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Analysis of structural changes and anti-inflammatory capacity in soybean protein isolates conjugated with anthocyanins

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Abstract

This study investigated the covalent cross-link at alkaline (pH 9.0) between different concentrations of anthocyanins and soybean protein isolate (SPI). The structure of the product was analyzed by SDS-PAGE, fluorescence spectrum, and circular dichroism. In order to test the effects of SPI on the anti-inflammatory ability of anthocyanins, in vitro inflammatory cell model was set by RAW264.7 cell line. Results showed that fluorescence quenching of soybean protein isolates was static strengthened. The α-helix content of the secondary structure gradually decreased with the content of the β-sheet and random coil increasing. A super molecular weight subunit was observed, and the complex of soybean protein and anthocyanins can markedly reduce the secretion of TNF-α and NO at the dose of 200 μg/mL when the concentration of anthocyanins is greater than 0.167 mg/mL.

Keywords: anthocyanins; soybean protein isolate; conjugates; inflammatory.

Practical Application: The study provides a new idea for the further development of SPI-anthocyanins conjugations as anti-inflammatory food and medicine.

1 Introduction

Soybean protein isolate (SPI) has a specific spatial structure composed of several amino acids. SPI is commonly utilized in food as additives to enhance processing ability, such as foaming, gelling, emulsifying, and structuring attributes (Jian et al., 2016). Whereas its processing characteristics still need improvement to meet the need of the modern food industry.

Anthocyanin is one of the most important flavonoids as a native bioactive component found in fruits and vegetables (Strack & Wray, 1994). They are also used to decrease the risk of chronic diseases, such as cancer, inflammation, and diabetes, partly due to their potent antioxidant and bioactivities (Yoshida et al., 2010). However, anthocyanins are unstable and rapidly degraded under neutral conditions (Brouillard, 1988). Their utilization as a bioactive ingredient in foodstuff is currently limited (Macz-Pop et al., 2006). Some of the molecular structure and functional properties of non-covalent protein–polyphenols complexes have been revealed. Recent studies found that relatively stable derivatives can be found by forming protein-polyphenols complexes stabilized by covalent bonds (Kanakis et al., 2013; Neilson & Ferruzzi, 2011; Yang et al., 2016). At alkaline pH, the ortho-diphenol and para-diphenol of anthocyanins can be oxidized irreversibly by the nucleophilic groups of the protein molecule. The physicochemical properties of proteins such as size, charge, solubility, and bioactivities were then changed (Labuckas et al., 2008), the bioactivities and bioavailability of plant polyphenols and other catechin derivatives may be affected by the covalent interaction between polyphenols and proteins. The formation of the corresponding quinone resulted in the formation of high molecular weight brown-colored pigments named tannins (Prodpran et al., 2012). It is, therefore, necessary to understand the interaction between polyphenol and protein characteristics (Arroyo-Maya et al., 2016). Further, the possible nutritional consequence of protein–polyphenols interactions may be affected by the amounts of essential amino acids in the food systems (Sathe, 2012). In order to evaluate how covalent reactions with protein modify the physicochemical properties and bioactivity of food polyphenols, effective methods are needed to monitor the formation of covalent complexes in foodstuff.

The objective of the present paper was to show the influence of conjugation and estimate the influence on the structure change of different conjugated anthocyanins ratios at alkaline pH. SDS-PGAE, circular dichroism spectroscopy (CD), and fluorescence spectroscopy were introduced to determine SPI-anthocyanins conjugation structure and functional properties. Another objective of this paper was to study the possible effects of conjugated anthocyanins on the inflammatory capacity *in vitro* of SPI-anthocyanins conjugations.

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2 Materials and methods

2.1 Materials

Soy protein with a protein content of 92.3% was purchased from the market. Black rice extract powder contented 25% anthocyanins was purchased from Run Shaanxi Days of Biological Technology Co., Ltd (Shanxi, China). RAW 264.7 murine macrophage cell line was purchased from the Cell Bank of the Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute (RPMI-1640 medium), phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). DMSO, lipopolysaccharides (LPS) from E. coli strain 0111:B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Griess reagent was purchased from Beyotime (China), TNF-α Elisa reagent was purchased from Haling Biological Technology Co., Ltd (Shanghai, China). Deionized water (DI) was used for the preparation of all solutions. The other reagents were analytically pure.

