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Gliadins as a potential carrier of health-promoting phenolic acids: fluorescence study of gliadin – phenolic acids complexation**

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Abstract. Due to the health benefits attributed to the consumption of phenolic acids, these compounds can be used as natural pro-health food or beverages ingredients. To improve polyphenols stability during food processing and storage, they could be encapsulated in colloidal systems *e.g. via* complexation with proteins. The process of gliadin protein and phenolic acids complexation was investigated with application of fluorescence spectroscopy. Strong fluorescence quenching of gliadin was observed as a result of phenolic addition and two types of quenching mechanism were determined: dynamic (for coumaric acid) and static (for caffeic, ferulic and sinapic acid). This result indicates that coumaric acid, unlike to other analysed acids, does not form complexes with gliadin. The binding constant for caffeic, ferulic and sinapic acid – gliadin interaction is between 0.4 and 8.7 x 10^3 M^{-1}. These three acids induced changes in protein conformation and tryptophan microenvironment. The calculated number of binding sites suggests that caffeic, ferulic and sinapic acids interact with gliadin to form 1:1 complexes. Analysis of thermodynamic parameters suggested that van der Waals and/or hydrogen bonding interactions play major role in the interactions between caffeic, ferulic as well as sinapic acid and gliadin.

Keywords: gliadin, phenolic acids, fluorescence quenching complex formation, binding constant, binding sites

1. INTRODUCTION

Phenolic acids are ubiquitous group of bioactive compounds present in various plant sources such as vegetables, fruits or cereals (Karasawa and Mohan, 2018; Stuper-Szablewska and Perkowski, 2019). As secondary metabolites they are distributed in different parts of plants and affect plant`s color, flavor and taste as well as astringency which contribute to the sensory and organoleptic properties of the food products. Phenolic acids have attracted attention due to their numerous beneficial biological functions. Due to their strong antioxidant activity they exhibit anti-inflammatory, anti-allergic, cardioprotective or anticancer properties (Rashmi and Negi, 2020). It is reported that phenolic acids possess much higher *in vitro* antioxidant activity than common antioxidant vitamins (Tsao and Deng, 2004). Due to the beneficial effects of polyphenols on human health, they can be considered as natural ingredients used to create functional food or beverages either in the forms of natural extracts or as pure compounds. One of the method to improve the stability of these pro-health compounds could be their complexation with compounds which are commonly found in food products.

Gliadins account for 40-50% of the total storage protein of wheat and can be extracted from wheat gluten with 70% ethanol (Urade *et al*., 2017). Based on electrophoretic mobility, they are classified as α-/β-, γ- and ω-gliadins with their molecular weight ranging from 30 to 88 kDa (Barak *et al.*, 2015). One of the unique feature of gluten proteins is that they contain exceptionally high amounts of glutamine (26-53%) and proline (10-29%) (Sivam *et al.*,

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2010). Proteins rich in proline can form complexes with polyphenols and the stability of these complexes as well as binding constant depend on the type of polyphenol. For example, quercetin can interact with gliadin reversibly *via* hydrophobic interaction or hydrogen bonds, depending on pH, whereas some procyanidins form stable complexes with these proteins (Dias *et al.*, 2015; Wang *et al.*, 2020). Insoluble complexes were obtained as a result of interactions of gliadin and polyphenol extracts from artichoke leaves, cranberries and apples (Pérot *et al.*, 2017).

Protein contains three aromatic amino acids which play role of natural fluorophores: phenylalanine, tyrosine and tryptophan. Intrinsic fluorescence of proteins in solution is commonly measured in order to detect changes in protein conformation. Fluorescence quenching is the decrease of the fluorescence quantum yield of fluorophore caused by some interaction with external quencher molecule that induces rapid deexcitation of excited electronic state. In this study, we consider fluorescence quenching originating from: collisions between fluorophore and quencher (dynamic quenching), formation of the complex between fluorophore and quencher (static quenching) as well as quenching caused by resonance energy transfer (FRET) between fluorophore and quencher (Lakowicz, 2006).

The aim of the experiment was to determine the magnitude and nature of interactions between phenolic acids and gliadin. Polyphenols can can form covalent or/and noncovalent bonds with proteins. One of the major factor influencing the ability of hydrophobic proteins to bind other compounds is their biding affinity. Four phenolic acids belonging to hydroxycinnamic group were analysed: coumaric, caffeic, ferulic and sinapic acid. Complexation of phenolic acids with gliadin was studied with application of steady-state fluorescence spectroscopy based on the protein fluorescence quenching.

