Influences of pH on binding mechanisms of anthocyanins from butterfly pea flower (Clitoria ternatea) with whey powder and whey protein isolate

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Abstract: The interactions between whey proteins and delphinidin-based anthocyanins in the water extract of Clitoria ternatea dried flower was investigated by measuring fluorescence quenching of whey powder proteins (WP) and whey protein isolate (WPI) by anthocyanins. Within the temperature range between 35 to 45 °C and buffer pH range between 2.5 to 11.0, the binding of whey proteins with anthocyanins in C. ternatea petal was spontaneous and governed by hydrophobic interactions. The formation of the protein-anthocyanin complex was influenced by the pH and non-protein constituents, i.e. lactose and minerals. In addition to hydrophobic interactions, electrostatic interactions played an important role in the complex formation mechanisms of whey proteins in WPI with anthocyanins. Nonetheless, the formation of the protein-anthocyanin complex of proteins in WPI did not involve electrostatic interactions. This investigation indicated the influences of non-protein constituents on the protein and anthocyanin microenvironments, which determined the types and binding strength of non-covalent forces involved in their interactions.

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PUBLIC INTEREST STATEMENT

The needs to avoid the use of synthetic colourants in foods have increased during the past decades. This study attempts to encourage health-promoting butterfly pea petal as the source of natural blue colourant in dairy products. This work presents the forces involved in the binding of blue anthocyanins with whey proteins over the acidic to alkaline pH. Information on the types of binding force and binding strength at the temperature below the denaturation temperature of whey proteins helps design the microenvironment surrounding proteins and anthocyanins delphinidin derivatives for desirable binding characteristics and conformations before heat treatment. The information can be of further use in pasteurized high-protein drinks consisted of whey powder and whey protein isolate containing anthocyanins and the encapsulation of anthocyanin by whey proteins.
the complex formation. This study also suggested the means for tailoring the binding of delphinidin-based anthocyanin with whey protein via non-covalent forces to formulate macromolecular encapsulation of delphinidin-based anthocyanins by altering the composition of non-protein constituents and pH at the temperature below 45 °C.

Subjects: Food Additives & Ingredients; Food Chemistry; Food Engineering

Keywords: Clitoria ternatea; fluorescence; protein; ternatin; whey

1. Introduction

*Clitoria ternatea* is commonly known as butterfly pea. It is used as a traditional Ayurvedic medicine in Asia. The pharmacological activities from the roots, seeds, flowers, and leaves included neuropharmacological actions, anti-inflammatory activity and anti-diabetic activity. These biofunctional properties result from the primary metabolites such as proteins and amino acids, as well as secondary metabolites, i.e., pentacyclic triterpenes, polyphenols, and phytosterols distributed in different parts of the *C. ternatea* plant (Mukherjee et al., 2008).

The dark blue colour of *C. ternatea* extracted from the petal has been used as a natural colourant in various foods and drinks in Southeast Asia. The flavonoid compounds of *C. ternatea* water extract mainly included flavonol glycosides and anthocyanins (Kazuma et al., 2003). The significant anthocyanins responsible for blue colour in *C. ternatea* petals were ternatins, which are (poly)acetylated delphinidin derivatives (Kazuma et al., 2003; Mukherjee et al., 2008). Their structures were characterised as malonylated delphinidin 3, 3′, 5′-triglucosides having 3′, 5′-side chains with alternative D-glucose and p-coumaric acid units (Kazuma et al., 2003; Terahara et al., 1998; Vuong, 2021).

The water extract of *C. ternatea* is famous as a blue beverage in some countries. *C. ternatea* water extract health-promoting effects have become more evident in terms of biofunctional activities (Chayaratanasin et al., 2019; Jeyaraj et al., 2020; Mukherjee et al., 2008; Pasukamonset et al., 2016). However, the studies on the interactions between *C. ternatea* water extract with other food constituents is limited and deserve further investigation.

