



Interaction between wheat gliadin and quercetin under different pH conditions analyzed by multi-spectroscopy methods

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ABSTRACT

Polyphenols have been known to have significant binding affinity for proteins, and the specific condition (such as pH) could affect the degree of binding, the formation of covalent bond, and non-covalent interaction. In this study, characteristics of binding quercetin (Q) to wheat gliadin (G) which is a strong food allergen, were studied from pH 2.0 to pH 9.0. The results showed that Q quenched the fluorescence intensity of G by dynamic and static quenching modes and the stoichiometry of binding was close to 1. Intermolecular binding distances were smaller than 8 nm. Thermodynamic parameters suggested that hydrophobic force took charge of the formation of complexes at pH 2.0–4.0, whereas hydrogen bonds and van der Waals forces at pH 5.0–9.0. Analyses of the Fourier transform infrared and the Raman spectra along with synchronous fluorescence spectra revealed secondary and tertiary structural alterations and microenvironmental changed around protein fluorophores upon complexation with Q. The *gauche-gauche-trans* conformation increased at the expenses of the *gauche-gauche-gauche* conformation and the transition from β -turn and random coil to α -helix and β -sheet at pH 5.0 might decrease the allergenicity of G. These results provided new insights into G/Q interactions at different pH values, which may have potentials in decreasing allergen immunoreactivity.

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1. Introduction

Gliadins (G), a gluten protein as the major storage protein fraction of wheat flour, are a family of heterogeneous polypeptides with an average molecular weight from 25 to 100 kDa. G plays a very important role in dough rheological properties and structure [1,2]. Wheat gliadins are monomeric and disulphide-bonded proteins, which are rich in α -helices and β -turns [3]. Besides, it is important to note that the peculiar sequence (rich in prolines and glutamines) and structure of G have been confirmed to be responsible for strong anaphylaxis such as coeliac disease and food allergy [4]. Many studies have explored the allergenic mechanisms of G, giving the approaches to decrease allergen immunoreactivity by modifying protein structural elements, such as β -turns and disulphide bonds [5,6].

Plant polyphenols, a class of secondary plant metabolites, exist widely in plants and vegetables. They are powerful antioxidant molecules with anti-bacterial, anti-carcinogenic, and anti-allergic activities. Recently, much attention has been paid to the interactions between allergen proteins such as wheat gliadins and peanut protein and plant

polyphenols, with the purpose to render them less allergenic [7]. Pérot et al. [8] reported that cranberry polyphenols could reduce allergenicity of gliadins by masking immunological epitopes via reducing mouse anti-gliadin IgE recognition. Taddei et al. [6] also found that β -turns content of gliadins decreased through making gliadins bind with plant polyphenols (malvin and pelargonin), which finally decreased the allergenicity. Caffeic, chlorogenic and ferulic were bound to peanut proteins to form insoluble complexes, which decreased the allergic response [9,10]. Therefore, plant polyphenols are promising additives which manipulate the structure of G.

Quercetin (Q, 3,5,7,3',4'-pentahydroxyflavone), one of the major dietary flavonoids, consists of two aromatic rings (A and B) linked by an oxygen containing heterocycle (ring C). The multiple hydroxyl groups in the molecular structure of Q are known to interact with proteins in the food matrix [11]. Studies have proved that hydrogen bonding and van der Waals forces were predominating in β -casein-Q binding process [12], whereas the driving forces for bovine serum albumin-Q association were hydrophobic interaction and hydrogen bond [13]. Wang & Wang [14] revealed that Q could bind to the hydrophobic pockets of soy protein isolate particles through hydrophobic interactions and hydrogen bonding. Liu et al. [15] had demonstrated that zein-Q covalent complexes exerted stronger antioxidant activity than native zein. Thus, Q may be prospective to improve the properties of proteins.

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During food processing, many factors, such as pH, temperature, ionic strength, may influence the interactions between proteins and polyphenols. The surface charge properties of proteins and polyphenols would change by varied pH values, subsequently influencing the type of interactions [16]. Study indicated that polyphenols prevented the formation of hot aggregates of lactoferrin at neutral pH in dairy products [17]. By properly controlling the pH value, the precipitation from the interaction of proteins and polyphenols could be avoided during the food (beers, wines, beverages, juices) processing or marketing [18]. Moreover, the covalent binding extent of α -lactalbumin to epigallocatechin gallate reached to the highest at pH 8.0, and would be negatively affected by the lower pH [19]. Hence, pH may be a critical factor during protein-polyphenols interactions. The interactions of gliadins with anthocyanidins and 3-ethoxycarbonylcoumarin have been analyzed at acidic and neutral pH [20,21]. However, to the best of our knowledge, interactions of G with Q under different pH environments have been scantily investigated, and at specific pH whether decrease the allergenicity were unclear.

In order to have a better understanding of the interactions existing between G and Q at different pH conditions, we investigated the binding characteristics of G/Q interactions by multiple spectroscopic methods. The quenching of intrinsic fluorescence of G induced by Q was used to determine the thermodynamic parameters involving in their binding. The changes of protein structure, the environment of specific amino acids and the conformation of disulphide bridges were evaluated by FTIR and the Raman spectra. This study might be helpful for the reduction of G allergenicity under different pH conditions.

2. Materials and methods

2.1. Materials

Wheat gliadins (G, Lot #SLBS6538) and quercetin (Q, Lot #SLBV2993) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other chemicals, reagents, and solvents were of analytical grade (Chengdu Kelon, Chengdu, China). Water purified by a Milli Q system (MiLLi-Qbiocel, Millipore, USA) was used for all the experiments.

2.2. Sample preparation

G/Q complexes were prepared by a method of antisolvent precipitation according to the procedure of Wu et al. [22] with some modifications. Briefly, 1.0 g G and 0.0075 g Q were dissolved in 100 mL 70% (v/v) ethanol-water solution under ultrasound-assisted stirring. 250 mL 1% acetic acid solution was added and the final solution was homogenized with 10,000 rpm for 30 s. The solvent was removed by rotary evaporation, the final dispersion was subjected to centrifugation at 1000 rpm for 10 min to separate larger aggregates if any. G being treated under the same condition in the absence of Q. The dispersion at pH 2.0–9.0 was adjusted with 5 mol/L HCl and 5 mol/L NaOH. Dispersions (may be with a few precipitations) dried by freeze-drying apparatus to obtain the solid powder.