A comprehensive understanding of the complexation process between proteins and phenolic acids is essential to improve the functional properties as well as biological activity of these compounds and expand their application in the food field. The gliadin – phenolic acids complexes can be regarded as a natural system that stabilizes polyphenol molecules and protects these sensitive compounds during food processing and storage. Moreover, the good biological adhesion of gliadins in the human intestinal tract makes them effective carrier of active compounds. Gliadin-phenolic acids complexes could be applied in emulsions, films, gels or other active substance delivery systems.

2. MATERIAL AND METHODS

2.1. Materials

Wheat gliadins, p-coumaric, caffeic and ferulic acids were purchased from Merck (Poland). Sinapic acid was purchased from Apollo Scientic (UK). Ethanol was obtained from Avantor (Poland). All reagents were at least of analytical grade.

2.2. Samples preparation

Commercially available wheat gliadins were dissolved in 70% (v/v) ethanol. Assuming an average molecular weight of gliadin (40 kDa), the protein concentration in the stock solution was 600 μM. For fluorescence measurement, the concentration of protein was fixed (16 μM) and increasing volume of phenolic acids (quenchers) was added to gliadin. We set the gliadin concentration to 16 μM, so that the absorbance of protein did not exceed 0.1 and quality of protein emission spectrum was satisfying. If absorbance intensity is higher than 0.1, process of self-absorption can occur in the system (Lakowicz, 2006). Phenolic acids were added to gliadins in the following concentrations: 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 μM.

Before fluorescence measurement, the protein solution without phenolic acids (control sample) as well as mixtures of gliadin-phenolic acids were incubated with continuous stirring at desired temperature (293 K, 303 K, 313 K) for 20 min.

2.3. Steady state fluorescence

Fluorescence emission spectra were collected using FluoroMax-4P spectrofluorometer (Horiba Jobin Yvon, USA) equipped with water thermostatted cell holder and magnetic stirrer. The excitation wavelength was set at 280 nm (tryptophan excitation) and the slits of both monochromators were set to 5 nm. Measurements were performed at three temperatures: 293 K, 303 K and 313 K. Fluorescence emission spectra were analysed with application of ORIGIN software (v.9.0 PRO, OriginLab Corporation, USA). Samples containing different concentrations of acids were prepared separately (not by adding successive portions of acid to the same protein sample) and measured at 293 K, 303 K and 313 K. The experiments were repeated three times.

2.4. Determination of binding and thermodynamic parameters

The Stern-Volmer (K_{SV}) quenching constants were determined using the Stern-Volmer equation (Lakowicz, 2006):

$$\frac{F_0}{F} = 1 + K_{SV}[P] = 1 + \tau_0 k_q [P], \quad (1)$$

where: F_0 and F refers to the gliadin fluorescence intensity in the absence and in the presence of phenolic acid, respectively, $[P]$ is the concentration of phenolic acid, τ_0 is the

average fluorescence lifetime of fluorophore (tryptophan) in absence of quencher and k_q is the biomolecular quenching constant.

The static quenching constant K (binging constant) and binding site number, n, were calculated from the following relationship (Acharya *et al.*, 2013):

$$\log\frac{F_0-F}{F} = \log K + n\log\,[P]. \quad (2)$$

Changes in entropy (ΔS) and enthalpy (ΔH) were determined based on Vant`t Hoff equation (Joye *et al.*, 2015):

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}, \quad (3)$$

where: R is the gas constant and T is temperature. The change in free energy (ΔG) can be estimated from (Wang *et al.*, 2015):

$$\Delta G = \Delta H - T\Delta S. \quad (3)$$

2.5. Statistical analysis

Fluorescence emission spectra were recorder for each gliadin-phenolic acid system in triplicate. Based on the spectra, F_0/F and log $[(F_0\text{-}F)/F]$ parameters were calculated, averaged and average parameters with standard deviation error bars were used to obtain Stern-Volmer and Van`t Hoff plots. Linear function was fitted to a scatter plots in order to calculate binding constants, number of binding sites and thermodynamic parameters.