Food proteins have been investigated as a potential biopolymer-based nanoparticle carrier for phenolic and polyphenolic compounds recently. The water- and oil-insoluble resveratrol could be encapsulated by sodium caseinate, zein, and gliadin (Acharya et al., 2013; Davidov-Pardo & McClements, 2014) using a spontaneous complex formation via hydrophobic interactions. He et al. (2016) also reported that the non-covalent forces (van der Waals forces, H-bonding, and hydrophobic interactions) between caseins and grape skin anthocyanin extracts, calculated based on malvidin-3-O-glucoside, had a positive effect on the thermal, oxidation and illumination stabilities of anthocyanins and colours.

The interactions between milk proteins and polyphenols resulted from the non-covalent forces and are dependent mainly on the structure and concentration of polyphenols and proteins (Yildirim-Elikoglu & Erdem, 2017). External factors such as sugar, temperature, pH, and ionic strength also affect the interactions between proteins and phenolic compounds and polyphenolic compounds (Naczk et al., 2006; Rawel et al., 2005; Wang et al., 2007). Casonova et al. (2018) investigated the interactions between cyanidin-3-O-glucoside (C3G) and 20 nm-sized sodium caseinate nanoparticles at pH 7 and pH 2 by fluorescence spectroscopy. They reported that the electrostatic interactions dominated the binding at pH 7, while hydrophobic effects were the main force at pH 2.

There are many forms of dairy ingredients in the markets, i.e., skim milk powder (SMP), whey protein concentrate (WPC), whey protein isolate (WPI), and whole dry whey or whey powder (WP). These dairy ingredients contain different protein, fat, lactose, and ash contents (Wijayanti et al., 2014). Bovine
whey proteins are one of the highest nutritious food supplements available for commercial use because of their high content in branched-chain amino acid (BCAA) and essential amino acids (EAA) (Walzem et al., 2002). Whey proteins are consist of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin, and other minor whey proteins (Walzem et al., 2002). Whey products such as WP, WPC, and WPI are used in various foods because of their excellent emulsifying, gelling, and foaming properties (Gerrard et al., 2002).

The complexation mechanisms of individual whey protein, such as β-LG and BSA, to the (poly) phenolic compounds, have been investigated extensively (Khalifa et al., 2018; Oancea et al., 2017; Xu et al., 2019). However, the data on the mixed proteins and (poly)phenols applicable in the real food system that may have a wide range of pH and contain other carbohydrates and minerals are quite limited and merit further investigation. The reversibility of protein-anthocyanin complex and the consequences of binding on physicochemical properties and bioactivities of proteins and (poly) phenols are crucial for designing the controlled release characteristics of encapsulated anthocyanins.

We hypothesised that the pH could determine the binding mechanisms of whey proteins to anthocyanins since they could ionise and possess different charges. Simultaneously, the anthocyanin chromophore could adopt different forms and exhibit different colour when pH altered. In this study, we reported the binding mechanisms of whey proteins from whey powder (WP) and whey protein isolate (WPI) with delphinidin-based anthocyanins from C. ternatea flower. The insights could help rationalise the ingredient selection for biomolecular-based encapsulation of blue anthocyanin pigments in the future.

2. Materials and methods

2.1. Materials and reagents

Packaged dried butterfly pea flowers (C. ternatea) of a local brand (distributed by Siam Macro, Co., Bangkok, Thailand) were purchased from a local market in Bangkok, Thailand, repacked and kept in the sealed aluminium bag at −20°C until used. The whey protein isolate (WPI) powder (Milk Specialties, MN, USA) was purchased from a local distributor (Bangkok, Thailand) contained 85.7% protein (Lowry et al., 1951), 2.65% ash, and 7.23% moisture content (AOAC, 2009). Imported whey powder (WP), locally packed by the domestic wholesale distributor (Multimax Co., Bangkok, Thailand), contained 24.18% protein (Lowry et al., 1951), 8.04% ash, and 3.01% moisture content (AOAC, 2009). The reducing sugar, calculated as lactose, was determined by the dinitrosaliclic (DNS) method described by Miller (1959) after the protein was precipitated by absolute EtOH using the method described by Richmond et al. (1982). The WP contained reducing sugar of 49.52% while the WPI contained 2.92% reducing sugar.

