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Research article

Effect of sugar and $CaCl_2$ concentration on fluorescence quenching characteristics of whey proteins by delphinidin derivatives from butterfly pea flower

Tung Thanh Vuong[a], Subin Srivichai[a], Parichat Hongsprabhas[a,b,*]

[a] *Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand*

[b] *Center of Excellence on Agricultural Biotechnology (AG-BIO/MHESI), Bangkok 10900, Thailand*

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Abstract

The interactions were investigated between the flavonoid delphinidins from butterfly pea (*Clitoria ternatea*) dried flower and commercial whey protein isolate (WPI) in the presence of osmolytes commonly used in food products, namely sucrose, lactose and $CaCl_2$. Ternatin-B2 (T-B2) was used as a representative anthocyanin quencher since it was the major delphinidin derivative in the water extract of butterfly pea as determined using liquid-chromatography mass spectrometry-ion trap-time of flight. Fluorescence-quenching studies of whey proteins in the WPI revealed the underlying thermodynamics parameters, indicating that the binding of whey proteins to anthocyanins was spontaneous and exothermic within the temperature range 35–65°C, with 0–150 mM added sucrose or lactose or 0–500 mM added $CaCl_2$. H-bonds were the main force responsible for the formation of the whey protein-anthocyanin complexes. Increased temperature lowered the binding constant (K_a) and the number of binding sites (n) on the protein molecules for anthocyanins ($p < 0.05$). Increasing the sucrose concentration did not significantly affect the K_a and n values ($p \geq 0.05$). However, the n values significantly increased when the lactose concentration was above 75 mM. Furthermore, increasing the $CaCl_2$ concentration above 375 mM increased the Ka and n values ($p < 0.05$). Understanding the binding mechanisms in the formation of whey protein-anthocyanin complexes under different temperatures, osmotic pressures and ionic strengths could help rationalize the selection of ingredients for biomolecular encapsulation of delphinidin derivatives using whey protein isolate as a carrier.

* Corresponding author.
E-mail address: parichat.h@ku.th (P. Hongsprabhas)

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Introduction

Bovine whey proteins are soluble proteins separated from casein during cheese and casein manufacturing. The major whey proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and immunoglobulins and about 80% of whey proteins are composed of β-LG and α-LA (Schmidt et al. 1984). Whey proteins are an excellent source of branched-chain amino acids compared to caseins and may affect glucose metabolism and muscle protein synthesis (Graf et al., 2011).

The stability of whey protein globular structure is influenced by sugars and salts solubilized in a water solvent as the addition of both osmolytes causes changes in the water medium, such as a change in osmotic pressure and surface tension, which subsequently change the water structure and its solvent properties (Arakawa and Timasheff, 1982a, b). Sugar and salt osmolytes can also affect the molecular structure of proteins by reacting with proteins directly. The changes in the water solvent and protein properties may enhance or limit protein stability and solubility, depending on the types and the concentrations of sugar and salt used (Arakawa and Timasheff, 1982a, b).

The conformation of globular whey proteins such as β-LG and BSA can be stabilized using glucose, lactose and sucrose (Arakawa and Timasheff 1982a). Arakawa and Timasheff (1982a) demonstrated that sugars increased the water surface tension. From the thermodynamic point of view, subsequent surface free energy perturbation plays a leading role in preferential protein interaction with sugar molecules. The stabilizing effect of sugars arises from preferential H-bonds between protein and sugar rather than the H-bonds between protein and water (Arakawa and Timasheff, 1982a). Therefore, the replacement of H-bonds helps to maintain the native conformation of globular whey proteins when sugar is present.

$CaCl_2$ is also known as a salting-in or destabilizing salt since it binds to proteins and increases the surface tension of water (Arakawa and Timasheff, 1982b). Ca-induced aggregation of whey proteins in whey protein isolate (WPI) was found to be a slow and time-dependent process (Zhu and Damodaran, 1994). The rate and the extent of whey protein aggregation were maximum at 20–40 mM of calcium ions (Zhu and Damodaran, 1994) due to both charge dispersion and Ca-crosslinking. Subsequent interactions such as protein-ligand binding may be enhanced for molecular encapsulation of ligands in the presence of Ca^{2+} ion. For example, bovine α-LA can be used as a carrier for Ca^{2+} and flavonoids such as genistein and kaempferol (Mohammadi and Moeeni, 2015).

It is apparent that different osmolytes could influence protein conformations and their susceptibility for further interactions with other compounds. However, there has been limited study on milk minerals such as calcium in the interactions between whey proteins and anthocyanins and this merits further investigation.

The butterfly pea flower (*Clitoria ternatea*) is consumed as a fresh flower or a beverage in Asia (Jeyaraj et al., 2020). The polyacylated delphinidins (ternatins) are responsible for the blue-color (Kazuma et al., 2003) and for the health-promoting bioactive compounds of butterfly pea flower petal, namely antioxidative, antibacterial, antifungal, anti-inflammatory, antiproliferative, antidiabetic activities (Jeyaraj et al., 2020).