3. RESULTS AND DISCUSSION

3.1. Fluorescence quenching studies and quenching mechanism analysis

Proteins contain three aromatic amino acids which are responsible for their natural intrinsic ultraviolet fluorescence: tyrosine, phenylalanine and tryptophan. Due to efficient excitation energy transfer, the emission of protein is originating mainly from the tryptophan residues (Lakowicz, 2006). Figure 1 shows the fluorescence emission spectra of the gliadin ethanolic solutions where a strong fluorescence band at 348 nm occurs. This band is assigned to tryptophan moiety of the gliadin protein and its location is in agreement with our previous studies (Welc *et al.*, 2022b). Fluorescence emission spectra were also recorded for gliadin-phenolic acid systems as a function of increasing phenolic acids concentration. As can be noticed, upon addition of phenolic acids to the gliadin solution, their fluorescence intensity strongly decreased. There are many molecular interactions between fluorophore (tryptophan) and quencher (phenolic acid) which can be responsible for such fluorescence quenching, including ground-state

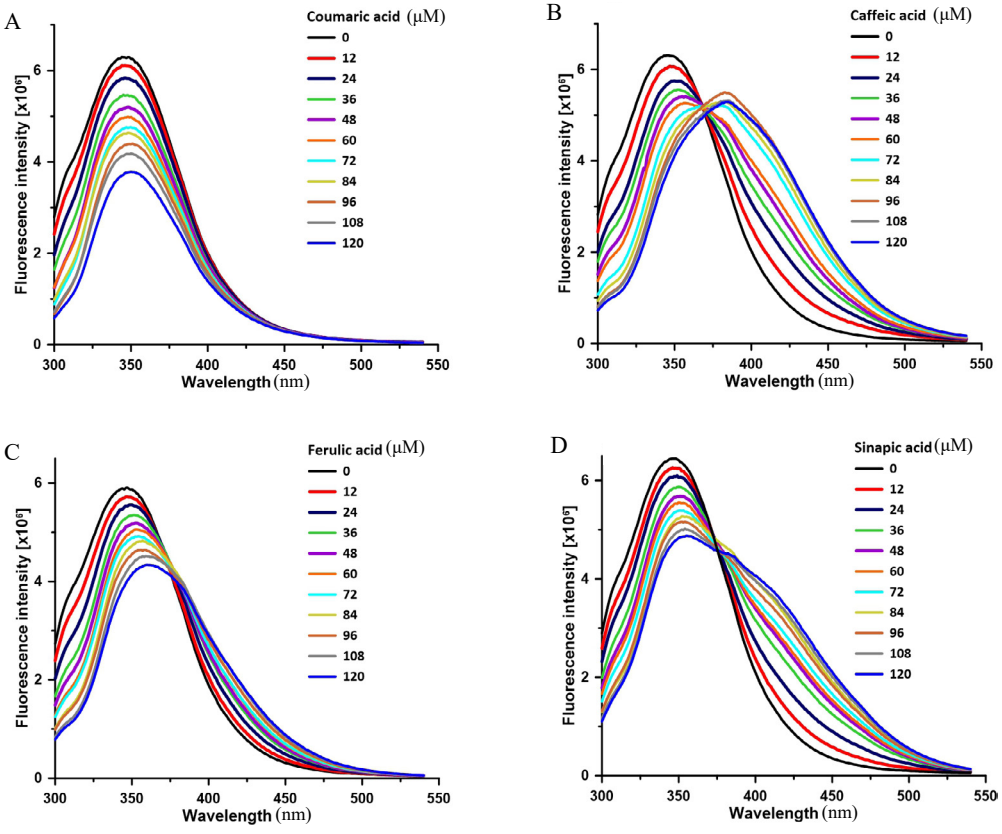

Fig. 1. Fluorescence emission spectra of gliadin dissolved in ethanol 70% in the presence of increasing phenolic acids concentration at 293 K. Phenolic acid concentration (a-k): 0, 12, 24, 36, 48, 60,72 ,84, 96, 108 and 120 µM. The analysed spectra were averaged over spectra registered in three independent experiments.