Bovine serum albumin was purchased from Fluka BioChemika, Fluka Chemic GmbH, Buchs, Switzerland and used as a standard for protein determination using the Lowry method without further purification. Copper sulfate, potassium sodium—tartrate and sodium carbonate of analytical grade were purchased from UNIVAR Ajax Finechem (Seven Hills, NSW, Australia). Folinic-Ciocalteau phenol reagent, phosphate-buffered saline and 6-hydroxy-2,5,7,8-tetramethoxychinone-2-carboxylic acid (Trolox) were obtained from Fluka (Biochemika, Sigma–Aldrich, Buchs, Switzerland). Sodium carbonate, gallic acid, potassium persulfate, citric acid, sodium dihydrogen phosphate, di-hydrogen phosphate, sodium hydroxide, and sodium hydroxide (Merck KGaA, Darmstadt, Germany) of analytical grade were used. 2,2’-Azino-bis (3-ethylbenzthiazolone-6-sulphonic acid (ABTS) was purchased from Sigma-Aldrich (Germany), and crocin was from Sigma-Aldrich (Tokyo, Japan).

3. Preparation of butterfly pea flower extract and its chemical composition

One gram of dried flower was extracted by 100 mL of distilled water at 80 °C, 30 min in a water-bath. The blue water extract was filtered through Whatman no. 1 filter paper as described by
Pasukamonset et al. (2016). The extracts were characterised for total phenolic compounds, monomeric anthocyanin, and antioxidant capacities as follows:

### 3.1. Total phenolic compounds

An aliquot of 50 μL of an extract was mixed with 1.25 mL of Folin—Ciocalteu phenol reagent (0.2 M) and allowed to react for 5 min using the method described by Wong et al. (2006). Then 1.25 mL of saturated Na₂CO₃ solution was added and allowed to react for one h. Gallic acid was used to prepare a standard curve. The absorbance was read at 725 nm using a Tecan microplate reader (Infinite 200 PRO, Grodig, Austria). The concentration of total phenolic compounds was expressed as mg gallic acid equivalent (GAE)/g of plant material on a dry weight basis.

### 3.2. Monomeric anthocyanin

Monomeric anthocyanin in the extract was determined using the method described by Lee et al. (2005). The anthocyanin pigment reversibly changes colour with a change in pH, i.e., the coloured oxonium form exists at pH 1.0, and the colourless hemiketal form predominates at pH 4.5. Subsequently, the difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. The extract was diluted with pH 1.0 buffer containing 0.025 M potassium chloride adjusted by HCl, and 0.4 M sodium acetate buffer pH 4.5. The absorbance of samples was read at 520 and 700 nm using distilled water as a blank. The anthocyanin content was expressed as polyacylated anthocyanin ternetin B2 (T-B2) equivalent, as shown in Eq.1.

\[
\text{Monomericanthocyanin (mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \\
\text{(Eq.1)}
\]

where \(A = [(A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}]; MW = 1,638 \text{ g/mol for T-B2}; DF = \text{dilution factor}; l = \text{pathlength in cm}; \varepsilon = 29,000 \text{ molar extinction coefficient of T-B2, in L/ mol.cm}; \text{and } 10^3 = \text{factor for conversion from g to mg}. \text{The calculated concentration of monomeric anthocyanin was expressed as T-B2, the major blue anthocyanin in dried butterfly pea flower (Vuong, 2021), and reported as mg T-B2 equivalent/g of plant material on a dry weight basis.}

### 3.3. ABTS radical scavenging antioxidant activity

The antioxidant activity based on the electron transfer (ET) mechanism was assessed using the ABTS assay described by Re et al. (1999). The green-blue stable ABTS⁺ was generated by oxidising the 7 mM ABTS (2 mL) with 1 mL of 2.45 mM potassium persulfate and allowing the mixture to react in the dark at room temperature for 16 h before use. The ABTS⁺ solution was diluted 40 times with phosphate-buffered saline (PBS) pH 7.4, to obtain the absorbance at 734 nm of 0.70 assessed by a Tecan microplate reader. Then, antioxidants (Trolox or extract) arrested ABTS⁺ radical, leading to a decrease in absorbance at 734 nm. The absorbance was recorded at precisely 1 min. The antioxidant activity was reported as μmol Trolox equivalent (TE)/g of plant material on a dry weight basis.