2.3. Fluorescence spectroscopy

The fluorescence spectra of protein ($\lambda_{\text{ex}} = 295$ nm, $\lambda_{\text{em}} = 300$ –500 nm) were recorded on a F-2500 spectrometer (Hitachi, Japan) by placing a quartz cuvette (1 cm path length). The final protein concentration was 5 $\mu\text{mol/L}$, and Q concentration was 0, 1.5, 2.5, 5, 10, 15, 30, 50 $\mu\text{mol/L}$, respectively. To evaluate the effect of temperature on G/Q interactions, the fluorescence quenching titration experiments were conducted at three different temperatures at 293 K, 303 K, and 313 K, respectively. The inner filter effect correction in the fluorescence data was conducted as described by Lakowicz [23]. The synchronous fluorescence measurements were performed at 293 K by scanning the protein samples in the wavelength ranging from 250 to 500 nm. The

spectral behavior of tyrosine (Tyr) and tryptophan (Trp) amino acid residues of G was recorded at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm ($\Delta\lambda = \Delta\lambda_{\text{ex}} - \Delta\lambda_{\text{em}}$), respectively. The excitation and emission slit widths were kept 5.0 nm.

2.4. UV-visible absorption spectroscopy

The absorption spectral measurements were carried out on a UV-vis spectrometer (UV-2450, Shimadzu, Kyoto, Japan) with a 1 cm optical path length. The spectra of G and G/Q mixtures (the protein concentration was 0.2 mg/mL in 70% (v/v) ethanol-water solution were determined, with 70% ethanol solution as a blank. The wavelength scanning range was 200–500 nm at a fast speed.

2.5. Determination of binding distance

According to Forster's energy transfer theory [24], which has been widely used in the research of the interactions between polyphenols and proteins, the distance (r) between the donor (G) and acceptor (Q), as well as the efficiency of transfer of energy (E) could be obtained from Eq. (1):

$$E = 1 - F/F_0 = R_0^6 / (R_0^6 + r^6) \quad (1)$$

where F and F_0 are the fluorescence intensities of G in the presence and absence of Q, and R_0 is the Forster critical distance where the efficiency of the energy transfer is at 50% and described by Eq. (2):

$$R_0^6 = 8.78 \times 10^{-25} K^2 \sigma N^{-4} J \quad (2)$$

where K^2 (as 2/3) is the spatial factor of orientation of the transition dipoles; N (as 1.336) is the refractive index of the medium; σ (as 0.118) is the fluorescence quantum yield of the donor; J is the integral overlap between the donor emission and acceptor absorption. And J is determined following Eq. (3):

$$J = \sum F(\lambda) \varepsilon(\lambda) \lambda^2 \Delta\lambda / \sum F(\lambda) \Delta\lambda \quad (3)$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ ; $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ .

2.6. Fourier transform infrared (FTIR) spectroscopy

The infrared spectra of dried samples were recorded on a FTIR spectrometer 100 (Perkin-Elmer, USA). Each spectrum was collected in the wavenumber range of 4000–500 cm^{-1} and averaged over 32 scans with 4 cm^{-1} resolutions. The FTIR spectra were smoothed and normalized, and their baseline corrected automatically using the built-in software of the spectrometer.

2.7. Raman spectroscopy

The molecular vibration mode of the sample was analyzed by Raman spectroscopy, and the dried powder was placed on a smooth glass slide for testing as follows: microscope objective was 20 $\times$; spot size was 1 μm ; excitation wavelength was 785 nm; grating was 400 line/mm; slit width was 50 μm ; laser power 15 mW; the number of background exposure was 512 s; scanning range was 400–4000 cm^{-1} ; resolution was 1 cm^{-1} ; and the number of scanning was 32. The spectra were analyzed by normalization with phenylalanine as an internal standard. The OMNIC 8.2 (Thermo Fisher Scientific Inc., Waltham, MA, USA) software was used in spectrogram baseline correction, smoothness and standardization for original data.

2.8. Statistical analysis

All the experiments were performed under the same conditions in triplicate. The software of OriginPro 8.5 (OriginLab Corp., Northampton, MA), PeakFit 4.12 and OMNIC 8.2 were used for the curves plotting and statistical processing. Statistical comparisons were performed using the Tukey's HSD multiple comparison test and analysis of variance (ANOVA). The level of significance used was $p < 0.05$.

3. Results and discussion

3.1. Fluorescence quenching during G-Q interactions

Information about changes in the tertiary structure of proteins was obtained using fluorescence spectroscopy. Protein intrinsic fluorophores (Trp and Tyr) were highly sensitive to its microenvironmental polarity.

G exhibited a strong fluorescence peak around 340 nm, a characteristic of proteins containing Trp residue. Trp residue was generally considered to be located in the hydrophobic pockets of globular proteins. With the increasing concentration of Q, fluorescence intensity of G progressively decreased at designated pH values (Fig. 1), suggesting that the ability of Q to quench the fluorescence of G in a dose-dependent manner, which was in agreement with previous findings [14]. The aryl groups of polyphenols hydrophobically bound to the hydrophobic pockets of proteins [25]. When the concentration of Q reached 50 $\mu\text{mol/L}$, 85%–89% of the protein fluorescence was quenched at pH 2.0–9.0, which indicated that strong interactions existed between G and Q. The lower λ_{m} and fluorescence intensity for G at pH 3.0 (relative to that at pH 7.0) reflected that as pH changed from 7.0 to 3.0, the tightening of protein structure through intramolecular interactions, such as hydrogen bonds, seemed to bury Trp in a more hydrophobic environment. This suggested that when the surface charge of proteins changed from a negative to positive value, the protein molecules underwent a structural unfolding and subsequent rearrangement [26].

At pH 3.0, pH 5.0, pH 7.0 and pH 9.0, a distinct blue-shift (about 10 nm) was observed (Fig. 1). A blue shift was indicative of the changes of Trp towards a more hydrophobic microenvironment and might be related to strong affinity interactions (hydrogen bond interaction, hydrophobic interaction and electrostatic interaction). Moreover, the neutral and alkaline systems (pH 7.0–9.0) had a higher fluorescence peak than acidic systems (pH 2.0–6.0), which indicated the former treatment of protein increased the number of OH groups exposed [13]. Also, the

separation of G emission band (pH 6.0–9.0) was more pronounced in the case of Q molecule with more OH groups. The observation was related to more exposure of Trp residue and unfolding of G structure [27]. These were consistent with our FTIR spectroscopy results (Section 3.5) that showed major secondary structural changes for G upon Q complexation. Similar fluorescence phenomena were observed in previous reports [15,22].