Although butterfly pea flower extract has potential use as a natural blue colorant for the food industry, little is known on the interactions of butterfly pea flower anthocyanins in the presence of nutrients such as proteins, carbohydrates and minerals. Such interactions could influence the biological activity and bioavailability levels of both proteins and anthocyanins due to the changes in the conformation and reversibility of the interactions (Acharya et al., 2013; Casanova et al., 2018; Naczk et al., 2006).

Fluorescence-quenching measurement is one of the most versatile approaches to investigate small ligands binding to proteins as fluorescence emission occurs in protein fluorophores, mainly originating from tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues (Lakowicz, 2006). When these residues interact with small ligands like anthocyanin, the intrinsic fluorescence is often lowered, with the decrease in the quantum yield of fluorophore in the presence of ligand being divided into two classes of quenching: static quenching and dynamic quenching (Lakowicz, 2006). Static quenching results from complex formation between protein and ligand, determined by the decrease in the quenching constant when the temperature increases; in contrast, dynamic quenching results from the collision of fluorophore and quencher and increasing the temperature thus increases the quenching constant (Lakowicz, 2006).

Recently, Vuong and Hongsprabhas (2021) reported the influence of pH levels between 2.5 and 11.0 on the binding mechanisms of delphinidin derivatives extracted from *C. ternatea* flower on the whey proteins in whey powder (WP) and whey protein isolate (WPI) in the buffered systems. They found that protein-anthocyanin complex formation involved hydrophobic interactions and electrostatic interactions over such a pH range in the buffered systems. In addition to pH, the high contents of indigenous lactose and minerals in

WP, compared to those in WPI, played significant roles in determining the strength of the binding forces and the number of binding sites on the whey protein molecules accessible by the delphinidin derivative.

The current study further explored the influences of sugar and calcium osmolytes on the binding characteristics of whey proteins in WPI to the anthocyanins in the extract of *C. ternatea* dried flower. It was hypothesized that the protein-stabilizing effect of sugar and the protein-destabilizing effect of $CaCl_2$ could further influence protein conformation and subsequent interactions with anthocyanins. The current study attempted to characterize the leading forces involved in the binding mechanisms in the presence of osmolytes commonly used in food products in distilled water instead of a buffered system to minimize the influences of the ionic strength of the buffer. Understanding the influences of osmolytes in the interactions of whey protein-anthocyanin complex could help design the affinity strength between protein and anthocyanin and process parameters for novel delivery systems of delphinidin derivatives using whey protein isolate as a carrier.

Materials and Methods

Raw materials

Dried butterfly pea (*C. ternatea*) flowers were purchased from a local market in Bangkok, Thailand and kept in a sealed aluminum bag and at -20°C until used. The whey protein isolate (WPI) powder was purchased from Milk Specialties (Eden Prairie, MN, USA). It contained 85.7% protein (Lowry et al., 1951), 2.65% ash and 7.23% moisture content (Association of Official Analytical Chemists, 2000). The reducing sugar was determined using the dinitrosalicylic method described by Miller (1959) after the protein had been precipitated using absolute EtOH (Richmond et al., 1982) using lactose for the calibration curve. The WPI contained 2.92% lactose equivalent.

Profiling anthocyanins in water extract of butterfly pea flower

Extraction of anthocyanins from butterfly pea dried flower

The water extract of butterfly pea flower was prepared using a dried flower-to-distilled water ratio of 1:100 (weight per weight basis) at 80°C for 30 min and passing through a Whatman no. 1 filter paper (Vuong and Hongsprabhas, 2021). The monomeric anthocyanin in the extract was determined using the method described by Lee et al. (2005). The absorbance of samples at the wavelengths of 520 nm and 700 nm was recorded using a TECAN microplate reader (Infinite 200 PRO; Grodig, Austria). The anthocyanin content was expressed as ternatin B2 (T-B2) equivalent, as shown in Equation 1:

$$Monomeric\ anthocyanin\ (mg/L) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \quad (1)$$

where $A = [(A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}]$; MW = 1,638 g/mol for T-B2; DF is the dilution factor; l = pathlength (measured in centimeters); ε is the molar extinction coefficient = 29,000 L/mol.cm; and 10^3 is the factor for conversion from grams to milligrams. The calculated concentration of monomeric anthocyanin was expressed as T-B2, the major blue anthocyanin in dried butterfly pea flower determined using liquid-chromatography mass spectrometry-ion trap-time of flight (LCMS-IT-TOF) described below.

Profiling of anthocyanins in water extract of butterfly pea dried flower

The anthocyanins in the water extract of *C. ternatea* were analyzed using the method described by Srivichai and Hongsprabhas (2020). Briefly, the extract was passed through a C-18 Sep-Pak cartridge (VertiPak™ C18-LP; Vertical Chromatography Co., Nonthaburi, Thailand) previously activated with MeOH, followed by 0.1% HCl (volume per volume; v/v) in deionized water. The flavonoid anthocyanins adsorbed onto the Sep-Pak column while sugars, acids and other water-soluble compounds were removed by washing the mini-column with 5 mL 0.01% HCl.