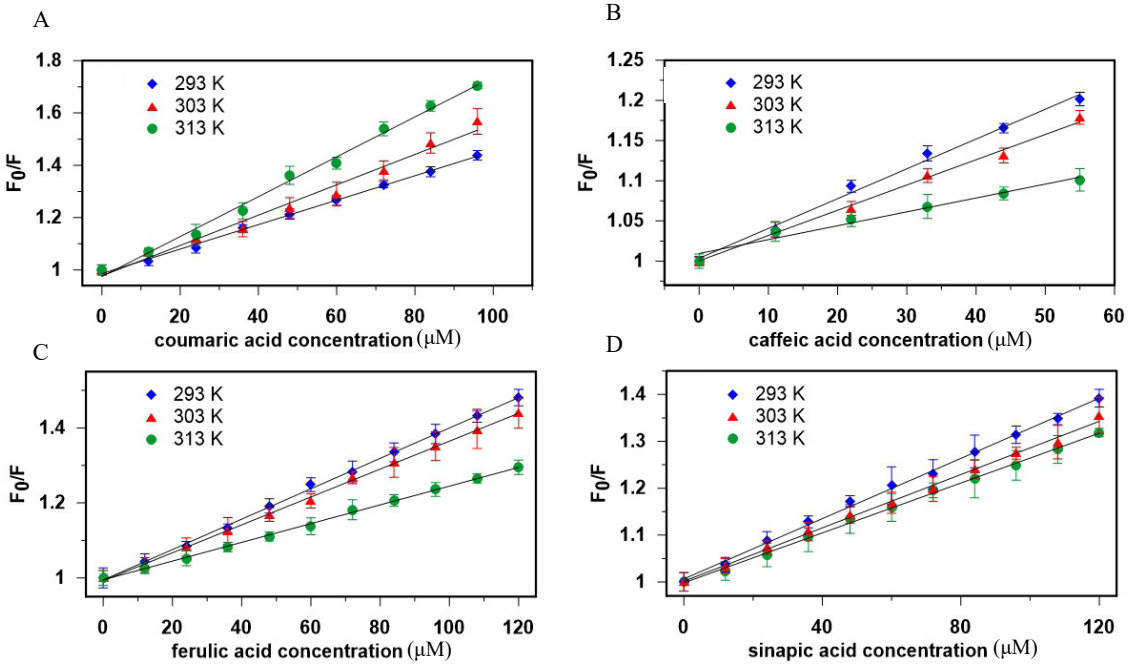

Fig. 2. Stern-Volmer plots describing gliadin fluorescence quenching caused by phenolic acids at three different temperatures. F_0 and F represent gliadin fluorescence intensity in the absence and presence of phenolic acids, respectively. Only the fluorescence spectra that had a distinguishable maximum of protein emission bands were analysed according to the Stern-Volmer equation. At higher phenolic acids concentration maximum of gliadin emission band disappears (see Fig. 1).

complex formation (static quenching), excited-state collisional quenching (dynamic quenching) or resonance energy transfer (Wang *et al.*, 2015). In order to determine the quenching mechanism of gliadin induced by phenolic acids, the fluorescence quenching data were analysed using Stern-Volmer relationship (Eq. (1)). Since at higher phenolic acids concentration maximum of protein emission band disappears (Fig. 1), only the fluorescence spectra that had a distinguishable maximum were analysed according to the Stern-Volmer equation. Based on this equation, K_{SV} can be obtained from the slope of a plot F_0/F as a function of [P]. Stern-Volmer plots as a function of phenolic acids concentration at three different temperatures are presented in Fig. 2. As can be noticed in the case of all analysed phenolic acids-gliadin systems, Stern-Volmer plots are linear function of phenolic acids concentration. The linearity indicates that in the system containing gliadin and analysed phenolic acids only one type of quenching mechanism is dominating (Ghali, 2010). To distinguish between static and dynamic quenching mechanisms, analysis of gliadin fluorescence quenching were performed at three different temperatures. The values of constant quenching and biomolecular quenching calculated for phenolic acids – gliadin interactions are presented in Table 1.