### 3.4. Crocin bleaching assay

The Crocin bleaching assay described by Tubaro et al. (1998) and Di Majo et al. (2008) was used to evaluate the antioxidant activity of the extract based on the hydrogen atom transfer (HAT) mechanism. The assay was based on the comparison of Crocin bleaching rates in the absence and presence of the antioxidant. The standard antioxidant Trolox (0.1–1 mM) was used to quantify the antioxidant capacity determined from the plot between relative bleaching rate and Trolox concentration. The antioxidant capacity was expressed as μmol Trolox equivalent (TE)/g of plant material on a dry weight basis.

### 4. Effect of pH on the binding of anthocyanins in the water extract of butterfly pea flower with proteins from WPI and WP

Before performing the quenching experiment, the protein solutions (10 mg protein/mL) prepared by solubilising WPI or WP in distilled water at 25 °C were used to determine the maximum
fluorescence wavelength of proteins from WPI and WP. The excitation wavelength was 280 nm. A TECAN Microplate reader monitored the intensity of emission wavelength between 310 and 850 nm at 25 °C to determine the wavelength for the maximum fluorescent intensity of whey proteins in WPI and WP. The Stern-Volmer quenching constant \(K_{sv}\) was calculated as the slope of the regression curve of \(F_0/F\) against \([Q]\) in the linear range shown in Eq. 2.

\[
\frac{F_0}{F} = 1 + K_{sv}[Q] \quad \text{(Eq. 2)}
\]

where \(F_0\) and \(F\) are fluorescence intensity emitted at 332 nm in the absence and presence of quencher respectively, \(K_{sv}\) is Stern-Volmer quenching constant, and \([Q]\) is the concentration of anthocyanin in the extract calculated based on T-B2 as described above.

The binding characteristics of proteins in WPI and WP with C. ternatea ternatin was calculated based on T-B2 in the buffered systems between pH 2.5–11.0 were determined. The 0.1 M citrate buffer was used to adjust the reaction solvent pH to pH 2.5 and 3.5, while the 0.2 M phosphate buffer was used to adjust the reaction solvent pH to 7.5 and 8.5. For alkali pH, the 0.1 M carbonate buffer was used to adjust the pH to 11.0.

WPI and WP suspensions containing 10 mg/mL protein were prepared in distilled water. Two hundred and fifty µL of protein suspension was added with 250 µL of buffer pH 2.5, 3.5, 7.5, 8.5, and 11 to alter microenvironment and protein conformation before mixing with C. ternatea extract. The 5 mg/mL protein solution (500 µL) at different pH was further mixed with 500 µL of C. ternatea extract containing 5.3, 10.6, 21.2, 31.8, 42.4, and 53.0 µM T-B2 equivalents. After mixing, the final concentration of protein was 2.5 mg/mL, and the final concentrations of T-B2 equivalents were 2.65, 5.3, 10.6, 15.89, 21.19, and 26.48 µM at respective pH.

The reaction was allowed to proceed at 35 °C (308.15 K) and 45 °C (318.15 K) in a water-bath for 30 min before reading fluorescence intensity. The excitation wavelength was 280 nm, and the emission wavelength was 332 nm. The fluorescence intensity was recorded to calculate binding constant \(K_a\), the number of the binding site \(n\), and thermodynamic parameters \(\Delta G\), \(\Delta H\), and \(\Delta S\) as described by Lakowicz (2006). The binding constant and the number of binding sites were calculated from a double logarithmic curve described in Eq. 3:

\[
\log \frac{F_0 - F}{F} = \log K_a + n \log [Q] \quad \text{(Eq. 3)}
\]

where \(F_0\) and \(F\) are fluorescence intensity in the absence and presence of anthocyanin quencher, respectively, \(K_a\) is the binding constant, \(n\) is the number of the binding site, and \([Q]\) is the concentration of anthocyanin calculated as T-B2.