To further elaborate the underlying mechanism, the effect of temperature on the Stern-Volmer quenching constant was performed at three different temperatures, i.e., 293 K, 303 K, 313 K. The mechanisms of quenching were usually classified into dynamic quenching and static quenching. Temperature increase led to a decrease in the static quenching constant while an increase in the dynamic quenching constant. F_0/F and $[Q]$ in the Stern-Volmer plots obtained at tested temperatures showed linear correlation (Fig. 1 (inset)), and the slope increased with the temperature increasing (such as 303 K $\rightarrow$ 313 K at pH 3.0 and 293 K $\rightarrow$ 303 K at pH 5.0, Table 1), which indicated that dynamic quenching occurred between G and Q. And values of K_q (in the range of $0.63\text{--}2.01 \times 10^{13}$ L/mol/s) were higher than the diffusion collision-controlled limit ($\sim 10^{10}$ L/mol/s), which suggested that there was static quenching during G and Q interactions. Actually, results in Fig. 1 (inset) was not a strictly straight line, but an ascending curve that gradually bent to the ordinate axis as the quencher Q concentration increased. Our study only selected the linear zones (the lower Q concentration zones) for regression analysis in order to determine the values as listed in Table 1. The results indicated that at all pH values the quenching of G by Q was governed by both dynamic and static quenching mechanisms. A similar phenomenon was found by the research of Joye, Ludescher & McClements [28] about the complexes of gliadin with resveratrol. They reported K_q values raised from 35 $^{\circ}\text{C}$ to 40 $^{\circ}\text{C}$ from 1.34×10^{12} L/mol to 1.53×10^{12} L/mol.

3.2. Binding forces between G and Q

Plotting $\text{Log}[(F_0 - F)/F]$ against $\text{Log}[Q]$ provided information about the association constant, K_a and the stoichiometry of binding, n for G/Q interaction at different pH, through the intercept with the y-axis and its slope, respectively (Fig. 1), and the values were listed in Table 1. High K_a calculated for G/Q represented high affinity interactions, and the crosslinking affinity of G/Q was stronger than electrostatic repulsion force of G at acidic conditions [29]. However, K_a had a large difference at different pH, which demonstrated that pH was an important factor influencing the association constant. The influence factors were

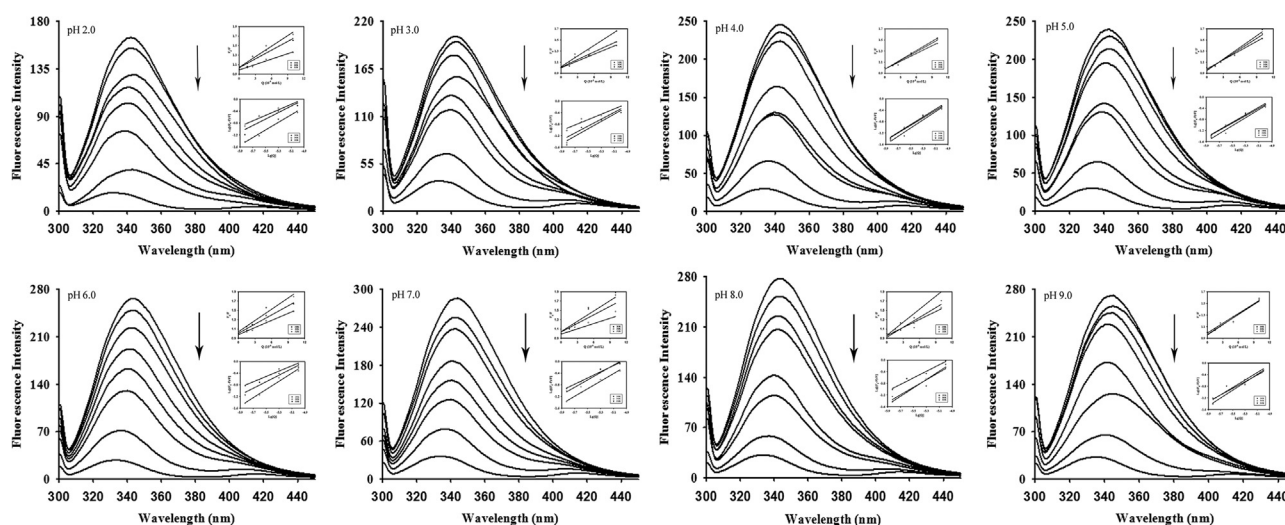


Fig. 1. Fluorescence quenching of wheat gliadin (G) by increasing concentrations (curves from top to bottom: 0, 1.5, 2.5, 5, 10, 15, 30, 50 $\mu\text{mol/L}$) of quercetin (Q). The effective G concentration used was 5 $\mu\text{mol/L}$. Inset Figs represent Stern-Volmer plot of F_0/F as a function of Q concentration and plot of $\text{Log}[(F_0 - F)/F]$ as a function of $\text{Log}[Q]$ concentration at three temperatures, i.e., 293 K, 303 K and 313 K. F_0 and F represent the fluorescence intensity in the absence and presence of Q, respectively.

Table 1

The quenching constant and thermodynamic parameters of the formation of wheat gliadin-quercetin (G/Q) complexes at different pH and three temperature.

pH	T (K)	K_q (L/mol/s)	K_a (L/mol)	n	ΔG (kJ/mol)	ΔS (kJ/mol/K)	ΔH (kJ/mol)
2.0	293	6.12×10^{12}	1.95×10^5	1.0831	-29.68	0.55	132.06
	303	7.38×10^{12}	3.26×10^4	0.9192	-26.18	0.52	
	313	3.76×10^{12}	8.80×10^5	1.2683	-35.62	0.54	
3.0	293	4.11×10^{12}	1.22×10^6	1.2812	-34.14	1.04	270.03
	303	6.41×10^{12}	3.14×10^4	0.9345	-26.08	0.98	
	313	4.62×10^{12}	5.32×10^5	1.1987	-34.31	0.97	
4.0	293	5.57×10^{12}	3.40×10^6	1.352	-36.64	1.36	361.07
	303	5.23×10^{12}	2.07×10^6	1.3178	-36.63	1.31	
	313	4.68×10^{12}	7.27×10^6	1.4342	-41.12	1.28	
5.0	293	6.98×10^{12}	1.07×10^7	1.4387	-39.42	-0.24	-110.53
	303	5.53×10^{12}	4.77×10^7	1.5709	-44.54	-0.22	
	313	6.36×10^{12}	2.94×10^7	1.5276	-44.75	-0.21	
6.0	293	6.65×10^{12}	3.84×10^5	1.3354	-36.94	-0.60	-221.48
	303	5.25×10^{12}	7.99×10^6	1.4292	-40.04	-0.60	
	313	7.70×10^{12}	2.54×10^4	0.8947	-26.39	-0.62	
7.0	293	8.55×10^{12}	1.88×10^5	1.0616	-29.58	-0.43	-155.23
	303	4.93×10^{12}	1.54×10^6	1.2949	-35.89	-0.39	
	313	9.35×10^{12}	2.02×10^6	1.2614	-37.78	-0.38	
8.0	293	9.32×10^{12}	3.62×10^5	1.1223	-31.18	-0.70	-235.54
	303	5.74×10^{12}	8.81×10^6	1.4372	-40.29	-0.64	
	313	7.04×10^{12}	1.44×10^6	1.2904	-36.91	-0.63	
9.0	293	5.70×10^{12}	2.62×10^5	1.1432	-30.39	-0.52	-184.03
	303	5.92×10^{12}	3.17×10^6	1.3516	-37.71	-0.48	
	313	5.58×10^{12}	1.53×10^5	1.2793	-31.06	-0.49	