2.2 Preparation of SPI samples

Preparation of SPI samples was according to the work of Torrezan et al. (2007) with some modifications. Soybean flour was defatted by Hexane (1:3 w/v) 3 times. Then the defatted flour was dispersed in DI (1:20). The pH of the dispersion was adjusted to 8.0 with 0.5 M NaOH, followed by precipitation at pH 4.5 with 0.5 M HCl. The precipitate dispersion was adjusted to pH 7.0 with 0.5 M NaOH and lyophilized. The protein content of SPI was determined by the Kjeldahl method (N × 6.25).

2.3 Purification of anthocyanins

Purification of anthocyanins was performed according to the work of Sui et al. (2016) with some modifications. In brief, the black rice extract powder was dissolved in DI to make a concentrated anthocyanins aqueous solution. The solution was filtered through a 0.45 μm PVDF filter. Sep-Pak C18 cartridges (WAT023635, Waters, USA) connected with a vacuum pump were conditioned by passing 15 mL of methanol and 20 mL of 0.01 M aqueous HCL. Afterward, the filter liquor was loaded onto cartridges. Wash cartridges with 15 mL of 0.01 M aqueous HCl and then 20 mL of ethyl acetate. Purified anthocyanins were obtained by washing cartridges with 40 mL of methanol. The methanol content was removed by using an evaporator. Purified ANC powder was stored at -20 °C until use.

2.4 Synthesis of SPI and anthocyanins

SPI-anthocyanins conjugates were prepared following with some modifications (Rawel et al., 2002b). SPI (1.0 g) was dissolved in 50 mL of DI, and the pH value of the solution was adjusted to 9.0 with 0.5 M NaOH. The solution was stirred by a magnetic agitator at 20 °C for 30 min to ensure complete dispersion. Purified ANC was then added to the solution, and the pH value was adjusted to 9.0 again, making the final volume 100 mL. After continuously stirring 8 h by a magnetic agitator at a speed of 150 rpm, the solution was ultrafiltrated with a 3 kDa ultrafiltration centrifuge tube and dried by an evaporator. In order to investigate the optimum concentration, ratios of SPI: anthocyanins were approximate: 10: 1, 20: 1, 40: 1, 60:1, and 80: 1 (w/w) during the reaction. The control group was prepared under the same condition without the addition of anthocyanins compound.

2.5 Determination of free anthocyanins

The untreated SPI-anthocyanins solution was ultrafiltrated with a 3 kDa ultrafiltration centrifuge tube for 8 min at 3500 g. The filtrate was filtered through a 0.22 μm PVDF filter and treated by ultrasonic. Free ANC content was checked using high-performance liquid chromatography (Waters e2695, USA) with a C18 reversed-phase column (250 × 4.6 mm, Sunfire, Waters, Wexford, Ireland) at 520 nm.

2.6 Solubility

The solubility of SPI-anthocyanins conjugates was determined following a previous method with slight modification (Morr et al., 1985). 250 mg of samples were dissolved in 25 mL of DI and the concentration of anthocyanins were 0.125, 0.167, 0.250, 0.500, 1.000 mg/mL (80:1, 60:1, 40:1, 20:1, 10:1) respectively. The solution was then stirred for 30 minutes at room temperature and centrifuged for 15 minutes at 4000 g. The protein content in supernatants was determined by the micro Kjeldahl method. Total protein content in the sample was determined after the dissolution of the samples in 0.5 M NaOH.

2.7 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the work of Liu et al. (2015) with some modifications. SPI-anthocyanins conjugates were mixed with twice the volume of the loading buffer. An aliquot of 5 μL of each sample was loaded on a 5% stacking gel and a 10% polyacrylamide resolving gel with a constant voltage at 120 V. The gels were stained with Coomassie Brilliant Blue R250 for protein visualization and scanned by a Gel Doc™ EZ Imager (BIO-RAD).