The dominant forces responsible for the bindings of protein fluorophore with anthocyanin quencher were determined based on the signs and values of thermodynamic parameters: Gibbs free energy change \(\Delta G\), enthalpy change \(\Delta H\) and entropy change \(\Delta S\). The Gibbs free energy change can be calculated from \(K_a\) as described in Eq. 4

\[
\Delta G = -RT \ln K_a \quad \text{(Eq. 4)}
\]

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 Eq. 3 at each temperature.

The change in enthalpy \(\Delta H\) when there is an increase, or a decrease of temperature from \(T_1\) to \(T_2\), was calculated by applying the van’t Hoff equation shown in Eq 5

\[
\ln \left( \frac{K_{a2}}{K_{a1}} \right) = \frac{-\Delta H}{R} \left( \frac{1}{T_2} - \frac{1}{T_1} \right) \quad \text{(Eq. 5)}
\]
where $T_1$ and $T_2$ are absolute temperatures at which $K_{a1}$ and $K_{a2}$ were calculated.

The entropy change ($\Delta S$) was calculated as described in Eq. 6.

$$\Delta G = \Delta H - T\Delta S \quad \text{(Eq. 6)}$$

There are four major non-covalent forces between small molecules and proteins: H-bonding, van der Waals force, hydrophobic interactions, and electrostatic interactions. Identifying the dominant force involved in the binding was based on the signs and values of thermodynamic parameters. For example, the negative $\Delta G$ and the negative $\Delta H$ indicate that the binding process is spontaneous and exothermic. When the $\Delta H$ is positive and the $\Delta S$ is also positive, the binding process is endothermic and hydrophobic interactions are the main force. However, when the $\Delta H$ is negative and the $\Delta S$ is negative, the H-bonding is the leading force. The electrostatic interactions play a significant role in the binding when the $\Delta H$ is negative and the $\Delta S$ is positive (Acharya et al., 2013; Davidov-Pardo & McClements, 2014; Fisicaro et al., 2004; Joye et al., 2015).

5. Statistical analysis
The experiments were carried out in three separate trials. The data were analysed by using analysis of variance (ANOVA) at a 95% significance level. Tukey test determined significant differences among mean values. All statistical analyses were performed using Graphpad Prizm 8.4.2 (GraphPad Software Inc., San Diego, CA, USA).

6. Results and discussion

6.1. Chemical composition of the water extracts from butterfly pea flower
Table 1 shows that the water extract contained total phenolic content around 30 mg GAE/g dried flower (d.b.) and monomeric anthocyanin content calculated as ternatin B2 was around 8.7 mg/g dried flower (d.b.), which were relatively high and within the range reported by Rabeta and Nabil (2013).

The C. ternatea flower water extract was dark blue in distilled water (DW), as shown in Figure 1. However, the colour of the extract was dependent on the pHs. At a pH of 2.5, the extract and the extract-whey protein mixed solutions exhibited a pink colour. Raising the pH to 3.5 altered the colour of the extract and the mixed solutions to purple. At pH 7.5 and 8.5, however, the extract and mixed solutions colour turn vivid greenish-blue. The colour of all samples turned olive green at pH 11.0. Note that the colour of the extract in the buffers at pH 7.5 was more greenish than the dark blue colour of the extract in DW, which could be due to the higher ionic strength of buffer than DW.

