processing. Several mammalian *β*-glucosidases have been identified in the small intestine, including a broad specificity cytosolic *β*-glucosidase enzyme, which

suggests that the intestinal mucosa plays an important role in the deglycosylation of isoflavones. Fortunately, it was found that the pancreatin from porcine pancreas contaminated *β*-glucosidase contaminated had to be contained with the activity of 0.09 U/g, which can be used to assess the bioconversion of isoflavone from glucosides into aglycones forms during *in vitro* intestinal digestion. The bioaccessibility of isoflavones trapped in protein aggregates was monitored at 37°C in the presence of pancreatin in the porcine pancreas during in vitro digestion, expressed by the release of free aglycones. In contrast with the glycoside forms, total aglycone content was increased during whole *in vitro* digestion, in which the genistein was the major fraction. In addition, it showed that the profiles and content of isoflavones remain unchanged during gastric digestion implying the protection of SPI aggregates particles to isoflavones at extremely low pH (Table 2).

In Vitro Fermentation

The pharmacokinetics of absorption, distribution, metabolism (bioconversion in the gut and biotransformation in the liver), and elimination all contribute to the bioavailability and subsequent effectiveness of isoflavones. Therefore, understanding bioavailability is important for evaluating the potential health benefits of isoflavones, which may assist in the interpretation of the high variability of results in clinical trials. *In vitro* fermentation studies and incubation of isoflavone glucosides with human fecal samples have shown that the isoflavone glucosides are hydrolyzed into aglycones by glucosidases from fecal bacteria and can be further metabolized into dihydrodaidzein, dihydrogenistein, or equol by anaerobic intestinal bacteria (Rekha and Vijayalakshmi, 2010). *β*-glucosidases (*β*-glucoside glucohydrolase, EC 3.2.1.21) comprise a heterogeneous group of enzymes that can cleave the *β*-glucoside linkages of di and/or oligosaccharide or other glucose conjugates. *β*-glucosidases play an important role in many biological processes. What's more, probiotic microorganisms have been found to possess *β*-glucosidases, which can play an important role in improving the biological activity of soymilk.

Lactobacillus plantarum was selected to determine the biological transformation *in vitro* fermentation of aggregate. Table 3 indicates that the conversion ability of aggregate from glucoside isoflavones to isoflavones aglycone showed obvious differences. The sample used for comparison was SPI directly mixed with isoflavones. From the table, the conversion ability of SPIG was stronger than that of isoflavones in the mixture after fermentation for 48 h. There was a downward trend of aglycones in the mixture after fermentation for 48 h, similar results were reported for the fermented soymilk by probiotic bacteria and yeast and *β*-glucosidase activity in fermented soymilk for 48h at 37°C had played a key role. The reduction and the increase in the content of isoflavones forms relied on the hydrolytic reaction catalyzed by *β*-glucosidase produced by bacterial strain (Rekha and Vijayalakshmi, 2010). In this study, the pH change tendency of the two kinds of soy milk was similar from pH4.4 to pH 2.7 after fermentation for 6-48 h (not shown). The titration acid of mixed soymilk (49.63 at 48 h) was higher than SPIG soymilk (39.84 at 48 h) (not shown). SPIG, different from previous studies, appeared more content of aglycones than the mixture within 48 h after fermentation, the ability of daidzin transformation was higher than that of genistin, which may attribute to the fact that after heating near pI of soy protein, SPI and isoflavones were closely combined, which facilitated glycosidic isoflavone accumulation gradually after slow release and might lead to a small scope increase of bioconversion rate in the late fermentation.

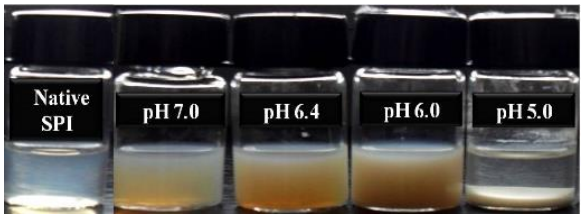

Fig. 1: Dispersions of the native SPI and SPIG synthesized by heating at 120°C with different pH

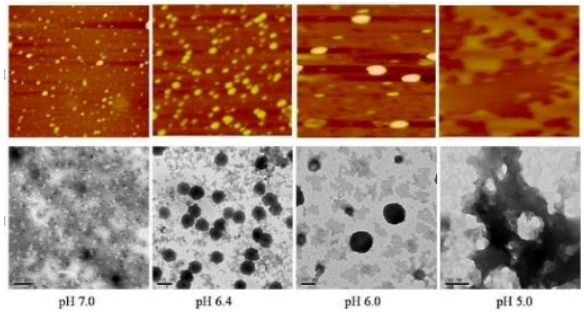

Fig. 2: AFM (100 × 100 nm) and TEM images (2 × 2 μm) of SPIG prepared by heating at 120°C and different pH