Anthocyanins were recovered using 50% EtOH (v/v) containing 0.1% HCl (v/v) and stored at -20°C. Samples were passed through a 0.45 µm filter before analysis. Chromatographic analyses were performed on an LCMS-IT-TOF (Shimadzu; Tokyo, Japan) coupled with a Prevail™ C18 column (5 µm, 150 × 4.6 mm). The linear gradient elution program used 0.5% (v/v) aqueous trifluoroacetic acid (phase A) and 80% (v/v) acetonitrile in MeOH (phase B) at a flow rate of 0.05 mL/min. The wavelength of the ultraviolet-visible detector was set at 520 nm.

The mass spectrometry (MS) parameters were: capillary voltage, 4.5 kV; interface temperature, 200°C; heat block temperature, 200°C; and gas flow (N_2), 1.5 L/min. The instrument was operated in positive ion mode, scanning in the range *m*/*z* 100–2500, using a collision gas (argon) pressure of 50% and a collision energy of 50% (Srivichai and Hongsprabhas, 2020).

Fluorescence quenching study of whey proteins using extract of butterfly pea flower

Effect of T-B2 equivalent concentration on fluorescence quenching characteristics of whey proteins

WPI solution containing 10 mg/mL protein was prepared in distilled water. The pH of the WPI solution was 5.5. The maximum fluorescence wavelength of protein fluorophore was determined using the TECAN microplate reader to scan the fluorescence emission intensity in the range 310–850 nm at 25°C when the excitation wavelength was 280 nm. The maximum fluorescence intensity of whey proteins was recorded at 332 nm.

The binding characteristics between whey proteins and anthocyanins in the water extract in the range 35–65°C were determined by mixing 0.5 mL of protein solution (10 mg protein/mL) with 0.5 mL of extracts containing different concentrations of anthocyanin, based on the molecular weight (MW) of T-B2. The final quencher concentrations [Q] after mixing with protein solution were 0 µM T-B2, 2.65 µM T-B2 equivalent, 5.3 µM T-B2 equivalent, 10.6 µM T-B2 equivalent, 15.89 µM T-B2 equivalent, 21.19 µM T-B2 equivalent and 26.48 µM T-B2 equivalent. The final protein concentration was 5 mg/mL.

The reaction was allowed to proceed at 35°C (308.15 K), 45°C (318.15 K) and 65°C (338.15 K) in a water-bath for 30 min. The Tecan microplate reader was used to evaluate the fluorescence intensity using the excitation wavelength at 280 nm and the fluorescence emission wavelength at 332 nm. The binding constant (K_a), the number of binding sites (n) and thermodynamic parameters were calculated based on a double logarithmic plot as shown in Equation 2 (Lakowicz, 2006):

$$Log\ \frac{F_0 - F}{F} = Log\ K_a + nLog\ [Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of anthocyanin quencher, respectively, K_a is the binding constant, n is the number of binding sites and $[Q]$ is the concentration of anthocyanin calculated as T-B2 equivalent.

Non-covalent forces involved in complex formation of whey proteins and butterfly pea anthocyanins

Four major non-covalent forces are involved in small molecules and protein binding: H-bonds, van der Waals force, hydrophobic interactions and electrostatic interactions. Determination of the dominant force involved in the binding was based on thermodynamic parameter signs and values. For example, a negative ΔG and a negative ΔH indicate that the binding process is spontaneous and exothermic. When ΔH is positive and ΔS is also positive, the binding process is endothermic and hydrophobic interactions are the main force. However, when ΔH is negative and ΔS is negative, H-bonds are the leading force. The electrostatic interactions play an important role in the binding when ΔH is negative and ΔS is positive (Fisicaro et al., 2004; Acharya et al., 2013; Davidov-Pardo and McClements, 2014; Joye et al., 2015).

The Gibbs free energy change can be calculated from K_a as described in Equation 3:

$$\Delta G = -RT\ ln\ Ka \quad (3)$$

where R is the gas constant (8.14 J/mole K), T is the absolute temperature (K), and K_a is the binding constant obtained from Equation 2 at each temperature.

The change in enthalpy (ΔH) when there is an increase or decrease in temperature from T_1 to T_2 was calculated by applying the van't Hoff equation shown in Equation 4:

$$Ln\ \left[\frac{K_{a2}}{K_{a1}}\right] = \frac{-\Delta H}{R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \quad (4)$$

where T_1 and T_2 are the temperatures at which K_{a1} and K_{a2} are measured.

The entropy change was calculated as described in Equation 5:

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

Effect of osmolyte concentrations on formation of whey protein-anthocyanin complexes

WPI solution containing 10 mg/mL protein was prepared in distilled water. An amount (250 µL) of protein solution was mixed with 250 µL of sugar (sucrose or lactose) solutions containing 30 mM, 150 mM, 300 mM, 450 mM and 600 mM sugar to generate different osmotic pressures (π) in the protein solutions before mixing with *C. ternatea* extract. The osmotic pressure of the osmolytes was calculated using Equation 6:

$$\pi = iMRT \quad (6)$$

where π is the osmotic pressure (atm), i is the van't Hoff factor of osmolyte (1 for sugar and 3 for $CaCl_2$ due to dissociation), M is the molar concentration of the solute

(mol/L), R is the universal gas constant (0.08206 L·atm/mol·K), and T is the absolute temperature (K).