2.8 Circular dichroism (CD) measurements

CD spectra were recorded with a JASCO J-815 Dichrograph ranging from 190 to 260 nm at 100 nm/min scan speed following our previous study (cite study). Samples of SPI-anthocyanins conjugation were diluted to a final protein concentration of 0.25 mg/mL in PBS (0.01 M sodium phosphate, pH 7.0) and centrifuged for 5 min at 4000 g. Measurements were made under a constant nitrogen flush, 1.0 nm bandwidth, and 1 mm path length quartz cell at room temperature. The secondary structure was analyzed by using software CDpro for protein secondary structure analyses from CD spectroscopic data.

2.9 Fluorescence spectroscopy

The fluorescent measurements were performed on a fluorescence F-4500 FL Spectrophotometer (Hitachi) using an emission wavelength at 280 nm according to the method of

Arroyo-Maya et al. (2016) with some modifications. Intrinsic fluorescence emission spectra ranged from 250 to 400 nm were recorded. The concentrations of SPI-anthocyanins conjugates were adjusted to 0.2 mg/mL. Scanning parameters were optimized using slit widths of 5 nm for excitation and 5 nm for emission at a speed of 60 nm/min. Each measurement was performed in triplicate.

To establish which of the two well known quenching mechanisms (dynamic or static) was operative, the intensity data were analyzed (Equation 1) (Fu & Lakowicz, 2006).

$$F_0 / F = 1 + K_{SV}[Q] \quad (1)$$

Here, F_0 and F represent the fluorescence intensities of the protein alone and in the presence of a given concentration of quencher $[Q]$, respectively. The Stern-Volmer constant, K_{SV}, is given by the product of the quenching bimolecular rate constant, k_q, and the lifetime of the fluorescent group being quenched, τ_0. For free chromophores, k_q is of the order of $10^{-10} mol^{-1}s^{-1}$, a typical value for a diffusion-controlled reaction.

In order to further explore the mechanism of anthocyanin binding with SPI, static quenching formula can be used to calculate apparent binding constant Kb and binding site number n by double-logarithm regression equation (Equation 2).

$$\lg\left[\frac{F_0 - F}{F}\right] = lgK_b + nlg[Q] \quad (2)$$

2.10 FI determination of tryptophan

The FI of tryptophan was determined by using a F-4500 FL Spectrophotometer (Hitachi) according to the work of Chen & Barkley (1998). Aliquot of each sample was diluted with 8 M urea to a protein concentration of 0.2 mg/mL. Emission wavelength ranged from 300 to 450 nm was recorded (slit 40 nm). The content of tryptophan was quantified at wavelengths of (295, 340). Corrections were made for the buffer blank.

2.11 Zeta-potential measurements

The Zeta-potential was measured by Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK) at 20 °C according to the work of Patil et al. (2007). The Zeta potential of the samples was measured as a function of the anthocyanins ratio. Aliquot of each samples was diluted to a protein concentration of 0.5 mg/mL with 0.01M PBS (pH 7.0). All measurements were carried out separately at least six times at each anthocyanins ratio.

2.12 Cell test

Cell culture and cell viability for assay

The RAW 264.7 cells were grown in DMEM medium supplemented with 10% FBS, 4.5 mM L-glutamine, 100 U/mL of penicillin, 100 mg/mL of streptomycin maintained at 37 °C in a humidified atmosphere containing 5% CO_2. A trypan blue exclusion protocol assessed cell viability as described by Strober (2001) with slight modification, and cell numbers were determined with a hemocytometer.

Dosage of SPI-anthocyanins conjugations was determined by seeding cells in a 96-well plate at a density of 1×10^5 cells per well and treating cells with 50, 100, or 200 μg/mL of each conjugation. Then the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 24 h according to Mosmann (1983). Viability was quantified as the absorbance value at 550 nm after 24 h.