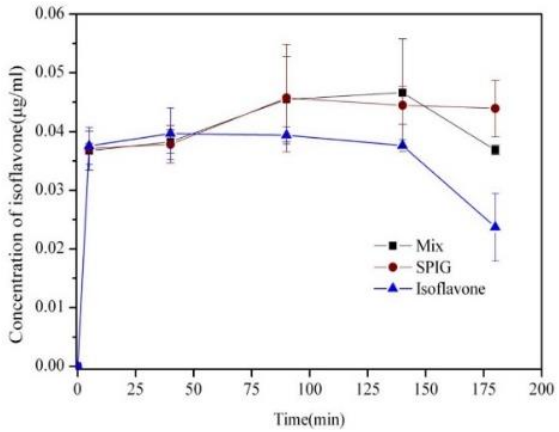

Fig. 3: Mean blood concentration−time profile of total isoflavones in Sprague-Dawley rats following oral administration

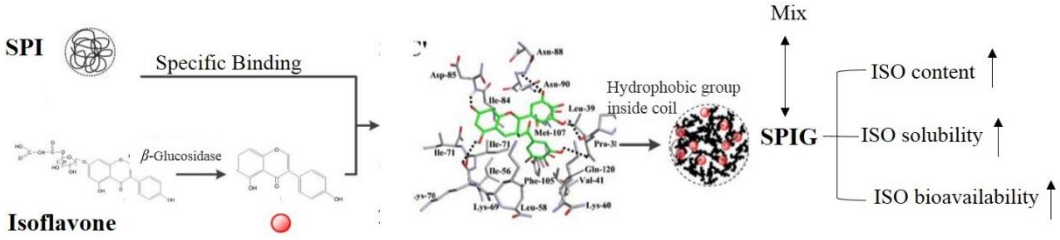

Fig. 4: The schematic diagram for the combination of soy protein and isoflavone complex

Table 1: Physiochemical, colloidal properties and fluorescence quenching spectra of soy protein-isoflavones complex prepared by different combinations of temperature and pH

Samples	Z-Ave (d.nm)	PDI	ζ-potential (mV)	Overall (mg/g)	Binding (mg/g)	Total (mg/g)	K_q($\times10^{12}$) (L· mol^{-1}s^{-1})	K_a	n
Native SPI	63.96±2.56^{a}	0.451±0.021^{b}	-28.6±0.3^{d}	0.57±0.19^{a}	0.72±0.22^{a}	1.29±0.41^{a}	/	/	/
25°C pH 7.0	106.40±3.8^{b}	0.520±0.110^{b}	-29.4±0.4^{d}	12.08±0.02^{d}	1.04±0.19^{a}	13.24±0.21^{b}	3.54±0.32^{a}	1.02±0.22	0.313±0.014
25°C pH 6.4	110.50±3.2^{bc}	0.582±0.020^{b}	-29.6±0.1^{d}	13.18±0.08^{e}	1.10±0.26^{a}	14.28±0.34^{bc}	3.81±0.14^{a}	1.12±0.42	0.326±0.045
25°C pH 6.0	112.20±5.5^{bc}	0.601±0.042^{b}	-29.8±0.1^{cd}	12.29±0.14^{d}	1.24±0.07^{a}	13.53±0.22^{b}	4.22±0.16^{a}	1.17±0.86	0.323±0.036
90°C pH 7.0	116.30±8.4^{bc}	0.221±0.103^{a}	-29.1±0.7^{d}	9.22±0.05^{b}	5.08±0.25^{b}	14.30±0.30 ^{bc}	8.59±0.56^{b}	/	/
90°C pH 6.4	126.30±5.9^{c}	0.204±0.011^{a}	-30.5±1.6^{cd}	9.84±0.12^{c}	5.61±0.16^{bc}	15.45±0.28^{cd}	9.14±0.22^{b}	/	/
90°C pH 6.0	146.80±2.6^{d}	0.252±0.074^{a}	-32.1±0.2^{bc}	9.12±0.23^{b}	6.08±0.07^{bc}	15.20±0.37^{cd}	9.42±0.17^{b}	/	/
120°C pH 7.0	117.50±6.3^{bc}	0.122±0.041^{a}	-31.0±1.0^{cd}	9.23±0.23^{b}	6.19±0.45^{bc}	15.42±0.68^{cd}	8.72±0.11^{b}	2.42±0.33	0.589±0.011
120°C pH 6.4	154.80±1.9^{d}	0.160±0.033^{a}	-34.2±0.2^{ab}	9.60±0.02^{bc}	6.42±0.77^{c}	16.02±0.79^{d}	9.28±0.44^{b}	2.89±0.09	0.601±0.004
120°C pH 6.0	280.10±2.6^{e}	0.201±0.012^{a}	-35.4±0.6^{a}	9.40±0.11^{bc}	6.68±0.56^{c}	16.08±0.67^{d}	9.45±0.31^{b}	3.03±0.14	0.612±0.032