The 5 mg protein/mL mixed solution (500 µL) was further mixed with 500 µL of *C. ternatea* water extract containing 5.3 µM T-B2 equivalent, 10.6 µM T-B2 equivalent, 21.2 µM T-B2 equivalent, 31.8 µM T-B2 equivalent, 42.4 µM T-B2 equivalent and 53.0 µM T-B2 equivalent. After mixing, the final concentration of protein was 2.5 mg/mL and the final concentration of added sugar was in the range 0–150 mM.

The binding characteristics of the whey proteins with *C. ternatea* extract in the presence of $CaCl_2$ were investigated by preparing a WPI solution containing 10 mg/mL protein in distilled water. An amount (250 µL) of protein solution was mixed with 250 µL of salt solutions containing 100 mM $CaCl_2$, 500 mM $CaCl_2$, 1,000 mM $CaCl_2$, 1,500 mM $CaCl_2$ or 2,000 mM $CaCl_2$ to alter the osmotic pressure (π) and the ionic strength (I) of the protein-$CaCl_2$ solutions. The 5 mg protein/mL mixed solution (500 µL) was further mixed with 500 µL of *C. ternatea* water extract containing 5.3 µM T-B2 equivalent, 10.6 µM T-B2 equivalent, 21.2 µM T-B2 equivalent, 31.8 µM T-B2 equivalent, 42.4 µM T-B2 equivalent and 53.0 µM T-B2 equivalent. After mixing, the final concentration of protein was 2.5 mg/mL and the final concentration of added $CaCl_2$ was in the range 0–500 mM.

The protein-sugar mixed solutions or the protein-$CaCl_2$ mixed solutions containing final concentrations of anthocyanin of 2.65 µM T-B2 equivalent, 5.3 µM T-B2 equivalent, 10.6 µM T-B2 equivalent, 15.89 µM T-B2 equivalent, 21.19 µM T-B2 equivalent and 26.48 µM T-B2 equivalent were incubated at 35°C (308.15 K), 45°C (318.15 K) and 65°C (338.15 K) in a water-bath for 30 min before reading the fluorescence intensity at an excitation wavelength of 280 nm and an emission wavelength of 332 nm. The fluorescence intensity was recorded to calculate the binding constant (K_a), number of binding sites (n) and thermodynamic parameters, as described above.

Statistical analysis

The experiments were carried out in three separate trials. The data were analyzed using analysis of variance at the 95% significance level. Tukey's test was used to determine significant differences among mean values. All statistical analyses were performed using the Graphpad Prizm 8.4.2 software (GraphPad Software Inc.; San Diego, CA, USA).

Results and Discussion

Profiling anthocyanins in water extract of butterfly pea flower

Fig. 1 shows the flavonoid anthocyanin chromatograms of the *C. ternatea* flower water extract. LCMS-IT-TOF identified the anthocyanins in the water extract as ternatin C2 (peak 3), ternatin B4 (peak 4), ternatin B3 (peak 6), ternatin D3 (peak 7), ternatin B2 (peak 8) and ternatin D1 (peak 9), as summarized in Table 1. All these anthocyanins had a delphinidin-based structure and were polyacylated. These results were in good agreement with those recently reviewed by Jeyaraj et al. (2020).

Peak 3 had a molecular ion precursor $[M]^+$ at 1491 and five fragment ions MS^2 m/z at 1243 ([M–malonoylglucoside]$^+$), 1080 ([M–malonoylglucoside – glucose]$^+$), 773 ([M–malonoylglucoside–glucose–*p*-coumaroylglucoside]$^+$) and 611 ([M–malonoylglucoside–di-glucose–*p*-coumaroylglucoside]$^+$). The MS data suggested that this anthocyanin had one delphinidin unit, five glucose units, one malonic acid unit and two *p*-coumaric acid units. Peak 3 was tentatively identified as ternatin C2, as reported by Terahara et al. (1998).

Peak 4 had a molecular ion precursor $[M]^+$ at 1329 and five fragment ions MS^2 m/z at 1081 ([M–malonoylglucoside]$^+$), 919 ([M–malonoylglucoside–glucose]$^+$), 773 ([M–malonoylglucoside –glucose–*p*-coumaric acid]$^+$), 611 ([M –malonoylglucoside–di-glucose–*p*-coumaric acid]$^+$) and 465 ([M–malonoylglucoside–di-glucose–di-*p*-coumaric acid]$^+$). The MS data suggested that this anthocyanin contained one delphinidin unit, four glucose units, one malonic acid unit and two *p*-coumaric acid units. Peak 4 was tentatively identified as ternatin B4, as reported by Terahara et al. (1996).