multispect: (1) The conformation of G changed at different pH values and (2) the degree of Q dissociation and the charging of G were different, which might result in different electrostatic forces. At 293 K and 303 K, K_a value at pH 5.0 was much greater than that at pH 3.0, which suggested that higher temperature might strengthen the combination of G and Q at pH 5.0. Additionally, values of n for all pH at three temperatures were approximately 1, which revealed that G and Q interacted at the molar ratio of 1. At pH 5.0, larger values of K_a and n indicated that pH 5.0 might be a suitable pH for better binding of G and Q.

To investigate the major binding force between G and Q, free-energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) were obtained. There were several forces between G and Q, including hydrogen bonds, hydrophobic interactions, electrostatic forces, van der Waals forces, and even covalent binding [30]. According to the results from Ross and Subramanian [31], if $\Delta H < 0$ and $\Delta S < 0$, van der Waals forces and hydrogen bonds were the main force, if $\Delta H > 0$ and $\Delta S > 0$, predominant force was hydrophobic, and if $\Delta H < 0$ and $\Delta S > 0$, it was the electrostatic interaction.

Thermodynamic parameters were shown in Table 1. The negative ΔG suggested that reaction between G and Q was a spontaneous process at each pH. Moreover, hydrophobic force was the predominant force at pH 2.0–4.0 due to its positive sign of ΔH and ΔS , because of greater disordering of the organized solvent layers around the G and Q molecules at lower pH [32]. On the other hand, both hydrogen bonds and van der Waals forces were associated with the negative ΔH and ΔS values. High absolute value of ΔH even suggested the creation of covalent bonds between G and Q [33]. Generally, polyphenols could interact with proteins via the formation of a phenolic radical or quinone at alkaline pH, with the formation of covalent bonds. Furthermore, Q also possessed significant hydrophobic groups, which might have contributed to hydrophobic interactions with protein non-polar groups. Above results demonstrated the force of G/Q complex formation was not a single one, but mainly hydrophobic force and hydrogen bonds, which noticeably influenced by pH and was agreement with the previous findings [22,26]. Other complementary techniques would be needed to fully explore the exact interaction types and strengths between G and Q. These results might at different pH change the secondary and tertiary conformation of G, and the speculation would be explored in FTIR and Raman spectroscopy experiments.

3.3. Intermolecular binding distances between G and Q

Fluorescence resonance energy transfer usually results from the transfer of excitation energy from a donor in the excited state (G) to a nearby acceptor (Q) due to dipole–dipole interactions between the molecules. Energy transfer efficiency (E) can be used to evaluate the distance between the binding site of the acceptor and amino acids residue.

The significant overlap of the UV–vis absorption spectra and fluorescence emission spectra of Q were shown in Fig. 2, which suggested possibility of energy transfer between the molecules. As described above, values for J, E, R_0 and r were calculated according to Eqs. (1)–(3) and values were given in Table 2. The reliability of R_0 and r values calculation was evident from the satisfying criteria of $0.5 R_0 < r < 2 R_0$ [24]. Furthermore, r-values were within the range at all pH, smaller than 8 nm indicating non-radiative energy transfer occurring tended to result in a decrease in the fluorescence intensity. Also, larger value of r than that of R_0 supported the static quenching mechanism observed for G and Q interaction [34]. Of note, the order of the complexes interaction distances was pH 3.0 > pH 2.0 > pH 8.0 > pH 5.0 > pH 9.0 > pH 6.0 > pH 4.0 > pH 7.0, which were not consistent with K_q and K_a (Section 3.1). Increasing in molecular distance resulted in a decrease in the interactions force, and consequently decreased the extent of fluorescence quenching. Studies had shown that 3-OH and 4-OH located at the ortho-position of the polyphenol B ring exhibited positive synergistic interaction with proteins, and two hydroxyl groups were easily combined with proteins simultaneously, leading to a high degree to protein fluorescence quenching [14].

However, in our study, pH might have a large impact on the structure and number of multiple phenol groups. For instance, the binding distance and K_a at acidic conditions (pH 2.0 and pH 3.0) were significantly larger than those at neutral and alkaline conditions (pH 7.0 and pH 9.0). This observation might be a result of steric effects, where the binding site was not sufficiently large enough to accommodate the larger phenolic moiety. One hydroxyl group bond to a specific site of the G ($n \approx 1$), and the other group exhibited a steric hindrance effect, which was detrimental to the proximity of protein binding site, causing an increase of the binding distance. Our results suggested that pH was a critical factor that influenced protein-phenolic interactions.

3.4. Synchronous fluorescence spectroscopy

To gain a better insight of the microenvironmental changes around protein fluorophores upon Q binding, synchronous fluorescence spectra of G were recorded in the absence and presence of increasing Q concentrations at different pH values (Fig. 3). When $\Delta\lambda = 15$ nm, about 84% or 86% decrease in the fluorescence intensity at 281 nm and 2 nm red shift were observed at pH 3.0 or pH 5.0 (the Q concentration was 50 $\mu\text{mol/L}$). On the other hand, when $\Delta\lambda = 60$ nm, Q produced ~93% or 94% quenching in G fluorescence intensity at pH 3.0 or pH 5.0, which was accompanied by a 3 nm red (from 276 nm to 279 nm) (Fig. 3). The fluorescence intensity quenching of Trp residue ($\Delta\lambda = 60$ nm) was stronger than that of Tyr residue ($\Delta\lambda = 15$ nm), namely, Trp residue contributed more to the quenching of intrinsic fluorescence, which were related to the setting of wavelength and the relative content of two amino acids. A red shift of maximum emission wavelength indicated the increased exposure of Trp residue to a more polar environment. Furthermore, the quenching extent decreased ($\Delta\lambda = 60$ nm) as the order pH 8.0 > pH 9.0 > pH 7.0 > pH 6.0 > pH 5.0 > pH 4.0 > pH 3.0 > pH 2.0, which further supported by previous analysis (Section 3.1). The results suggested that Trp residue polarity of G might be pH-dependent.