Table 2: Overall release of isoflavones of Mix and SPIG during *in vitro* digestion in the presence of *β*-glycosidase(mg·L^{-1})

	Time(min)	Daidzin	Glycitin	Genistin	Total glucosides	Daidzein	Glycitein	Genistein	Total aglycones
Mix	0	47.30±1.22	70.67±0.17	4.66±1.85	122.63±3.24	154.68±2.55	40.46±1.64	9.36±5.220	204.49±9.410
	60	45.05±2.71	82.61±4.66	4.74±1.01	132.40±8.38	402.29±1.66	64.31±1.62	57.83±1.070	524.43±4.350
	70	36.15±0.18	26.51±3.22	4.26±0.51	66.92±3.91	315.24±0.94	48.49±7.68	44.77±4.790	408.51±13.41
	120	31.13±0.49	12.48±1.76	3.92±0.46	47.53±2.71	301.88±3.04	54.66±5.63	50.45±3.780	407.00±14.30
	180	5.12±1.03	13.69±0.13	4.11±1.73	22.92±2.89	330.35±6.89	54.06±4.40	55.41±2.140	439.76±13.43
SPIG	0	70.94±1.85	105.74±1.44	3.87±1.23	180.56±4.52	352.57±3.56	24.95±1.16	48.43±2.640	425.95±7.360
	60	51.89±2.11	82.11±2.33	5.42±1.56	139.42±6.00	347.81±1.45	27.07±0.89	43.75±4.780	418.62±7.120
	70	50.27±2.34	30.41±0.78	4.03±0.77	84.70±3.89	361.03±7.64	55.43±7.03	48.18±1.460	464.64±16.13
	120	32.30±4.24	17.48±1.34	4.04±0.24	53.82±5.82	371.92±5.09	61.35±1.11	55.05±3.240	488.32±9.440
	180	18.66±1.45	17.64±0.56	4.10±1.42	40.40±3.43	404.60±5.04	64.10±1.14	63.64±4.670	532.34±10.85

Table 3: Changes of isoflavones during fermentation *in vitro* with Lactobacillus casei LC-L134-1-M and Lactobacillus plantarum LP-L134-1-P, at 37°C for 48h