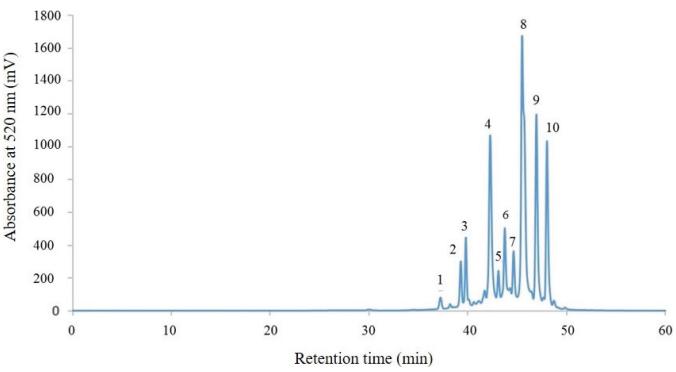

Fig. 1 Liquid chromatography profile of anthocyanins in water extract of *Clitoria ternatea* dried flower, where peak numbers correspond to anthocyanins in Table 1

Table 1 Mass spectrometric data and tentative identification of anthocyanins in water extract of *Clitoria ternatea* dried flower

Peak number	Retention time (t_R, min)	Area (%) ± SD	Molecular ion precursor $[M]^+$ (*m/z*)	MS^2 fragment ion (*m/z*)	Tentative anthocyanins in water extract of *C. ternatea* dried flower	Reference
1	37.24	1.02±0.10	627	Unknown	Unknown	
2	39.32	3.49±0.35	950	Unknown	Unknown	
3	39.95	4.92±0.49	1491	1491$[M]^+$→1243, 1081, 773, 611	Ternatin C2	Terahara et al. (1998)
4	42.20	18.50±1.89	1329	1329$[M]^+$→ 1081, 919, 773, 611, 465	Ternatin B4	Terahara et al. (1996)
5	43.06	1.89±0.19	465	Unknown	Unknown	
6	43.72	5.11±0.39	1638	1638$[M]^+$→ 1389, 1227, 1081, 919, 611	Ternatin B3	Terahara et al. (1996)
7	44.73	3.26±0.38	1167	1167$[M]^+$→ 919, 773, 611, 465	Ternatin D3	Terahara et al. (1998)
8	45.54	30.57±7.20	1638	1638$[M]^+$→1551, 1389, 1227, 1081, 919, 611	Ternatin B2	Terahara et al. (1996)
9	47.02	16.87±1.90	Unknown	Unknown	Unknown	
10	48.06	14.38±1.52	1783	1783$[M]^+$→1535, 1227, 919, 611	Ternatin D1	Terahara et al. (1998)

Peak 6 had a molecular ion precursor $[M]^+$ at 1638 and five fragment ions MS^2 *m/z* at 1389 ([M–malonoylglucoside]$^+$), 1227 ([M–malonoylglucoside–glucose]$^+$), 1081 ([M– malonoylglucoside–glucose–*p*-coumaric acid]$^+$), 919 ([M–malonoylglucoside–di-glucose–*p*-coumaric acid]$^+$) and 611 ([M–malonoylglucoside–di-glucose–di-*p*-coumaric acid]$^+$). The MS data suggested that this anthocyanin contained one delphinidin unit, five glucose units, one malonic acid unit and three *p*-coumaric acid units. Peak 6 was tentatively identified as ternatin B3, as reported by Terahara et al. (1996).

Peak 7 had a molecular ion precursor $[M]^+$ at 1167 and four fragment ions MS^2 *m/z* at 919 ([M–malonoylglucoside]$^+$), 733 ([M–malonoylglucoside–*p*-coumaric acid]$^+$), 611 ([M– malonoylglucoside–*p*-coumaric acid–glucose]$^+$) and 465 ([M–malonoylglucoside–di-*p*-coumaric acid–glucose]$^+$). The MS data suggested that this anthocyanin contained one delphinidin unit, three glucose units, one malonic acid unit and two *p*-coumaric acid units. Peak 7 was tentatively identified as ternatin D3, as reported by Terahara et al. (1998).

Peak 8 had a molecular ion precursor $[M]^+$ at 1638 and six fragment ions MS^2 *m/z* at 1551 ([M–malonic acid]$^+$), 1389 ([M–malonic acid–glucose]$^+$), 1227 ([M–malonic acid–di-glucose]$^+$), 1081 ([M–malonic acid–di-glucose–*p*-coumaric acid]$^+$), 919 ([M–malonic acid– tri-glucose–*p*-coumaric acid]$^+$) and 611 ([M–malonic acid–di-*p*-coumaric acid–tri-glucose– di-*p*-coumaric acid]$^+$). The MS data suggested that this anthocyanin contained one delphinidin unit, five glucose units, one malonic acid unit and three *p*-coumaric acid units. Peak 8 was tentatively identified as ternatin B2, as reported by Terahara et al. (1996).

Peak 10 had a molecular ion precursor $[M]^+$ at 1783 and four fragment ions MS^2 *m/z* at 1535 ([M–malonoylglucoside]$^+$), 1227 ([M–malonoylglucoside–*p*-coumaroylglucoside]$^+$), 919 ([M–malonoylglucoside–di-*p*-coumaroylglucoside]$^+$) and 611 ([M–malonoylglucoside–tri-*p*-coumaroylglucoside]$^+$). The MS data suggested that this anthocyanin had one delphinidin unit, five glucose units, one malonic acid unit and four *p*-coumaric acid units. Peak 10 was tentatively identified as ternatin D1, as reported by Terahara et al. (1998).