Nitrite assay

RAW 264.7 cells were seeded in each 24-well plate at a density of 1×10^5 cells per well with 500 μL of culture medium without antibiotic and incubated for 24 h. The cells were then treated with various concentrations of SPI-anthocyanins conjugates for 2 h before stimulation with LPS (1 μg/mL) for 24 h. To measure the nitrite concentration, 50 μL of each culture supernatant was mixed with standard Griess reagent following the manufacturer's instructions. The plate was then incubated at 37 °C for 15 min, and the absorbance at 540 nm was determined with the Tecan M200 PRO microplate reader.

TNF-α assay

Cells were plated and grown to confluency in 24-well plates. Following 24 h of treatment in 0.5 mL of medium, supernatants were collected and centrifuged for 30 sec at 3500 g. 50 μL of each culture supernatant was analyzed for TNF-α by ELISA as per the manufacturer's instructions. For samples containing LPS, the supernatant was diluted 50% with a culture medium before analysis.

2.13 Statistical analysis

The results were repeated ten times; Student's test and ANOVA/ posthoc test were performed. The digestions and other analyses were repeated at least three times and, where possible, evaluated by means and standard deviation. A maximum of 5% standard deviation from the averaged values was generally tolerated (when not otherwise specified). The averaged values are documented in the respective figures.

3 Results and discussion

3.1 Solubility analysis of SPI–anthocyanins conjugates

Solubility is an important factor for a functional ingredient protein and is critically necessary for food systems. The difference in protein solubility behavior underlined their structure and molecular parameters in the interaction with the anthocyanins. Figure 1 shows the solubility of soy protein conjugated with anthocyanins at pH ranging from 2 to 10, resulting in a decreased solubility of SPI. The high solubility of SPI-anthocyanins conjugations from pH 7 to 10 and pH 2 to 3 may correspond to high positive or negative charge levels, which caused a strong electrostatic repulsion between particles. At the isoelectric point (pH 4~5), SPI-anthocyanins conjugations had a low net charge. Therefore the electrostatic repulsion was not large enough. Solubility was highest for the untreated SPI. As a control, untreated SPI showed the maximum solubility of protein molecules produced

in specific processing settings. In comparison, the conjugations were formed, lyophilized, and then re-dissolved. Samples at the ratio of 80:1, 60:1, 40:1 showed no significant difference. Anthocyanins significantly decreased the solubility of SPI at the ratio of 20:1, 10:1. At alkaline pH, anthocyanins may be oxidized by autoxidation interacting covalently with the polar group of soybean globulin, reducing the polarity of protein in an aqueous solution. Thus the solubility of SPI decreased.

3.2 SDS-PAGE analysis of SPI–anthocyanins conjugates

The structural characteristics of SPI conjugated with anthocyanins were measured using SDS-PGAE, circular dichroism spectroscopy, fluorescence spectroscopy. As shown in Figure 2, anthocyanins induce a significant change in the composition of SPI subunits. The intensities of bands corresponding to SPI generally decreased with the ratio of SPI/anthocyanins. Some new bands attributed to the protein aggregates were observed at the top of the separating gel, which suggested that anthocyanins-induced protein aggregate stabilized by covalent bonds. Rawel et al. (2002a) reported that the gallic acid-soy protein interaction increases SPI's molecular weight.

3.3 Far-UV CD spectroscopy analysis

Circular dichroism (CD) can provide a sensitive indicator at the molecular level, such as changes in secondary structure and tertiary structure of proteins. The information of the secondary structure level of proteins was determined by using Far-UV CD. As shown in Table 1, the far-UV CD spectrum of SPI showed a broad negative peak ranging from around 200 to 240 nm. The band intensity of SPI-anthocyanins conjugation caused a slight change ranging from 200 to 230 nm, indicating some change in the secondary structure of the SPI after conjugation. The secondary structure elements of the samples were calculated by analyzing the CD spectra using software CDpro: SPI had 22.26% α-helix, 27.74% β-sheet, 19.77% turn, and 30.23% random coil; SPI-anthocyanins (10:1) conjugation had 19.25% α-helix, 28.55% β-sheet, 20.75% turn, and 31.45% random coil. These results showed that anthocyanins conjugation significantly changed the secondary structure of SPI, with a net increase in the amount of β-sheet and a net decrease in the amount of a-helix regions. A decrease in the amount of α-helix present after conjugation (Caffeic acid, Gallic acid, Flavone, Myricetin) was also reported by Rawel et al. (2002a) for SG.