		Daidzin (mg·L^{-1})	Glycitin (mg·L^{-1})	Genistin (mg·L^{-1})	Daidzein` (mg·L^{-1})	Glycitein (mg·L^{-1})	Genistein (mg·L^{-1})	Total (mg·L^{-1})
Total Mixture		46.67±0.16	13.32±0.67	14.94±0.17	7.08±0.83	8.83±0.33	6.06±0.69	96.90±2.85
	0 h	45.77±0.52	12.94±0.49	13.70±0.84	6.86±0.25	4.86±0.25	6.61±0.60	90.74±2.95
	6 h	42.86±0.63	12.82±0.96	11.88±0.09	6.51±0.68	4.86±0.91	6.65±0.42	87.58±3.69
	10 h	43.95±0.87	11.27±0.52	12.55±0.84	6.19±0.35	6.05±0.26	7.30±0.46	87.31±3.30
	19 h	43.11±0.12	11.20±0.96	12.27±0.53	5.63±0.26	6.55±0.53	7.96±0.59	86.72±2.99
	24 h	43.36±0.81	8.96±0.81	11.02±0.96	5.91±0.78	7.62±0.16	8.86±0.84	85.73±4.36
	28 h	43.32±0.20	7.96±0.28	10.59±0.83	5.93±0.39	8.29±0.88	9.15±0.04	85.24±2.62
	36 h	39.41±0.85	7.45±0.29	9.17±0.90	5.38±0.41	8.65±0.56	9.29±0.14	79.35±3.15
	48 h	38.83±0.25	6.19±0.06	8.90±0.32	5.00±0.50	10.27±0.86	9.12±0.15	78.31±2.14
SPIG	0 h	38.63±0.04	13.55±0.31	12.82±0.34	1.66±0.48	4.98±0.93	5.46±0.05	77.10±2.15
	6 h	38.53±0.19	12.18±0.40	12.48±0.54	1.72±0.27	4.01±0.58	5.77±0.65	74.69±2.63
	10 h	38.37±0.36	11.19±0.54	12.52±0.41	1.75±0.59	4.21±0.52	6.21±0.99	74.25±3.41
	19 h	38.04±0.55	9.36±0.93	11.27±0.05	2.36±0.96	6.09±0.92	6.40±0.72	73.52±4.13
	24 h	37.96±0.43	6.18±0.94	11.09±0.84	3.49±0.99	7.43±0.37	7.29±0.89	74.44±4.46
	28 h	37.80±0.80	6.87±0.58	9.56±0.19	3.50±0.53	7.82±0.31	7.79±0.61	73.34±3.02
	36 h	36.76±0.43	5.94±0.84	8.66±0.62	5.16±0.50	8.04±0.65	8.57±0.88	73.13±3.92
	48 h	35.10±0.36	5.23±0.77	7.99±0.16	8.37±0.07	7.05±0.29	9.38 ±0.31	73.12±1.96

Table 4: Pharmacokinetic parameters of isoflavones, Mix and SPIG

Groups	C_{max} (*μ*g/mL)	AUC_{0-24} (h *μ*g/mL)	Relative bioavailability (%)
ISO	2.24±0.35	16.89	100.00
Mix	3.13±0.32	20.62	122.08
SPIG	3.91±0.56	30.19	178.74

Bioavailability of Soy Isoflavones

Isoflavones with different protein matrices were used for the measurement of oral bioavailability in rats. The mean blood concentration-time patterns of isoflavones after single-dose oral administration are shown in Fig. 3 and 4. The results showed that the bioavailability of isoflavones was significantly elevated when it was encapsulated in the particles.

Pharmacokinetic parameters calculated from the non-compartmental analysis of a single dose of isoflavones with or without encapsulation are listed in Table 4. The rats fed isoflavones (50 mg/kg) encapsulated in artificial oil bodies possessed a high C_{max} (37±28 ng/mL) of blood isoflavones at t_{max} of 45±17 min, whereas those fed isoflavones (1000 mg/kg) without encapsulation displayed a relatively low C_{max} (15±12 ng/mL) at t_{max} of 50±32 min. Relative bioavailability calculated based on the area under the plasma concentration−time curve (AUC) was increased by 47.7 times when isoflavones were encapsulated in artificial oil bodies for animal feed.

Conclusion

A systematic study on interaction and improvement of bioavailability in ISO compounds. SPIG is prepared at 120°C and pH 6.0 and then compared with the normal-temperature mixture of SPI and isoflavone. Tests of physiochemical properties show that these nanoparticles are microstructurally spheric and possess high colloid stability: Potential -35.4±0.6 mV; Polydispersity Index (PDI) 0.59-0.60; particle size 280.1±2.6 nm. The powder of SPIG compounds contains 6.42-6.68 mg/g ISO, which is significantly higher compared with SPI or Mixture. Simulated digestion experiments are conducted by the addition of *β*-glucosaccharase into the intestinal environment in vitro. The results confirm that the ISO in the compound ingredients after heat treatment can be well released into the simulated intestinal fluid and converted to aglycon-typed ISO. Pharmacokinetic experiments confirm that the oral bioavailability of soy ISO by rats is the highest. The content, solubility, and bioavailability of ISO in the SPIG ingredients are largely improved, indicating the potent application as functional protein ingredients for menopausal women.

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Author's Contributions

Juan Yang: Contributed to the conception of the study. Experimented. Contributed significantly to analysis and manuscript preparation. Performed the data analyses and wrote the manuscript.

Weifeng Lin: Contributed to the conception of the study.

Xiaoquan Yang: Contributed to the conception of the study. Performed the data analyses and wrote the manuscript.

Zhining Bao: Performed the experiment.

Ethics

The authors declare their responsibility for any ethical issues that may arise after the publication of this manuscript.

Conflict of Interest

The authors declare that they have no competing interests. The corresponding author affirms that all of the authors have read and approved the manuscript.

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