The major anthocyanin in the *C. ternatea* dried flower in the current study was ternatin B2 (T-B2). Further calculation on fluorescence quenching of whey proteins by anthocyanin quencher was calculated based on the T-B2 equivalent concentration.

Fluorescence quenching study of whey proteins using water extract of butterfly pea flower

Fluorescence-quenching measurements elucidated the underlying forces involved in the formation of the whey protein-anthocyanin complex in distilled water. The results showed that the fluorescence quantum yield of protein fluorophores was quenched by *C. ternatea* extract (Fig. 2). In the absence of *C. ternatea* extract, WPI solution had the highest fluorescence intensity at 332 nm (Fig. 2A). The addition of *C. ternatea* extracts containing different anthocyanin concentrations, calculated as T-B2 equivalent, reduced the fluorescence intensity of whey proteins in a concentration-dependent manner (Fig. 2B).

Fig. 3 shows that increasing the temperature from 35°C (308.15 K) to 65°C (338.15 K) significantly decreased the binding constant *Ka* and the number of binding sites *n*. The magnitudes of the binding constant *Ka* were relatively high, indicating that the anthocyanins had a strong affinity

with proteins (Acharya et al., 2013). The value of *n* indicated a single or more than one binding site on the protein molecules. The *n* value was slightly greater than 1.0 when binding occurred in the range 35–45°C, possibly because of the involvement of both dynamic and static quenching; or more binding sites being available with slightly different binding constants (Acharya et al., 2013). Whey proteins are mixed proteins composed of β-LG, α-LA and BSA. The available Trp residue for quenchers could be different as the temperature increased from 35°C to 45°C. However, when the reaction temperature increased to 65°C, the number of binding sites decreased to less than 1.0 (Fig. 2C), probably due to more collisions of proteins and quenchers when the temperature was raised.

Table 2 reveals that the protein-anthocyanin complex formed simultaneously (negative *ΔG*). The *ΔG* values at this magnitude (30 kJ/mol) are considered weak, compared to the energy required for covalent bond formation (approximately 400 kJ/mol), according to Koolman and Roehm (2005). Therefore, it was likely that non-covalent binding was responsible for the formation of the protein–anthocyanin complexes in the distilled water. The bindings were exothermic (negative *ΔH*). The negative *ΔH* and negative *ΔS* suggested H-bond involvement as the primary force (Acharya et al., 2013; Chung et al., 2015, He et al., 2016) in the complex formation of delphinidin-based anthocyanins with whey proteins in distilled water.

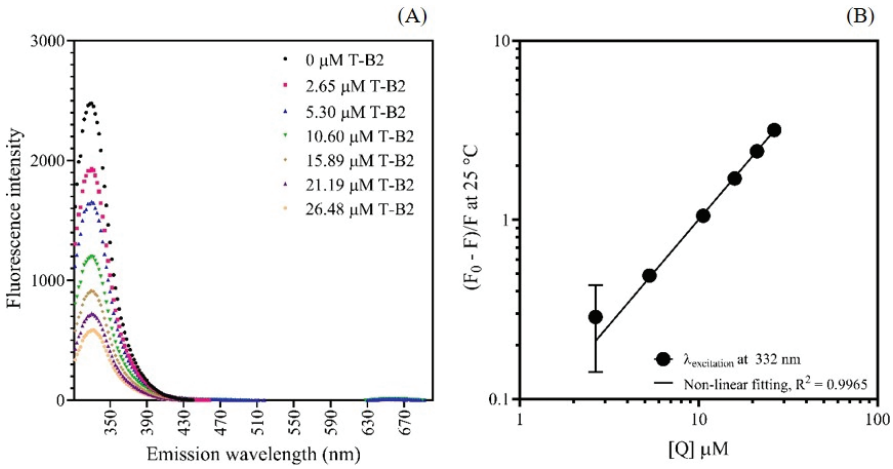

Fig. 2 Fluorescence quenching of whey proteins in WPI by water extract of *Clitoria ternatea*: (A) fluorescence spectra at different anthocyanin concentrations calculated based on ternatin-B2 (T-B2); (B) log-log plot of ($[F_0-F]/F$) as a function of anthocyanin quencher concentration ([Q]) at 25°C, where R^2 = goodness of fit on non-linear curve regression