3.5. UV–vis absorption spectroscopy

UV–vis absorption spectroscopy was used to investigating the interactions of G and Q. Generally, the intensity of peak represented the strength of interaction, and the movement of peak position was

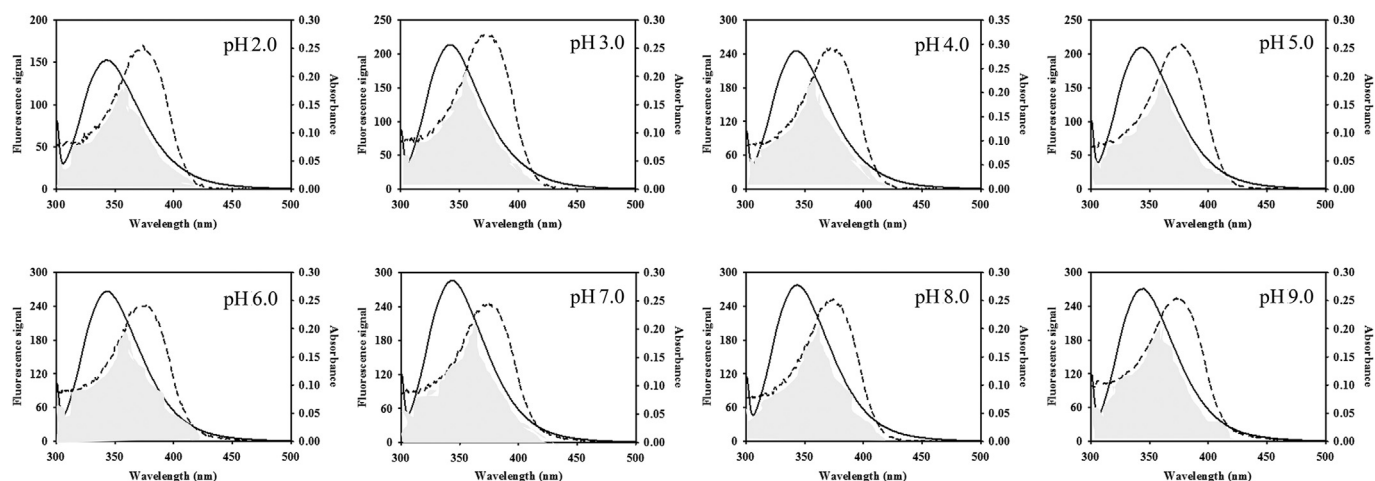


Fig. 2. Spectral overlap (the gray area) between the fluorescence spectrum of wheat gliadin (the solid lines) and absorption spectrum of quercetin (the dotted lines). The concentrations of both were 5 $\mu\text{mol/L}$ each. G: wheat gliadin; G/Q: wheat gliadin and quercetin complexes.

considered to be caused by the conformational change resulting from changes of hydrophobic amino acid residue microenvironment. There was a maximum absorption around 275 nm (Fig. 4A), which could be characterized as the absorption peak of Trp and Tyr residues, as well as absorbance by phenolic groups in Q used.

Absorption peak of G showed hyperchromism with 20 nm (275 $\rightarrow$ 255) blue shift in the absorption maximum in the presence of Q but showed a slight hyperchromism effect at different pH (Fig. 4A and C). The peak of G/Q at 255 nm almost disappeared at pH 9.0, indicating that pH affected the coordination of Q and G. Such changes indicated G/Q complex formation at different pH has led to microenvironmental alterations around protein chromophores. The structure changed in protein resulted in exposure of more Tyr and Trp residues to the surrounding solvent, which was helpful for the π - π^* transition of aromatic heterocycles. An additional absorption peak was observed around 372 nm at different pH, suggesting that during the complexes formation process Q had been oxidized and a protein conformational change might have occurred (Fig. 4C). At alkaline conditions, G/Q complexes exhibited higher absorbance, which was ascribed to the formation of covalent bonds between amino groups or sulphhydryl of the protein and quinones. However, the decrease of the absorbance at pH 9.0 might be associated with the abnormal folding of protein [35]. Additionally, the presence of the absorption near 220 nm in all the samples was ascribed to absorption peak of n - π^* on the peptide bond, which was related to the α -helix content of the protein.

3.6. FTIR spectroscopy

FTIR analysis was further conducted to identify the major interactions responsible for the G/Q complexes formation. The above observations suggested that at pH 3.0, pH 5.0, pH 7.0 and pH 9.0, Q induced more G conformational changes and structural modification, stronger interaction. Therefore, to have a knowledge of the structures and side chains of proteins, the above pH conditions were chosen for FTIR and subsequent Raman spectrum analyses. The infrared self-deconvolution with second derivative resolution enhancement and curve-fitting procedures were used to determine the protein secondary structures at different pH values.

Apart from the broad peak in the range of 3500–3000 cm^{-1} corresponding to the —OH group, G and G/Q complexes showed characteristic peaks around 1660 cm^{-1} (amide I, representative of C=O stretching), 1540 cm^{-1} (amide II, representative of C—N stretching coupled with N—H bending modes) and 1110 cm^{-1} (the stretching vibrations of —C—O— group) [15]. pH induced protein conformational changes (Fig. 5). The peak position of amide II showed a blue shift from pH 3.0 to pH 9.0 (1545 $\text{cm}^{-1} \rightarrow$ 1547 cm^{-1} for G and 1541 $\text{cm}^{-1} \rightarrow$ 1543 cm^{-1} for G/Q), which implied that the pH treatment altered the C—N or N—H bond of G, suggesting the glutamine was deamidated to glutamate at alkaline conditions [36]. Moreover, the intramolecular conformational difference in G/Q complexes implied existing specific interaction mode of G and Q at specific pH

Table 2

Intermolecular binding distances between wheat gliadin and quercetin (G/Q) at different pH.

pH	J ($\text{cm}^3/\text{L/mol}$)	R_0 (nm)	E (%)	r (nm)
2.0	4.80×10^{-14}	3.20	0.20	4.00
3.0	5.31×10^{-14}	3.24	0.19	4.11
4.0	5.69×10^{-14}	3.28	0.38	3.55
5.0	4.98×10^{-14}	3.20	0.26	3.83
6.0	4.94×10^{-14}	3.20	0.30	3.67
7.0	4.99×10^{-14}	3.20	0.41	3.41
8.0	4.95×10^{-14}	3.20	0.24	3.87
9.0	5.29×10^{-14}	3.24	0.28	3.77

