Effect of pH on the Interaction of Major Phenolic Compounds in Apple Juice with Gliadin

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Abstract. The effect of interactions between polyphenolic substances (Chlorogenic acid, epicatechin and gallic acid) and gliadin under different pH (pH 3.8/7.0) were analyzed by fluorescence spectrum. The results showed that adding polyphenols could induce static fluorescence quenching of gliadin solution at pH 3.8 and 7.0. Fluorescence quenching spectrum of chlorogenic acid-gliadin complex at pH 3.8 presented a red shift toward long wave, while other phenols spectra showed blue shifts toward the short wave. The binding ability showed a significant difference between acid condition and neutral condition, and the quenching constants (Kq and KSV) of all three polyphenols at pH 3.8 were larger than pH 7.0. Compared to epicatechin and gallic acid, pH value had a marked effect on binding ability of chlorogenic acid with gliadin.

1. Introduction

Apple juice is the second largest juice consumption in the world. Compared with clear apple juice, cloudy apple juice contains higher nutritional value and more natural taste. There is no clarification step during cloudy apple juice processing, therefore, the phenomena of turbidity and precipitation usually exist. These precipitation caused by non-microbial factors could bring unpleasant organoleptic state which seriously restrict the product quality. From previous studies [1], the major reason of non-microbial turbidity or precipitation is the interactions between proteins and polyphenols that create larger molecules. Other components, such as starch and pectin can also participate in the process of turbidity precipitation yet mainly passive co-precipitation by net flocculation [2]. It is known that polyphenol-protein aggregations are involved in hydrophobic interactions as well as hydrogen bonding [3, 4]. The difference in the molecular mass, special structure and environmental system of polyphenols led to different interactions with protein. The molecular weight of the main proteins in apple juice and that of the glutenohemic protein are close to each other between 25 kDa and 100kDa, which means they both belong to small single-peptide proteins, similar with gliadin. Gliadin could self-assemble by disulfide bond, hydrogen bond, hydrophobic interaction and electrostatic force, etc [5]. Moreover, it could adsorb small molecule compounds such as polyphenols.

Fluorescence spectroscopy is extensively used in the research of interactions between proteins and polyphenols to obtain binding constants, binding sites, types of forces or conformation and physiological function changes of proteins [6, 7]. Based on information like quenching constant (Ksv), the aim of this work was to determine the interaction of gliadin with three characteristic phenolic compounds
(Chlorogenic acid, epicatechin, gallic acid) at different pH values. The study on the interaction characteristics between gliadin and polyphenols is of great importance in production practice.

2. Materials and methods

2.1. Sample preparation.
Gliadin (G, MW=40KDa) was slightly dissolved in ethanol solution. Powdered gliadin were slowly dissolved in ethanol solution, potassium phosphate buffer adjusted to pH 3.8 and pH 7.0. Gliadin solution was diluted to 10 mol/L in buffer respectively, and vortex stirred at ambient temperature. Powder chlorogenic acid, epicatechin and gallic acid (chromatographic pure, Sigma-aldrich) were dissolved in potassium phosphate buffer solutions with pH 3.8 and 7.0 respectively. All solution vortex mixed at room temperature to ensure completed solubility. The standard reserve solution (1mg/mL) was stored in a refrigerator at 4℃ and prepared for further analysis.

2.2. Fluorescence spectroscopy.
10 mol/L gliadin sample solution was mixed with chlorogenic acid, epicatechin, and gallic acid at different concentrations, respectively. The concentration of gliadin in the mixed solution was 5 mol/L, and the concentration of three polyphenols was 0, 2.5, 5, 10, 20, 40, 60, and 80 mol/L. Hitachi f-7000 fluorescence spectrometer was used for the determination excitation wavelength $\lambda_{ex}=280$nm, emission spectral wavelength $\lambda_{em}$ range of 290nm ~ 450nm, excitation slit was 5.0nm and emission slit was 2.5nm.