3.4 Fluorescence spectroscopy analysis

Intrinsic fluorescence analysis was performed to evaluate the tertiary structural changes of SPI conjugation with anthocyanins. The dependence of the fluorescence emission spectra for different ratios of SPI-anthocyanins conjugate solutions (pH 7.4, 25.0 °C) was measured (Figure 3). These measurements show that the protein fluorescence intensity, dominated by the emissions of tryptophan and tyrosine residues present in its structure, decreases appreciably with the increasing anthocyanins ratio,

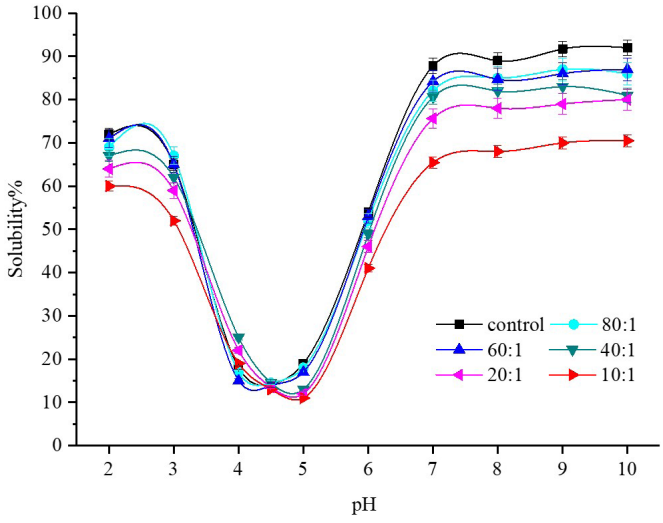

Figure 1. Solubility analysis of different ratio of SPI–anthocyanins conjugates.

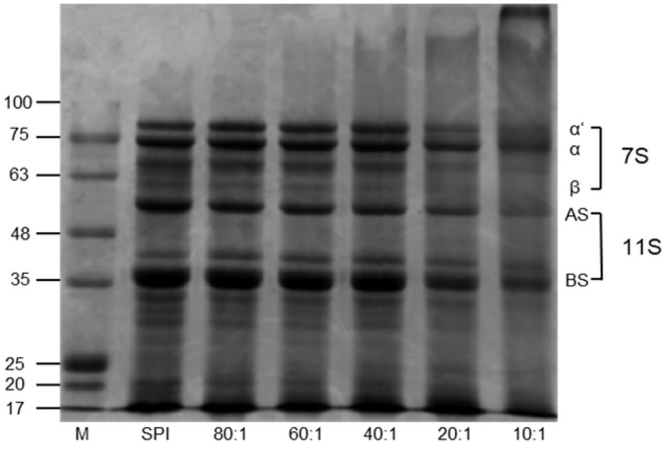

Figure 2. SDS-PAGE analysis of different ratio of SPI–anthocyanins conjugates.

Table 1. Secondary structure contents of different ratio of SPI–anthocyanins conjugates.

Group	α-helix	β-sheet	β-turn	Random coil
SPI (control)	22.26 ± 0.2^{a}	27.74 ± 0.0^{e}	19.77 ± 0.0^{a}	30.23 ± 0.1^{b}
80:1	21.43 ± 0.0^{b}	27.91 ± 0.2^{de}	18.89 ± 0.2^{a}	30.77 ± 0.0^{ab}
60:1	21.23 ± 0.1^{b}	28.39 ± 0.1^{cd}	19.86 ± 0.1^{a}	30.52 ± 0.2^{b}
40:1	21.02 ± 0.1^{b}	28.61 ± 0.2^{bc}	19.43 ± 0.1^{a}	30.64 ± 0.1^{ab}
20:1	20.95 ± 0.2^{b}	29.07 ± 0.1^{ab}	19.18 ± 0.2^{a}	30.80 ± 0.0^{ab}
10:1	19.25 ± 0.1^{c}	29.55 ± 0.1^{a}	19.75 ± 0.1^{a}	31.45 ± 0.2^{a}

Mean values in the same row followed by the different superscript letters are significantly different ($P < 0.05$).