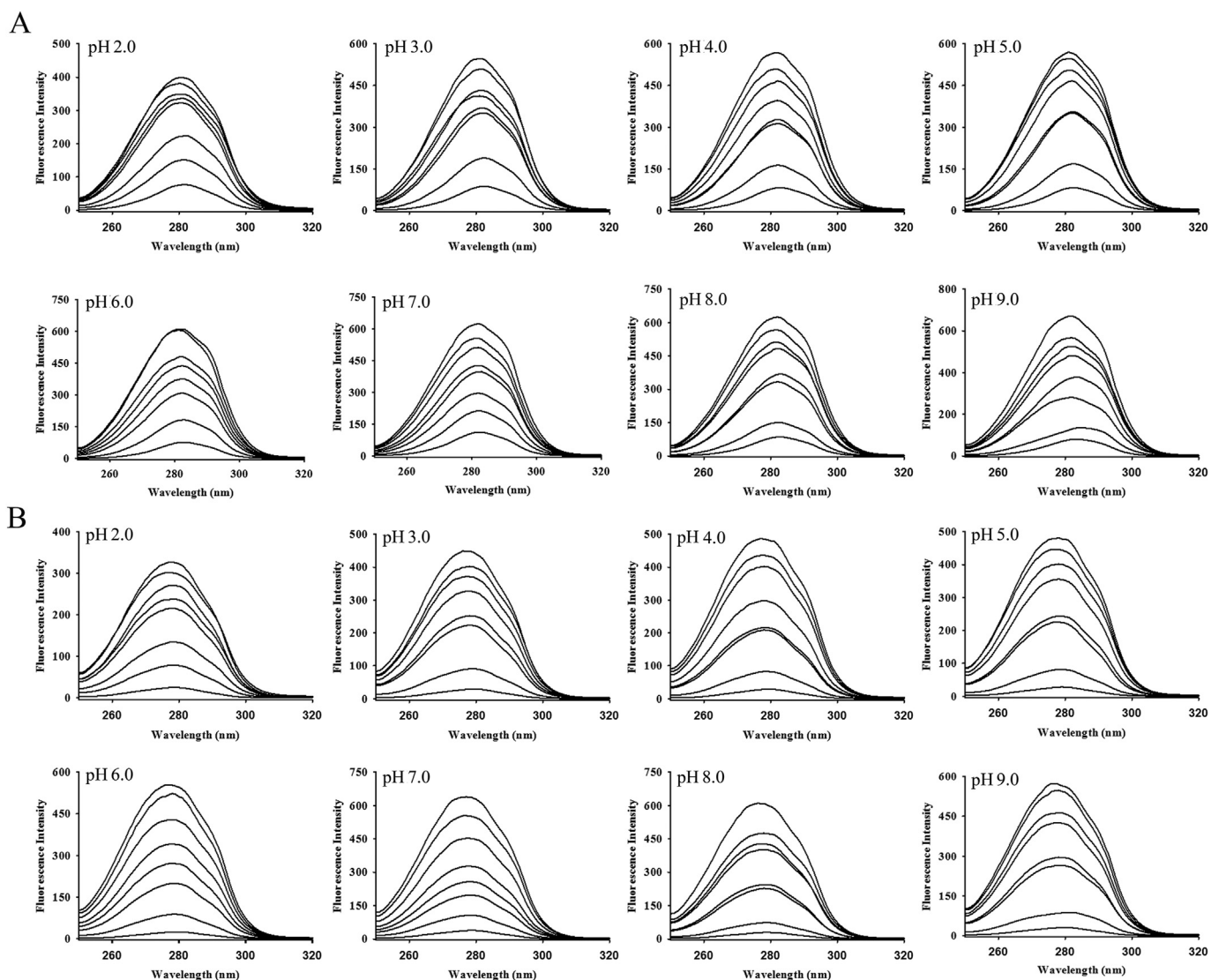


Fig. 3. Effect of quercetin (Q) on the synchronous fluorescence spectrum of wheat gliadin (G). A. The spectrum of tyrosine residue ($\Delta\lambda = 15$ nm). B. The spectrum of tryptophan residue ($\Delta\lambda = 60$ nm).

values. Additional evidence to supported Q interaction with C—N and N—H groups at different pH came from the shifting of the protein amide A band around 3410 cm^{-1} (N—H stretching mode) in G to 3412 cm^{-1} (pH 3.0), 3400 cm^{-1} (pH 5.0), 3362 cm^{-1} (pH 7.0), 3313 cm^{-1} (pH 9.0) upon Q interaction and the peak width became wider at pH 5.0–9.0, indicating the forming of hydrogen bonding, the result was in good agreement with fluorescence spectral analysis (Section 3.2). And Jia et al. [37] speculated that polyphenols were

incorporated into the cross-linked protein as bridging agents at pH 9.0, resulting in covalent interaction. The characteristic peaks at 2960 cm^{-1} corresponded to the stretching vibrations of C—H stretching coupled with hydrophobic interactions. These results demonstrated that G/Q complexes formations mainly were facilitated by non-covalent interactions (hydrogen-bonding or hydrophobic interactions) at pH 3.0, pH 5.0, and pH 7.0, whereas covalent interaction existing at pH 9.0.

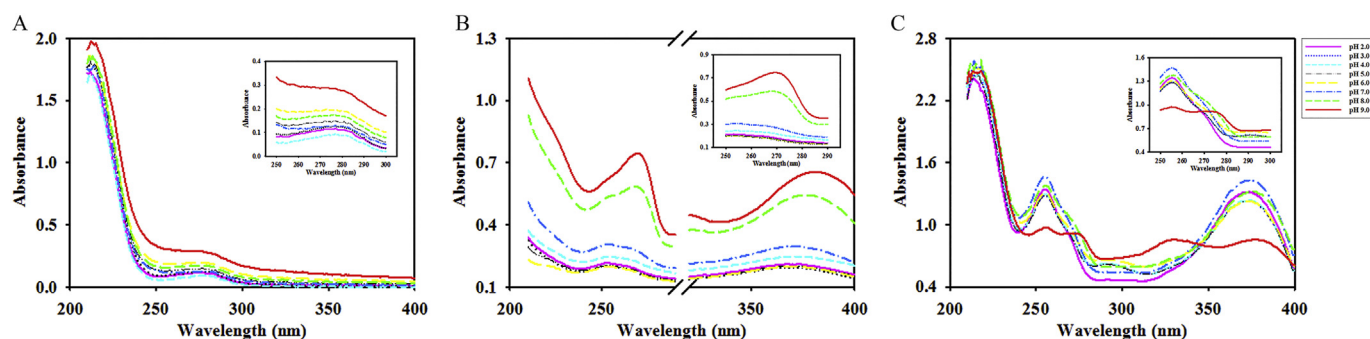


Fig. 4. Changes in the UV-visible absorption spectra. Letter A, B, C represent the spectra of wheat gliadin (G), quercetin (Q), G/Q, respectively.