Figure 2A shows that in the case of gliadin-coumaric acid system, an increase in F_0/F of gliadin fluorescence with coumaric acid concentration at higher temperatures is observed. The calculated K_{SV} constants increase from 4.67 x 10^3 at 293 K to 7.62 x 10^3 at 313 K (Table 1). These results suggest that gliadin fluorescence is quenched via dynamic mechanism because higher temperatures result in faster diffusion and hence larger amount of collisional quenching. Biomolecular quenching constant k_q can be calculated from Eq. (1) assuming that tryptophan average fluorescence lifetime (τ_0) in ethanol is about 5 ns (Albani,

Table 1. Stern-Volmer quenching constants and biomolecular quenching rate constants for the interaction of gliadin with phenolic acids at various temperatures

Phenolic acid	T (K)	K_{SV} (x10^3 M^{-1})	k_q (x10^{11} $M^{-1}s^{-1}$)	R
Coumaric	293	4.670	9.342	0.998
	303	5.812	11.621	0.991
	313	7.621	15.211	0.996
Caffeic	293	3.745	7.413	0.991
	303	3.192	6.231	0.992
	313	1.737	3.492	0.969
Ferulic	293	4.810	9.612	0.994
	303	4.413	8.862	0.995
	313	2.916	5.854	0.992
Sinapic	293	4.286	8.531	0.997
	303	3.879	7.589	0.996
	313	3.556	7.112	0.997

2014a). The value k_q obtained for gliadin-coumaric acid system was in the range between 9 and 15 x 10^{11} M^{-1} s^{-1}. It is reported that for dynamic quenching the maximum scattering collision quenching constant is about 1 x 10^{10} M^{-1} s^{-1}. Values of k_q smaller than the diffusion-controlled value can result from steric shielding of the fluorophore or a low quenching efficiency. Apparent values of k_q larger than the diffusion-controlled limit usually indicate some type of binding interaction (Lakowicz, 2006). Since k_q value obtained for gliadin-coumaric acid system is higher than typical for dynamic quenching mechanism, it is possible that due to collisions between coumaric acid and tryptophan, some kind of "temporary or transient complexes" are formed between these molecules. As can be also noticed, addition of coumaric acid to solution of gliadin does not change position of protein fluorescence emission band (Fig. 1A), which suggests that there is no binding reaction between protein and phenolic acid. It is well known that tryptophan is highly sensitive to its microenvironment and even subtle change in the microenvironment polarity results in shift of tryptophan band towards longer or shorter wavelengths (Lakowicz, 2006). The absence of this kind of shift indicates that the addition of coumaric acid does not induce conformational changes within gliadin protein and tryptophan microenvironment remains unchanged. This result is in agreement with our previous studies, which showed that coumaric acid is weakly bound to gluten network and their extractability is the highest compared to other acids within hydroxycinnamic group (Welc *et al.*, 2022a).

In the case of caffeic acid (Fig. 2B), the result showed that Stern Volmer quenching constant is inversely proportional to temperature and decreases from 3.74 to 1.74 x 10^3 as the temperature increases (Table 1). This observation is an experimental evidence that the static mechanism is the most probable interaction responsible for gliadin fluorescence quenching. Static quenching is related to the situation when the complex between quencher and fluorophore is formed in the ground electronic state of fluorophore (Wang *et al.*, 2015). Additionally, biomolecular quenching constant value was larger than the maximum diffusion-limited also indicates some type of binding interaction between caffeic acid and gliadin protein. These results suggest that the complex between gliadin and caffeic acid is formed directly after acid addition. At the same time, we observed a strong shift of gliadin fluorescence emission maximum towards longer wavelength (red shift) as a result of interaction with caffeic acid (Fig. 1B). In the sample containing caffeic acid at concentration 60 μM, the protein emission maximum is shifted from 345 nm to 356 nm. This red shift is likely due to conformational changes in gliadin caused by the formation of the caffeic acid-gliadin complexes, leading to the exposure of tryptophan to a more hydrophilic environment (Albani, 2014b). At caffeic acid concentrations higher than 60 μM, the emission maximum associated with tryptophan disappeared and a new maximum appears around 385 nm, corresponding to the spectrum of the caffeic acid, suggesting that tryptophan fluorescence of gliadin is mostly quenched by phenolic acid. The spectra of fluorescence emission of caffeic acid is presented in Fig. S2b in the Supplementary Material. Static fluorescence quenching and a large red shift in the emission spectra of gliadin can be connected with Forster resonance energy transfer (FRET). Overlapping electron levels of tryptophan and caffeic acid allow for efficient excitation energy transfer between these molecules (Lakowicz, 2006). The red shift towards wavelengths longer than 360 nm cannot be consistent with tryptophan fluorescence (Joye *et al.*, 2015). Since the sample was excited at 280 nm, which corresponds the maximum of gliadin absorption, the presence of emission band associated with phenolic acid could be explained as excitation energy transfer between gliadin and caffeic acid. Such a strong fluorescence quenching and a large red shift due to energy transfer were also observed in the case of gliadin-resveratrol and β-lactoglobulin-resveratrol complexes (Joye *et al.*, 2015; Liang *et al.*, 2008).