3. Results and discussions

Fluorescent chromogenic amino acid residues, such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) existed in proteins, result in endogenous fluorescence. The phenolic compounds bound to the protein caused decreasing on fluorescence intensity of protein solution. This phenomenon is called fluorescence quenching. Fluorescence spectrum scanning analysis was performed on the mixed solution to study whether the three polyphenols interact with gliadin at different pH values. The results were shown in Fig.1 (pH 3.8) and Fig.2 (pH 7.0). According to the two figures, under different pH conditions, gliadin had endogenous fluorescence at $\lambda_{ex}=280$nm, and gliadin had the strongest fluorescence intensity in the range of 320 to 360nm, with the largest contribution of Trp residues. Endogenous fluorescence intensity of gliadin was regularly decreased as the increasing concentration of polyphenols. Fluorescence intensity was quenched in varying degrees, and the wavelength of fluorescence peaks significantly shifted, indicated that the gliadin microenvironment had clearly changed. A red shift toward long waves was observed in chlorogenic acid and gliadin complex at pH 3.8. It illustrated that as the concentration raised, the gliadin molecules unfolded and structure became loose. Trp was exposed to the environment and surface hydrophobicity was increased. For pH 3.8 spectra, the complex of other two polyphenols and proteins had a blue shift toward the short wave, showed that the molecule of gliadin aggregated, and Trp was tightly wrapped and hydrophobicity was weakened [8]. All the three polyphenols had strong quenching effect on the protein. The addition of polyphenols at different pH value had different impacts on the fluorescence intensity, especially for chlorogenic acid-gliadin complex. The appearance of two fluorescence peaks at pH 7.0 indicated that pH value influenced their interacting mode and the complex structure. Furthermore, the emission wavelengths at different pH were different, and this further proved that pH affects the structure of the complex.
Fig 1. Fluorescence quenching of polyphenols interacted with gliadin at pH 3.8.
Fig 2. Fluorescence quenching of polyphenols interacted with gliadin at pH 7.0.
The mechanism of fluorescence quenching mainly includes static quenching, dynamic quenching and their coexistence. It is generally considered the impact diffusion rate constant \( K_q \) of the quenching agent (polyphenols) on biological macromolecules (gliadin) is greater than \( 2.0 \times 10^{10} \text{ L·mol}^{-1}\text{s}^{-1} \) as static quenching. Fluorescence quenching as a result of protein–polyphenol interactions was described by the Stern–Volmer equation [9]:

\[
\frac{F_0}{F} = 1 + K_{sv} [P] = 1 + K_q \tau_0 [P]
\]  

Fig. 3 showed the three kinds of polyphenols and gliadin in pH = 3.8 and pH = 7.0 reaction in Stern–Volmer curve. The epicatechin-gliadin, chlorogenic acid-gliadin and gallic acid-gliadin compounds curves all showed good linearity. Each curve slope was calculated by the corresponding \( K_{sv} \) and \( K_q \) values under corresponding pH value, and the results were shown in table 1. With the increase of the pH, the quenching constants of epicatechin, chlorogenic acid and gallic acid with gliadin all decreased. Moreover, the collision diffusion rate constant \( K_q \) is larger than \( 10^{12} \text{ L·mol}^{-1}\text{s}^{-1} \), which indicated that the quenching mechanism was static quenching [10]. The static quenching constant obtained by stern-volme equation could partly represent relative intensity of the static quenching effect. Under the same experimental conditions, the quenching constants of the three small polyphenol molecules with gliadin were different from each other. The quenching constants under acidic conditions (pH=3.8) were significantly greater than those under neutral conditions (pH=7.0), showed that the binding capacity of polyphenols and proteins were stronger under acidic conditions. The \( K_{sv} \) value of gallic acid-gliadin complex was the largest at pH 3.8, and this might due to structure difference or the binding strength of gliadin and the quenching constant increase.

![Fig 3. Stern-Volmer curves of three phenolic compounds interacted with gliadin at different pH](image-url)
**Table 1.** Quenching parameters of the reaction between three polyphenols and gliadin at different pH

| System                | pH  | $K_q$ | $K_{sv}$ |
|-----------------------|-----|-------|----------|
| Epicatechin-Gliadin   | 3.8 | 4.7   | 4.7      |
|                       | 7.0 | 4.1   | 4.1      |
| Chlorogenic acid-Gliadin | 3.8 | 4.6   | 4.6      |
|                       | 7.0 | 3.8   | 3.8      |
| Gallic acid-Gliadin   | 3.8 | 5.1   | 5.1      |
|                       | 7.0 | 3.9   | 3.9      |

4. **Conclusions**
The pH value had a certain effect on the binding ability of polyphenols in cloudy apple juice with gliadin. All three characteristic phenolic compounds of cloudy apple juice could combine with the luminescent groups of gliadin. The binding ability of the same polyphenol with protein at acidic condition was slightly stronger than that at neutral condition. The pH had more obvious effect on the interaction of chlorogenic acid with gliadin.

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