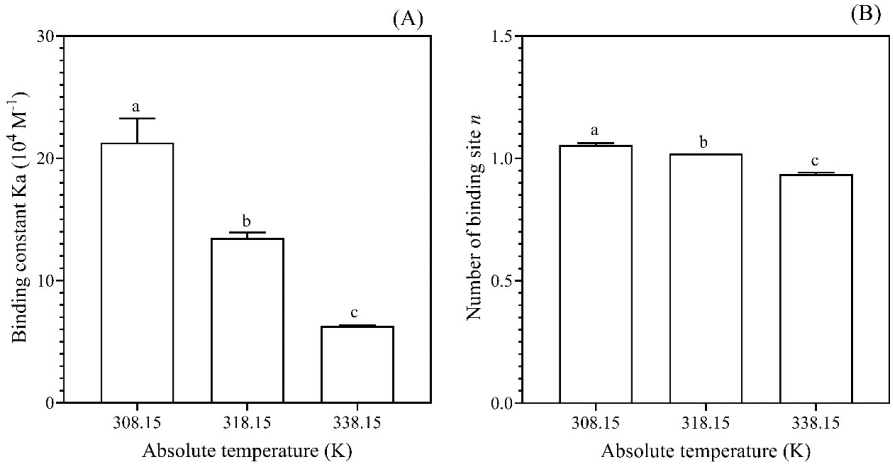

Fig. 3 Effect of absolute temperature on: (A) binding constant; (B) the number of the binding site on whey protein molecule for anthocyanin binding, where error bars indicate + SD and different lowercase letters above columns indicate significant ($p < 0.05$) differences

Table 2 Thermodynamic parameters of whey protein-anthocyanin interaction in distilled water calculated based on ternatin-B2

Temperature		ΔG (kJ/mol)^{ns}	ΔH (kJ/mol)	ΔS (J/mol K)
°C	K		T_1 = 35°C	T_2 = 65°C
35	308.15	-30.77±0.23	-32.948±4.728	-7.415±4.349
45	318.15	-30.59±0.09		
65	338.15	-30.44±0.00		

ΔG = Gibbs free energy change; ΔH = enthalpic change; ΔS = entropic change; T_1 and T_2 = temperature difference at which the ΔH was calculated. Value = means ± SD (n = 3 separate trials)

Whey protein solutions containing different sugar osmolyte concentrations were investigated for their binding characteristics with *C. ternatea* extract at 35 °C, to determine the influences of the medium properties on complex formation (Fig. 4). The K*a* values were within the range 11.3×10^4 $–16.8\times10^4$ M^{-1} in the lactose solution and 14.4×10^4–15.7×10^4 M^{-1} in the sucrose solution (Fig. 4A; $p \geq 0.05$). However, the sugar types significantly affected the number of binding sites on the whey protein molecules accessible by anthocyanins to form a complex (Fig. 4B). At a lactose concentration lower than 37.5 mM, the value of *n* on whey protein molecules was lower than that at a high lactose concentration and that of sucrose solutions ($p < 0.05$).

The microenvironment surrounding the protein molecules induced by sugar osmolytes could alter the protein conformation differently when different sugars were used. Arakawa and Timasheff (1982a) demonstrated that lactose had a stronger cohesive force than sucrose and increased the water surface tension to a greater extent than sucrose. In turn, this resulted in each sugar influencing the conformation of whey proteins differently. This study further illustrated that the conformational changes of whey proteins induced by different sugars could lead to different binding sites on the whey protein molecules accessible by anthocyanins.

The influences were further investigated of a divalent cation, ionic strength and osmotic pressure on the binding characteristics and complex formation of proteins and anthocyanins. Table 3 shows that increasing the concentration of $CaCl_2$ to above 375 mM (which provided an ionic strength (I) of 1.125 M and osmotic pressure (π) of 28.448 atm) increased the binding constants and the number of binding sites in the complex formation ($p < 0.05$). A high concentration of $CaCl_2$ could increase the affinity and strengthen the interactions of whey proteins and delphinidin-based anthocyanins. It was also possible that the formation of a Ca-flavonoid complex occurred at high $CaCl_2$ concentration as reported by Castilho et al. (2018) on quercetin and Ca^{2+}. However, more work is needed to elucidate the complexion sites in delphinidin-based anthocyanins.

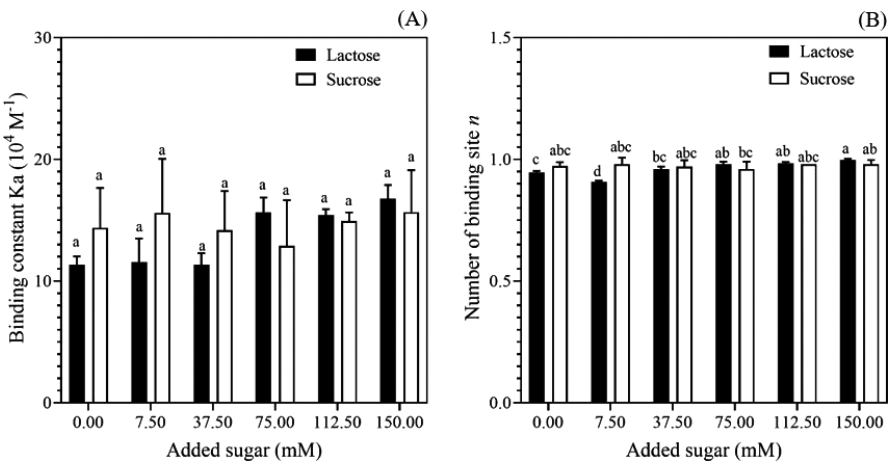

Fig. 4 Effect of sugar type and concentration on: (A) binding constant; (B) number of binding sites of whey proteins with *Clitoria ternatea* anthocyanins at 35°C calculated based on ternatin-B2T-B2. The osmotic pressure was calculated based on respective added sugar concentrations were 0.190 atm, 0.948 atm, 1.897 atm, 2.845 atm and 3.793 atm. Error bars indicate + SD and different lowercase letters above columns indicate significant ($p < 0.05$) differences.