Heat treatment at 72 °C for 15 s did not change the colours of the extract-whey protein mixed solutions compared to the colours of the extracts at respective pH. However, heat-induced small protein aggregates were observed in the extract-whey protein mixed solutions after heating,

| Table 1. Chemical characteristics of water extract from Clitoria ternatea dried flower |
|-----------------------------------------------|
| Characteristics                             | Means ± standard deviation (n = 3 extraction trials) |
| Water-extractable (poly)phenolic compounds   |                                                     |
| Total phenolic compounds                     | 30.48 ± 4.84 mg gallic acid equivalent/g dried flower |
| Monomeric anthocyanin content                | 8.67 ± 0.32 mg ternatin B2 equivalent/g dried flower |
| Antioxidant capacity                         |                                                     |
| ABTS assay                                  | 467.04 ± 30.45 µmole TE/g dried flower              |
| Crocin bleaching assay                       | 26.90 ± 2.18 µmole TE/g dried flower                |
suggesting irreversible protein denaturation and aggregation after heating. Such irreversible aggregation could involve covalent bond formation between protein-protein and protein-anthocyanin. The investigation on the complex formation via non-covalent forces between whey proteins and anthocyanins in the water extract of *C. ternatea* was then performed at the temperature below denaturation of whey proteins next section.

7. Effect of pH on the binding of anthocyanins in the water extract of butterfly pea flower for proteins in WPI and WP

Fluorescence quenching technique was used to monitor the bindings of protein (mainly via aromatic acid residues, particularly tryptophan) to anthocyanin using ternatin B2 (T-B2) as representative anthocyanin for the calculation. The T-B2 is polyacylated delphinidin derivative that was the major anthocyanin in dried butterfly pea flower extract previously reported (Vuong, 2021). The ternatins are also responsible for the blue colour of *C. ternatea* petal (Kazuma et al., 2003). Figure 2 shows that increasing the quencher concentration ([Q], calculated based on T-B2) lowered the fluorescence intensity of protein fluorophore in a linear trend. The quenching characteristics observed as the Stern-Volmer quenching constant ($K_{SV}$) values of proteins in WPI and WP were different ($P < 0.05$).

Figure 3A shows that raising the pH from acidic to alkali pH at 35 °C reduced the binding constants $K_b$ of anthocyanins for proteins ($P < 0.05$). The binding constant of the protein-anthocyanin complex in WP was remarkably higher than in the WPI within the pH range of 2.5 to 7.5 ($P < 0.05$). At and above pH 8.5, the binding constant $K_b$ values of proteins in WPI and WP were not different ($P \geq 0.05$).

Increasing pH from acidic to alkali pH significantly decreased the numbers of binding sites $n$ (Figure 3B, $P < 0.05$). Note that different pH altered the conformations and the surface charges of the proteins and the ionised forms of anthocyanin, which may influence anthocyanin binding natures with proteins (Yildirim-Elikoglu & Erdem, 2017).
The binding constant $K_a$ of anthocyanin with protein at different pH was also governed by the temperature shown in the heat map of Figure 4. Raising temperature from 35 °C to 45 °C drastically increased the binding constants of anthocyanin with protein in WPI at every pH investigated (Figure 4A). The binding constant of protein in WP at pH 2.5 drastically decreased when the temperature increased to 45 °C. However, the binding constant $K_a$ at pH 8.5 increased when the temperature increased from 35 °C to 45 °C. The different binding responses to pH and heat treatment between proteins in WP and WPI suggested different binding mechanisms in the protein-anthocyanin complex formation.

The pHs could determine different main forces involved in binding whey proteins with anthocyanins as the temperature increased from 35 to 45 °C. Figure 5 summarises the values of $\Delta G$, $\Delta H$, and $\Delta S$ during the complex formation of whey proteins and anthocyanins. Although the formation of whey protein—anthocyanin complex could simultaneously occur (negative $\Delta G$, Figure 5A), the types of the primary binding forces were significantly dependent on pHs ($P < 0.05$).

Within the pH range between 2.5 and 11 in buffered systems, the bindings of anthocyanins to proteins in WPI were an endothermic process (positive $\Delta H$, Figure 5B) and occurred spontaneously.
The positive ΔS (Figure 5C) meant that the solvent molecules surrounding the anthocyanin—protein complexes arranged themselves in a less orderly fashion. The hydrophobic interactions were most likely the leading force during complex formation when WPI was used (Fisicaro et al. 2004; Joye et al., 2015).