Stern-Volmer plots, describing interactions between gliadin and ferulic acid at three different temperatures are presented in Fig. 2C. As can be seen, the slope of a plot F_0/F versus acid concentration decreases with temperature. The calculated K_{SV} values decrease from 4.81 x 10^3 at 293 K to 2.91 x 10^3 at 313 K which indicates that ferulic acid and tryptophan in gliadin form ground-state complexes and the fluorescence is quench via static mechanism (Lakowicz, 2006). The maximum of the gliadin emission fluorescence band is shift towards longer wavelength as a result of interaction with ferulic acid. In the sample containing ferulic acid at concentration 108 μM, the protein emission maximum is shifted from 345 to 358 nm and the band at about 380 nm starts appearing. The band at 380 nm is assigned to ferulic acid (Fig. S2c) and its presence can indicate excitation energy transfer between protein and acid. Similar phenomenon was observed when higher concentration of caffeic acid was added to gliadin solution (Fig. 1B).

In the case of sinapic acid, the mechanism of fluorescence quenching was similar to caffeic and ferulic acids. The calculated K_{SV} values decrease with increasing temperature and the protein emission maximum is shifted from 345 to 355 nm for the sample containing sinapic acid at concentration 96 μM. Additionally, as in the case of caffeic and ferulic acids, the new band at 410 nm appears, which can be assigned to free sinapic acid fluorescence emission (Fig. S2d in the Supplementary Material). This band is probably associated with excitation energy transfer from tryptophan to free sinapic acid molecules. As mentioned above, overlapping electron levels of tryptophan and phenolic acids may result in efficient excitation energy transfer between these molecules.

In general, among the group of analysed hydroxycinnamic acids, three of them (caffeic, ferulic, sinapic acids) reveal similar mechanism of interactions with gliadin.

They form complexes with protein in the ground state and quench fluorescence *via* static mechanism. Moreover, the microenvironment of tryptophan changes towards more hydrophilic which suggest unfolding protein and exposure of tryptophan to ethanolic solution. At higher concentration of ferulic, caffeic and sinapic acids, excitation energy transfer occurs between protein and acid. It is possible, that after protein unfolding, phenolic acids locate in close distance from exposed tryptophan molecules and quench their fluorescence by energy transfer. FRET occurs when the donor and energy acceptor are located in the distance 2-10 nm (Lakowicz, 2006). Interestingly, coumaric acid quenched gliadin fluorescence by collisions with tryptophan and did not change the protein conformation. It is difficult to explain unambiguously differences in behaviour of these four acids belonging to one hydroxycinnamic group. However, in our previous papers we showed that changes in the secondary structure or gluten protein can be connected with chemical structure of the acids (Kłosok *et al.*, 2021, 2022; Welc *et al.*, 2022a).

3.2. Binding parameters of phenolic acids to gliadin

As showed above, a complex between gliadin and ferulic, caffeic and sinapic acids is formed in ground state of protein and gliadin fluorescence is quenched as a result of static mechanism. Therefore, it can be assumed that independent and equivalent binding sites occurs in the fluorescent macromolecule in these systems. The binding constant (K) and the number of binding sites (n) were determined from Eq. (2), as the linear regression of the plot $log\frac{F_0-F}{F}$ against $log\,[P]$. Figure 3 shows the plots of $log\frac{F_0-F}{F}$ versus $log\,[P]$ for gliadin-phenolic acid systems at three different temperatures. The calculated parameters are presented in Table 2. Since coumaric acid does not chemically bind to the protein and does not form stable complex with gliadin, binding constant and the number of binding sites were not calculated for this system. As can be noticed in Fig. 3, in the case of caffeic, ferulic and sinapic acids, the binding constant decreases as a function of temperature, which indicates that the formation of gliadin-phenolic acids complexes is less favourable and the complex stability decreases. The decreasing trend of K constant with increasing temperature is in accordance with K_{SV} dependence on temperature as showed above. The calculated K constant for caffeic, ferulic and sinapic acids is in the range 0.4-8.7 x 10^3 M^{-1} (Table 2). Joye *et al.* (2015) analysed binding between gliadin and another polyphenol – resveratrol and showed that constant binding value is about 10 times higher (Joye *et al.*, 2015). This discrepancy may be connected with difference in chemical structure of resveratrol and phenolic acids. Resveratrol consists of two phenolic rings bonded together by a double styrene bond which is responsible for *cis* and *trans* isomerization whereas phenolic acids contain only one aromatic ring and additional methylene functional groups. On the other hand, our results are in agreement with Dufour and Dangles (2005) who analysed flavonoid-bovine serum albumin (BSA) complexation. They showed that binding constant values for flavonoids such as rutin, genistein or naringenin are similar to those obtained in our studies for phenolic acids. Interestingly, since BSA is typical transport protein, a higher binding constant could