Table 3 Effect of $CaCl_2$ concentration on binding constant and number of binding sites of protein with *Clitoria ternatea* anthocyanin at 35°C calculated based on ternatin-B2

[$CaCl_2$] (M)	Ionic strength (M)	Calculated osmotic pressure of $CaCl_2$ solution (atm)	Binding constant × 10^4 (/M^1)	Number of binding sites
0	0.000		13.56±4.73^c	0.966±0.027^b
0.025	0.075	1.897	14.76±2.11^c	0.976±0.013^b
0.125	0.375	9.483	14.04±2.28^c	0.970±0.011^b
0.250	0.750	18.965	14.23±3.26^c	0.972±0.017^b
0.375	1.125	28.448	20.63±1.36^b	1.005±0.006^a
0.500	1.500	37.930	26.32±1.94^a	1.025±0.006^a

Means ± SD (n = 3 separate trials) superscripted by different lowercase letters are significantly ($p < 0.05$) different.

Fig. 5 shows that the complex formation was a simultaneous and exothermic process (negative ΔG and ΔH values). The negative values of ΔH and ΔS also suggested that despite the increase in osmotic pressure and ionic strength, the H-bonds remained the leading force in the formation of the whey protein-anthocyanin complex in $CaCl_2$ solution. The result from this study were different from Vuong and Hongsprabhas (2021) that was carried out in buffered systems using 0.1 M citrate buffer, 0.2 M phosphate buffer or 0.1 M carbonate buffer, in which hydrophobic interactions were the main forces involved in the formation of the whey protein-delphinidin complexes. This was probably because the Ca^{2+} ion could likely bind directly to whey proteins and alter their conformations (Arakawa and Timasheff, 1982b).

Further investigation is still necessary into the influences of anion types and concentrations on the conformation of whey proteins and subsequent binding mechanisms during whey protein-anthocyanin complex formation. Different anions could perturb the surface free energy of the protein molecules and their conformations differently (Arakawa and Timasheff, 1982b) and subsequently result in different binding mechanisms of proteins with anthocyanins.

In summary, this study further demonstrated that different types and concentrations of the osmolytes commonly used in food product could determine the leading force responsible for the formation of a protein-anthocyanin complex in distilled water, where the proteins solely governed the buffering capacity of the mixed solutions. Understanding the binding mechanisms of complex formation between whey proteins and delphinidin derivatives via H-bonds under different temperatures, osmotic pressures and ionic strengths could help in the design and rationalization of the types and concentrations of osmolyte to manipulate protein conformation and its further interactions with polyacylated delphinidin derivative via reversible H-bonds in the future.

Conflict of Interest

The authors declare that there are no conflicts of interest.

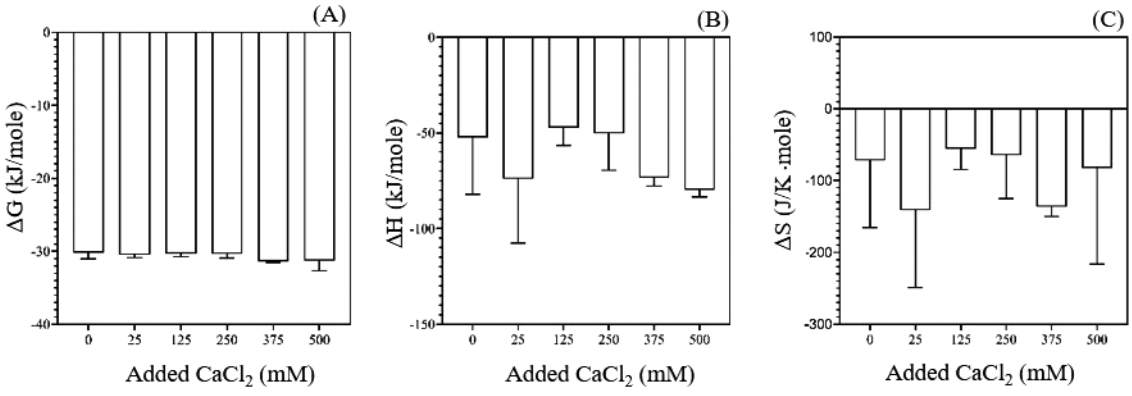

Fig. 5 Effect of added $CaCl_2$ concentration on: (A) Gibbs free energy change; (B) enthalpic change; (C) entropic changes in binding of whey proteins with *Clitoria ternatea* anthocyanins at 35°C calculated based on ternatin-B2, where error bars = + SD

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