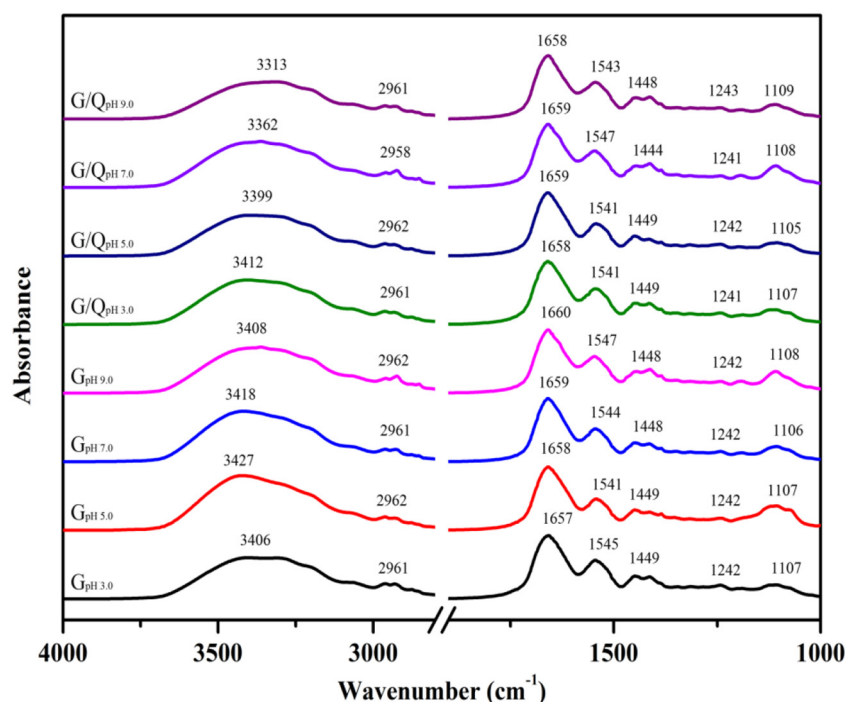


Fig. 5. FTIR spectra of wheat gliadin (G), wheat gliadin-quercetin (G/Q) complexes. The characteristic peaks were around 3410 cm^{-1} (N–H stretching mode), 1660 cm^{-1} (amide I, representative of C–O stretching), 1540 cm^{-1} (amide II, representative of C–N stretching coupled with N–H bending modes) and 1110 cm^{-1} (the stretching vibrations of –C–O–group).

Amide absorption bands on FTIR spectra, i.e., amide I–VII, amide A, and B, were widely used to qualitatively and quantitatively assess protein secondary structures. In particular, amide I band ($1600\text{--}1700\text{ cm}^{-1}$) was considered to be the most reliable indicator. The Fourier deconvoluted spectra of G and G/Q complexes showed several differences at different pH (Supplementary Fig. 1), which indicated that protein undergone conformational modifications upon pH treatment. The contents of α -helices and β -turns were 19.11%, 18.37% for G and 32.25%, 16.22% for G/Q at pH 5.0. Remarkably, higher α -helices content might produce a three-dimensional network with amorphous structure at pH 5.0, thereby lowering extensibility, which was similar to results reported by Linlaud et al. [38] and Liu et al. [39]. β -Turns gave allergen particles a specific conformation. It had been reported that in allergenic tetrapeptides, the presence of proline and serine contributed to the formation of the β -turns conformation [6]. Tozzi, Zanna, & Taddei pointed out that the decrease of the β -turns content could decrease the allergenicity of gliadins [21]. The order of β -turns content was as follows: pH 9.0 > pH 5.0 > pH 7.0 > pH 3.0 (G), pH 3.0 > pH 9.0 > pH 7.0 > pH 5.0 (G/Q). For G/Q complexes, the lower β -turns content at pH 5.0 could decrease the allergenicity of G. Furthermore, pH made the protein surface charge changed, and then affected the stability of electrostatic interaction and hydrogen bond, leading to the decrease of β -sheets content. Particularly, the complexes treated with pH 5.0 enhanced the transition from β -turns and random coil to α -helices and β -sheets, likely as a result of hydrogen bonding and hydrophobic effects [40]. The decrease of β -turns and random coil contents might be related to the bury of non-polar group and hydrophobic group due to a light aggregation of protein [41]. Above mentioned results suggested that pH 5.0 was favourable for the formation of G/Q complexes and this treatment might be an approach to decrease the allergenic power of G, which was in line with K_a analysis (Section 3.2).

3.7. Raman spectroscopy

Raman spectroscopy was further used to gain insight into the environment of specific amino acids (such as Tyr, Trp), and the conformation of disulphide bridges. Phenylalanine (1003 cm^{-1}) was used as a standard to normalize the vibrational intensity of molecular structures.

The Trp band at 760 cm^{-1} has been proposed as an indicator of the hydrophobicity of the environment of the indole ring. The hydrophobicity intensity was as follows: pH 9.0 > pH 7.0 > pH 3.0 > pH 5.0 (Fig. 6A). Studies had proved that the decrease of Raman normalized intensity at acidic conditions was associated with the unfolding of G, resulting in the exposure of hydrophobicity residues [42], and our result was consistent with this study. Furthermore, the spectral intensity at different pH was 0.30 (pH 3.0), 0.31 (pH 5.0), 0.28 (pH 7.0) and 0.59 (pH 9.0) for G and 0.42 (pH 3.0), 0.27 (pH 5.0), 0.44 (pH 7.0) and 0.86 (pH 9.0) for G/Q (Fig. 6A). The decrease in intensity observed at pH 5.0 was related to more exposure of Trp residue. Also, the intensity changed the band at 1340 cm^{-1} , which confirmed a change in Trp environment and was similar to the change at 760 cm^{-1} (except at pH 5.0). The addition of Q at different pH caused strengthening of 760 cm^{-1} and 1340 cm^{-1} , suggesting increment in the “buriedness” of Trp residues in protein, which contributed to the formation of a more ordered structure, and the effect was agreement with that observed by other authors upon the gliadins-anthocyanidins (3-ethoxycarbonylcoumarin and cyanidin) complexes [21].