Although hydrophobic interactions played significant roles in binding whey proteins to C. ternatea anthocyanins, the thermodynamic parameters suggested that proteins in WP could form a complex via additional forces. The formation of the complex at pH 2.5, 7.5, and 11.0 when WP was used is an exothermic process (negative ΔH, Figure 5B), accompanied by a positive entropy change (ΔS, Figure 5C). From the water structure standpoints, the positive ΔS suggested that hydrophobic interactions are the primary force during binding (Fisicaro et al. 2008). Nonetheless, electrostatic interactions between ionic species in the buffered system could also be responsible for the positive value of ΔS and a negative value of ΔH (Tian et al., 2003) at pH 2.5, 7.5 and 11.0. The alterations in the surface charges of proteins and the states of flavylium ions in anthocyanins at different pH were then considered. The anthocyanidins are known as flavylium cation, of which the hydrogen atoms are substituted with various groups and possess different colours. At pH 2, anthocyanin is in the form of flavylium cation (AH⁺). However, between pH 2 to 8, anthocyanin changes into a mixture of colour and colourless compounds with different charges, such as neutral quinonoid base (A) and anionic quinonoid base (A⁻) that could modulate the interactions with tryptophan in whey proteins when WP was used.
Moreover, some amino acid residues such as arginine, cysteine, serine, and threonine could be destroyed at pH 11.0, although tryptophan is relatively stable against alkali pH (Meussner et al., 2014). The surface charge and the conformation of proteins could then be different from those at neutral pH or mild alkali conditions, leading to different binding mechanisms with anthocyanin.

For proteins in WPI, the complex formation at pH 11.0 was an endothermic process driven by hydrophobic interactions observed as positive ΔH and positive ΔS. When WP was used, the negative ΔH and positive ΔS suggested that the formation of protein—anthocyanin complex was exothermic and driven by hydrophobic interactions and electrostatic interactions. The binding mechanisms of whey proteins in WP were governed by pH, high lactose content, and the ionic strength in the systems. The WP contained 8.04% ash, while the WPI contained 2.65% ash in the powder form.

This study has revealed that the protein—anthocyanin interactions at 35 and 45 °C, which is lower than the denaturation temperatures of whey proteins, were influenced by the reaction pH and non-protein constituents. The difference in the microenvironment surrounding the protein molecules prepared from the WPI and WP could alter protein conformation differently. The hydrophobic interactions are dominant binding forces due to the alterations of water structure at different ionic strength and pH. Electrostatic interactions could also be involved when the proteins and anthocyanins had different surface charge and conformation. These non-covalent forces are reversible (Yildirim-Elkoglu & Erdem, 2017), which offers the means for tailoring the release of anthocyanins from macromolecular whey protein matrices by altering the strength of protein—anthocyanin bindings.

The insights from this investigation are useful for rationalising ingredient selection for the encapsulation of anthocyanins in the future. Nonetheless, the fluorescence quenching studies demonstrated the molecular basis of protein-anthocyanin binding mechanisms at low protein concentration regime. The final concentration of proteins in this study was 2.5–5.0 mg protein/mL, which was relatively low, to minimise protein-protein interactions. Dairy-based food and beverage usually contained much higher protein content. The investigation on the complex formation of a more concentrated protein solution under the gastrointestinal tract physiological conditions is critical to practically control the release of anthocyanins and enhance their bioaccessibility and bioavailability.

8. Conclusions
The reaction pHs and the non-protein constituents regulated binding mechanisms of anthocyanins from C. ternatea with whey proteins at 35 and 45 °C. High lactose and mineral concentrations in WP resulted in the microenvironment favouring strong affinities between protein and anthocyanin via hydrophobic interactions. At pH 3.5, 7.5, and 11.0, hydrophobic interactions and electrostatic interactions were involved in the protein—anthocyanin bindings of proteins in WP. Overall, this study presented the insights on tailoring the interactions between whey proteins and delphinidin-based anthocyanins from C. ternatea flowers.

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Data availability
The data used in this study are available from the authors upon request.

Disclosure statement
The authors declare that there are no conflicts of interest.

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