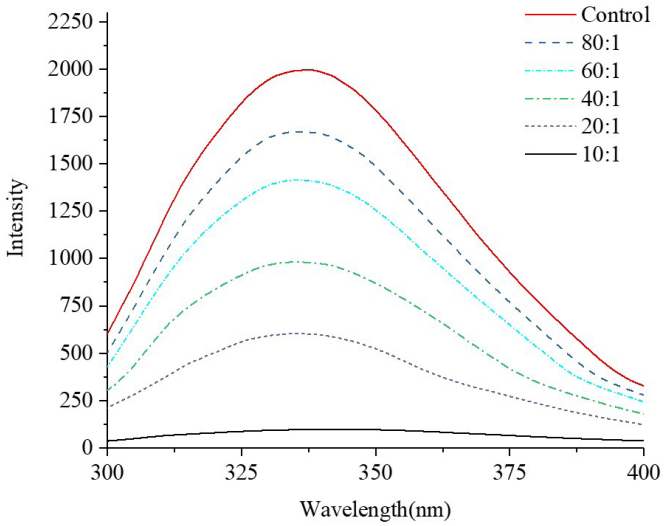

Figure 3. Fluorescence emission spectra of different ratio of SPI–anthocyanins conjugates.

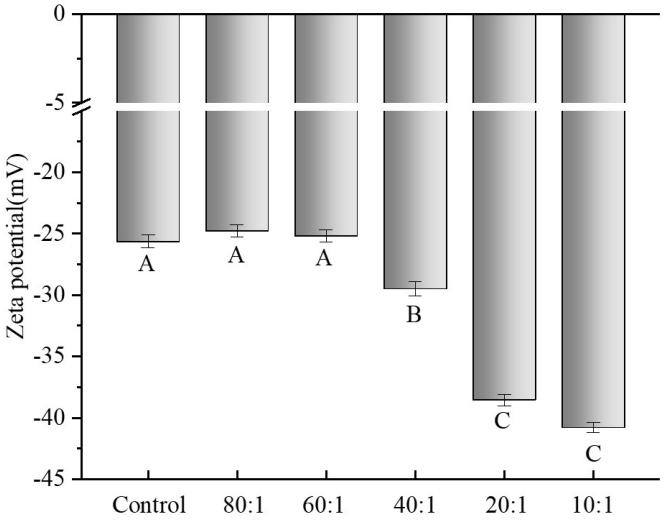

Figure 4. Tryptophan fluorescence determination of different ratio of SPI–anthocyanins conjugates.

whereas there was a redshift of the spectral maximum observed. The fluorescence intensity of SPI-anthocyanins is less than that of SPI alone, which suggests a quenching of fluorescence intensity due to the reaction of the anthocyanins' aromatic ring with tyrosine tryptophan of the protein. These data were in agreement with previous studies that covalent modification of protein with polyphenol decreases. (Ozdal et al., 2013; Rawel et al., 2002a; Wu et al., 2011).

The intensity data were plotted to establish which of the two well-known quenching mechanisms (dynamic or static) was operative (Table 2). This data agrees with the Stern-Volmer equation for the dynamic quenching of a single emitting chromophore (Fu & Lakowicz, 2006). The maximum quenching constant of all kinds of fluorescence quenching agents for biological macromolecules is about 2.0×10^{10} L/ (mol·s), if Kq is greater than 2.0×10^{10} L/ (mol·s), it is static. Instead, it is dynamic. The quenching constants of anthocyanins on SPI at temperature are shown in Table 1, indicating that anthocyanins have a significant effect on SPI. The quenching mechanism of SPI is mainly static quenching, which further indicates that anthocyanins and SPI forms a non-luminous complex, which results in a decrease in the fluorescence intensity of SPI.