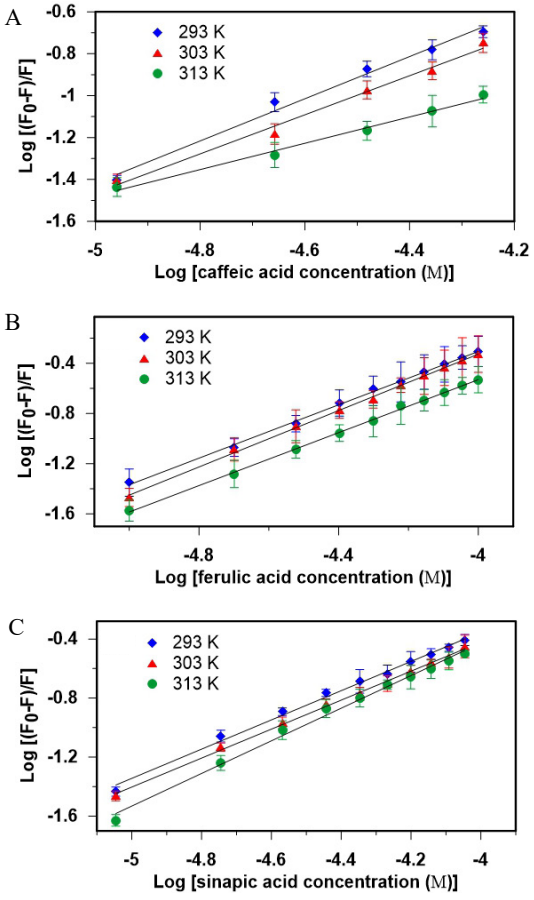

Fig. 3. Plots of log $[(F_0\text{-}F)/F]$ as a function of log of phenolic acids concentration at three different temperatures. F_0 and F represent gliadin fluorescence intensity in the absence and presence of phenolic acids, respectively.

Table 2. Binding constants (K) and number of binding sites (n) of competitive experiments of phenolic acids-gliadin system

Phenolic acid	T (K)	K (x10^3 M^{-1})	n	R
Caffeic	293	2.290	1.007	0.984
	303	1.263	0.933	0.986
	313	0.446	0.627	0.983
Ferulic	293	8.723	1.162	0.995
	303	5.811	1.123	0.996
	313	4.817	1.055	0.996
Sinapic	293	3.981	0.996	0.993
	303	1.126	0.992	0.997
	313	0.988	0.901	0.994

be expected in this system. According to the Authors, this range of K values indicates that ligands are bond reversibly and exhibit moderate affinities to protein. It is reported that if $K > 10^5$ M^{-1}, the interaction between ligand and target can be considered as strong (Butkus *et al.*, 2016). Therefore, it can be concluded that caffeic, ferulic and sinapic acid do not form strong bonds such as covalent bonds during complexation with gliadin proteins.

The values of binding sites (n) for gliadin protein, which were obtained from the slope of the linear function presented in Fig. 2, are approximately equal to one (Table 2). This result indicates that there is one type of available binding site for caffeic, ferulic as well as sinapic acids to gliadin. Moreover, it can be noticed that n value decreases with increasing temperature, which is in agreement with the trend of constant binding and confirm less favourable complex formation between gliadin and phenolic acids at higher temperatures. The value of n equal to 1 was obtained for gliadin – resveratrol and zein - resveratrol interactions (Joye *et al.*, 2015).