To evaluate the Tyr environment, the height of bands at about 855 cm^{-1} and 830 cm^{-1} (I_{855} and I_{830}) as obtained by curve fitting were used to calculate the I_{855}/I_{830} intensity ratio. If $I_{855}/I_{830} \geq 1$, Tyr residue was exposed to environment and participated in the formation of hydrogen bonding, in contrast, Tyr residue was embedded inside the protein, i.e. to a more buried state. The I_{855}/I_{830} intensity ratio was the ranges of 1.0–1.3, suggesting that phenolic OH groups acted as both donor and acceptor of moderate hydrogen bonds [43]. Upon treatment

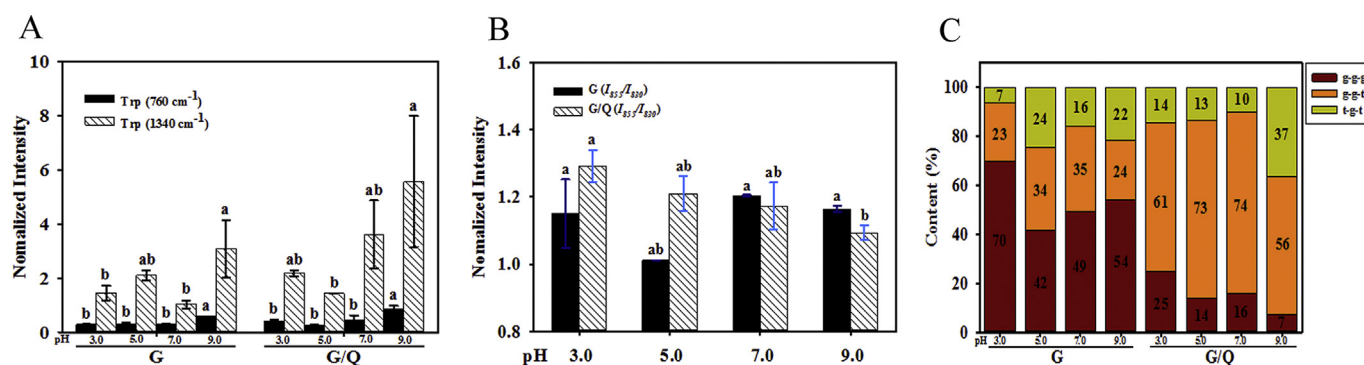


Fig. 6. Raman structure, normalized intensity and the conformation of disulphide bridges of wheat gliadin (G). A. The normalized intensity of tryptophan residue (760 cm^{-1} and 1340 cm^{-1}). B. The intensity ratios of tyrosine residue (I_{855}/I_{830}). Different letters indicate significant differences among different treatment pH for each spectral normalized intensity for G or G/Q, according to Tukey's HSD multiple comparison test ($p < 0.05$). C. Percentages of *gauche-gauche-gauche* (g-g-g), *gauche-gauche-trans* (g-g-t) and *trans-gauche-trans* (t-g-t) $C_{\alpha}-C_{\beta}-S-S-C_{\beta}-C_{\alpha}$ conformations, as obtained by fitting the $500\text{--}550\text{ cm}^{-1}$ Raman spectra of the analyzed samples.

with Q, the I_{855}/I_{830} intensity ratio increased at pH 3.0 and pH 5.0, whereas decreased at pH 7.0 and pH 9.0 (Fig. 6B), indicating that Trp residue trended to an exposed state at acidic conditions and a more buried state at neutral and alkaline conditions. For G/Q complexes, the degree of Trp residue exposed was pH 3.0 > pH 5.0 > pH 7.0 > pH 9.0, suggesting the stronger hydrogen bonding interaction at pH 3.0 and pH 5.0. These observations were also revealed in the analysis of synchronous fluorescence spectra (Section 3.4).

The contents of *gauche-gauche-gauche* (g-g-g), *gauche-gauche-trans* (g-g-t) and *trans-gauche-trans* (t-g-t) $C_{\alpha}-C_{\beta}-S-S-C_{\beta}-C_{\alpha}$ conformations were determined from the areas of bands at $500\text{--}510\text{ cm}^{-1}$, $515\text{--}525\text{ cm}^{-1}$ and $535\text{--}545\text{ cm}^{-1}$, respectively. The content of each conformation was calculated from the area of the individually assigned bands and expressed as fraction of the total area of the above-mentioned bands. It found that g-g-g was the lowest potential energy conformation (about 508 cm^{-1}), while g-g-t and t-g-t conformations were detected as shoulders [6], our results were consistent with it. The quantitative data (Fig. 6C) obtained by curve fitting (Supplementary Fig. 2) suggested that the treatment with Q induced a conformational rearrangement that involved an increase in g-g-t (such as from 23% to 61% at pH 3.0), a significant decrease in g-g-g (such as from 70% to 25% at pH 3.0) and a decrease of the content of t-g-t at pH 5.0 (from 24% to 10%) and at pH 7.0 (from 16% to 10%). An analogous decrease of the g-g-g conformation had been reported upon treatment of gliadins with malvin and oenin, respectively [6]. Ferrer et al. [43] had confirmed that g-g-g and g-g-t were typical inter-bond and intra-bond disulphide conformation. For G/Q complexes, content of g-g-g decreased indicating the inter-bond disulphide conformation was broken, and resulting in decrease of the disulphide bridges stability. The lower content of g-g-g at pH 9.0 might be related to the abnormal folding of protein and the disorder shift of polypeptide chain due to subunit aggregation, as reported by previous study [35]. Above observations suggested that pH conditions appeared to significantly influence the conformation of protein and the interaction of G/Q.

4. Conclusion

The study found that the quenching of wheat gliadin (G) by quercetin (Q) was governed by both dynamic and static quenching mechanism at pH 2.0–9.0. Hydrophobic force was the predominant force at pH 2.0–4.0, whereas hydrogen bonds and van der Waals forces at pH 5.0–9.0. Results demonstrated secondary and tertiary structural alterations and microenvironmental (Trp and Tyr residues) changed around protein fluorophores upon complexation with Q, as indicated by FTIR and the Raman spectra along with synchronous fluorescence spectra. Furthermore, lower β -sheets content at pH 5.0 might decrease the allergenicity of G. These results would be of importance for the development of wheat gliadin-based functional products enriched with

quercetin under different pH values. Nevertheless, further research is needed to evaluate the effect of Q modification on other functionalities of G under different pH conditions to develop their applications in food industries.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.saa.2019.117937>.

Author contributions

Qiming Wang: carried out the experiment, analyzed and interpreted the experimental data, and drafted the article. Yuwan Tang and Yaxuan Yang: helped collating experimental data. Yuhao Zhang, Lin Li, and Qiang Wang: designed the experiment. Jian Ming and Jichun Zhao: helped interpreting the data and revising the articles.

Declaration of competing interest

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

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