3.5 Tryptophan fluorescence determination

Tryptophan fluorescence determination also gave evidence to confirm these observations as shown in Figure 4. There is a notable decrease in the amount of free tryptophan with the increasing anthocyanins ratio. Remarkably, as the ratio of anthocyanins conjugated increase, the color of the solution gradually deepened which may cause self-quenching. This phenomenon can be attributed to the decrease in tryptophan FI and even that of solutions.

3.6 Zeta-potential analysis

The difference in the zeta-potential is due to the chemicals involved in the synthesis process. Soy protein isolate is a typical ampholyte with many polar (Carboxyl, amino, hydroxyl) and non-polar groups on the side chain. While the anthocyanins, an unstable molecule, will be covalently grafted to SPI, forming quinone compounds under alkaline conditions and changing SPI's surface charge. In order to determine the electrical properties changes of SPI, zeta-potential was measured as a function. As shown in Figure 5. The zeta-potential of re-dissolved samples at pH 7.0 was not significantly different at anthocyanins conjugated ratio of 80:1 and 60:1 to SPI. However, different behavior was found from the conjugated ratio at 40:1 in which samples have a greater negative charge (-29.48 mV) than SPI (-25.63 mV). Decreased solubility is likely related to the formation of the large aggregates generated during the interaction. The Zeta-potential significantly increased ($P < 0.05$) when concentration larger than 20:1. Electrostatic repulsion between molecules, which increases with surface charge, can reduce the aggregation between SPI molecules. Besides, more hydrophobic groups were exposed to the surface of SPI molecules. This makes the solution system more stable by hydrophobic interaction.

Table 2. Stern-Volmer quenching constants.

Tempereture/K	KSV/(L/mol)	Kq/(L/(mol•s))	R2
298	8.015×10^2	8.015×10^{10}	0.9646
308	6.808×10^2	6.808×10^{10}	0.9847
318	6.125×10^2	6.125×10^{10}	0.9779

3.7 In vitro anti-inflammatory capacity of SPI–anthocyanins conjugates

Anthocyanins are known for their anti-inflammatory properties (Chen et al., 2006). Previous research shows that a particular impact on the functionality of polyphenols was attributed to the type of protein and type of polyphenols (Manach et al., 2004). Therefore, it was imperative to evaluate if the biological activity of polyphenols was changed when conjugated with protein. Dosage of SPI-anthocyanins conjugations was tested in the range of 50-

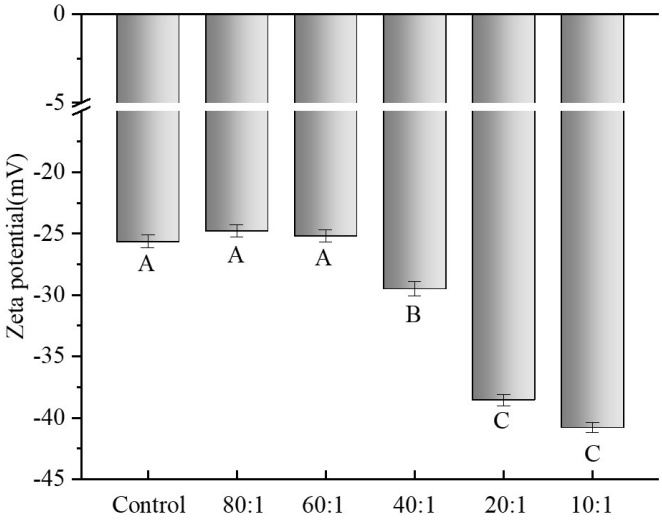

Figure 5. Zeta-potential analysis of different ratio of SPI–anthocyanins conjugates.