3.3. Thermodynamic parameters and binding forces

The interaction forces between small organic molecules such as phenolic acids and biomacromolecule such as proteins includes hydrophobic force, hydrogen bonds formation, van der Waals interaction, electrostatic interactions *etc*. According to Ross and Subramanian (1981), the signs and magnitudes of the thermodynamic parameters (ΔH and ΔS) can be associated with various individual types of interactions (Ross and Subramanian, 1981). Enthalpy change (ΔH) and entropy change (ΔS) in the reaction of gliadin with phenolic acids were calculated from Eq. (3). Van`t Hoff plots for interaction between protein and caffeic, sinapic and ferulic acids are presented in Fig. S1 in the Supplementary Material. $\Delta H$ was obtained from the slope of the Van`t Hoff relationship. The free energy change was estimated from Eq. (4). The thermodynamic parameters obtained for gliadin-phenolic acids interaction at different temperatures are presented in Table 3. As shown in Table 3, ΔH and ΔS for the binding reaction between gliadin and phenolic acids are negative values which indicates that the formation of phenolic acids-protein complexes is an exothermic reaction. The negative ΔG value shows that the binding between gliadin and caffeic, ferulic and sinapic acid occurs spontaneously. Additionally, if $\Delta S<0$ and $\Delta H<0$, van der Waals and hydrogen bonding interactions play major role in the reaction. Moreover, this result also suggest that the binding of caffeic, ferulic and sinapic acid to gliadin is enthalpy-driven and the favourable enthalpy change is offset partially by unfavourable entropy change (Ross and Subramanian, 1981). The interaction *via* hydrogen bonds formation was observed for zein-resveratrol complex formation or buttermilk protein – resveratrol combination (Joye *et al.*, 2015; Ye *et al.*, 2013). It is reported that higher abundance of hydroxyl groups, which is observed in bigger polyphenols, provides multiple sites for interaction and strengthen interactions with proteins (Xiao *et al.*, 2011).

In general, thermodynamic parameters suggest that the interaction between gliadin protein and ferulic, caffeic and sinapic acids can be mainly cause by van der Waals forces or/and hydrogen bonds. These interactions contributed to the stability of complex formed between these molecules as evidenced by the decrease of entropy value. The formation of hydrogen bonds between phenolic acids and gliadin polypeptide chains was confirmed by our previous experiments as well as by studies of Kłosok *et al.* (2022), Krekora *et al.* (2020) and Welc *et al.* (2022b).

4. CONCLUSIONS

Steady-state fluorescence analysis showed that phenolic acids belonging to hydroxycinnamic group can interact with gliadin in two ways. Coumaric acid quenched gliadin emission through a dynamic mechanism, as evidenced by the increasing values of the Stern-Volmer constant quenching with increasing temperature. This acid does not affect the tertiary structure of protein and tryptophan microenvironment. Caffeic, ferulic and sinapic acid formed complex with gliadin in the ground electronic state of

Table 3 Thermodynamic parameters of phenolic acids-gliadin interaction determined from linear Van`t Hoff plot: entalphy change (ΔH), entropy change (ΔS) and free energy change (ΔG)

Phenolic acid	T (K)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})	R
	293			-18.872	
Caffeic	303	-62.304	-148.235	-17.395	0.999
	313			-15.908	
	293			-22.112	
Ferulic	303	-30.187	-27.536	-21.884	0.999
	313			-21.563	
	293			-21.201	
Sinapic	303	-52.773	-136.331	-18.836	0.950
	313			-16.472	

protein. The calculated Stern-Volmer quenching constants were inversely proportional to temperature. The maximum of fluorescence band was strongly shifted towards longer wavelength indicating exposition of tryptophan to hydrophilic environment. The values of binding constants calculated for these three acids indicate reversible interactions between these molecules and gliadin. In the case of this three systems, the number of binding sites for gliadin was equal to 1 which suggest the presence of only one type of bidging site. Thermodynamic parameters obtained from Van`t Hoff plot equation showed, that phenolic acids-protein complexes formation is an exothermic reaction and binding process occurs spontaneously. Additionally, van der Waals and/or hydrogen bonding interactions play major role in the reaction between gliadin and caffeic, ferulic as well as sinapic acid.

Credit Author Statement:

Renata Welc-Stanowska: Conceptualization, Methodology, Investigation, Formal analysis, Writing-original draft, Writing review and editing
Konrad Kłosok: Investigation
Agnieszka Nawrocka: Conceptualization, Methodology, Supervision, Writing review and editing, Funding acquisition

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