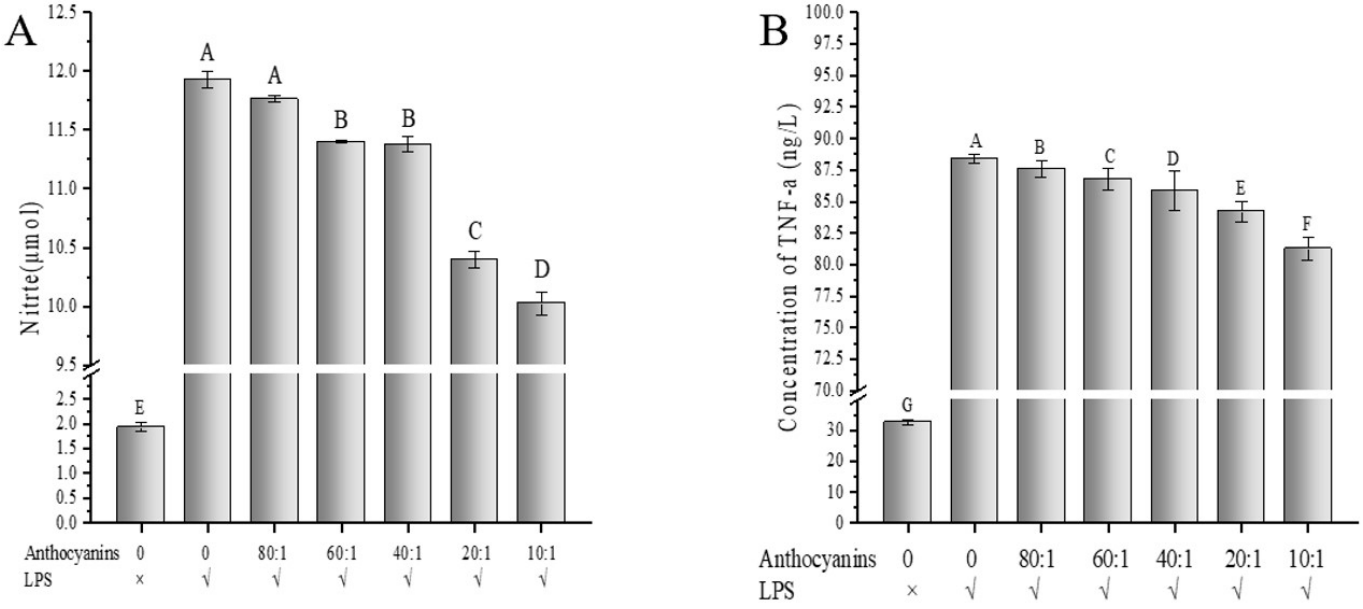

Figure 6. NO secretion and TNF-α production after treatment with anthocyanins or SPI-anthocyanins complex at 200 μg/mL.

250 μg/mL. Data showed no significant impact on cell viability, indicating that the addition of SPI-anthocyanins conjugations will not contribute to cytotoxicity at these concentration.

TNF-α induces a rapid transcription of genes that modulate inflammation. To establish if LPS could set a TNF-α induced inflammatory response *in vitro*, cells were exposed to LPS 10 ng/mL for 24 h. As shown in Figure 6a and 6b, the release of NO and the production of TNF-α was inhibited by SPI-anthocyanins conjugates. SPI-anthocyanins conjugates suppressed the LPS-induced NO levels and TNF-α levels in mouse macrophage RAW 264.7 cells in an anthocyanins concentration-dependent manner, which is in agreement with the study that whey protein-polyphenol inhibits LPS-induced NO levels (Schneider et al., 2016; Tanaka et al., 2014). In contrast, the low-binding-rate conjugates had suppression of the NO levels. These results also attribute to a higher anthocyanins reaction concentration forming more changes in the structure of SPI.

4 Conclusion

SPI-anthocyanins conjugations were formed under alkaline conditions, and their functionality was evaluated by CD studies, intrinsic fluorescence, ANS binding experiments, and cell culture, which indicated significant structural changes in SPI confirmation after the reaction. With the content of anthocyanins increasing, the solubility of SPI decreased, and the isoelectric point slightly shifted to a higher pH value. The hydrophobic property of SPI decreased after the formation of SPI-anthocyanins conjugation, and high-binding rate conjugation can significantly reduce the

expression of genes associated with chronic inflammation. These results suggest that SPI-anthocyanins can be made that benefit food quality and, at the same time, supply additional health benefits.

Conflict of interest

The authors have no conflicts of interest to be declared.

Acknowledgements

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