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Interaction between lysozyme and procyanidin: Multilevel structural nature and effect of carbohydrates

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ABSTRACT

The interaction of procyanidins with proteins has aroused extensive attention due to its important relationship with the bioavailability and astringent property of polyphenols. In the present work, we have investigated the interactions of lysozyme with procyanidin dimer (B3) using various biophysical approaches, which aims to provide insights into the mechanism of protein/polyphenol aggregation. Procyanidin B3 spontaneously binds lysozyme, inducing the multilevel structural changes in lysozyme and the formation of insoluble complexes. The relationship between lysozyme aggregation and the loss of enzymatic activity was monitored using dynamic light scattering and fluorescence quenching. The influences of two carbohydrates (gum arabic and sucrose) on lysozyme/B3 aggregation were also studied. Gum arabic effectively inhibited the formation of insoluble aggregates, but was unable to restore the fluorescence and activity of lysozyme. However, sucrose concomitantly decreased the aggregate size with the recovery of fluorescence and lysozyme activity. These results proposed two probable mechanisms by which these two carbohydrates inhibit protein/polyphenol aggregation.

1. Introduction

Polyphenols are secondary plant metabolites that enter the human diet mostly through fruits and vegetable-based beverages such as juices, beer, and wine. The regular consumption of polyphenols has been claimed to be beneficial for human health, mostly due to their biological functions, such as anti-carcinogenic, anti-allergic, and antioxidant properties (Xue, Feng, Cao, Cao, & Jiang, 2009). The physicochemical properties, biological activity and the important protective effects of polyphenols have been summarized in recent years (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). Moreover, various studies have demonstrated that polyphenols possess a prominent binding affinity for salivary proteins and digestive enzymes. This interaction between protein and polyphenols often lead to the disruption of the multilevel structures of proteins and formation of complex aggregates.

Many techniques including circular dichroism (CD) (Jöbstl, O'Connell, Fairclough, & Williamson, 2004), fluorescence spectroscopy (Soares, Mateus, & de Freitas, 2007), dynamic light scattering (DLS) and nuclear magnetic resonance (NMR) (Gonçalves, Mateus, Pianet, Laguerre, & de Freitas, 2011b) have been employed to understand the formation mechanism of protein/polyphenol aggregates in solution. Among these spectroscopic techniques, CD is a simple and fast method to evaluate secondary and tertiary structure of proteins by far-UV and near-UV CD, respectively. Therefore, it has been widely applied for studying protein conformational perturbation occurring after interaction with phenolic compounds (Gonçalves, Mateus, & de Freitas, 2010). Fluorescence spectroscopy, as a highly sensitive method for protein tertiary structural characterization, is the most commonly used technique to study the structural change in proteins upon association with polyphenols (Soares et al., 2007). In addition, the aggregates in solution resulting from the high binding affinity of polyphenols for proteins could also be characterized by light scattering such as DLS and nephelometry measurements, and electron microscopy techniques (Brás et al., 2010; Charlton et al., 2002) thus offering useful information on quaternary structure of protein to some extent. Recently, NMR combined with molecular modeling have also been used by some researchers to further decipher the binding process at molecular level (Gonçalves et al., 2011b). Hydrophobic effects, hydrogen bonding, and van der Waals interactions have been suggested to be involved in the formation of aggregates. However, a complete understanding of the aggregation mechanisms by which lead to the loss of enzymatic activity is still lacking. Further studies on the alterations of multilevel structure and aggregation behaviour is required to elucidate the mechanisms.

It is well known that the association of proteins with polyphenols is an important nutritional topic of study because this

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affinity is of particular relevance in disease processes, such as bacterial colonization, growth retardation, and tumor survival (Gonçalves, Soares, Mateus, & de Freitas, 2007; Soares et al., 2007). The protein/polyphenol complexes have also been considered to be responsible for the sensation of astringency on the human palate. Astringency is often perceived as an undesirable quality factor in some beverages such as fruit juices and red wine, if too intense (Jöbstl, O'Connell, Fairclough, & Williamson, 2004). Generally, this astringency is influenced by several factors, including the structures and concentrations of polyphenol, ionic strength, pH values, and the alcohol level (Fontoin, Saucier, Teissedre, & Glories, 2008). In addition, some studies have found that the presence of other co-substrates such as neutral and anionic polysaccharides also have the ability to disrupt the binding of polyphenols to proteins, thereby inhibiting aggregation and astringency (de Freitas, Carvalho, & Mateus, 2003; Luck et al., 1994). This phenomenon is also observed in the natural loss of astringency during fruit maturation, which is related to the increase of insoluble pectin during maturation. Therefore, insight into how polyphenol interact with proteins will be helpful to control the astringency and to improve the quality of wines (McRae & Kennedy, 2011).

Inspired by the phenomenon during the fruit ripening stage, the interaction between procyanidin B3 and lysozyme with and without carbohydrates is investigated in the present work. B3 is one of the most widely studied procyanidin dimer due to its abundance in red wine. Lysozyme, which is a globular basic protein found in saliva, attacks the cell walls and disrupts the membrane structures of bacterial (Croguennec, Nau, Molle, Le Graet, & Brule, 2000). Consequently, lysozyme has the advantage of blocking malolactic fermentation in winemaking process, controlling spontaneous lactic acid bacteria growth, and improving wine stability during storage or ageing (Granato et al., 2010; Guzzo, Cappello, Azzolini, Tosi, & Zapparoli, 2011). Lysozyme has been considered as an excellent model protein for studying the interaction between protein and polyphenols. A range of biophysical methods was employed to gain deeper insights into the multilevel structural changes and the aggregation mechanism by which procyanidin B3 interacts with lysozyme. This protein–polyphenol interaction leads to the loss of lysozyme activity. In addition, gum arabic and sucrose were introduced to influence the lysozyme/B3 system to evaluate the effects of these two carbohydrates on the interaction.

2. Materials and methods

2.1. Reagents and preparation of solutions

Chicken egg white lysozyme (ultrapure grade) was purchased from Amresco (Solon, OH, USA). Procyanidin dimer B3 ($\geqslant$98%) was kindly provided by JF-Natural (Tianjin, China). Lyophilized *Micrococcus lysodeikticus* powder was obtained from the China General Microbiological Culture Collection Center. Other reagents such as gum arabic, sucrose, disodium hydrogen phosphate, and monosodium orthophosphate were all of analytical grade (Kermel, Tianjin, China). Ultrapure water from a Milli-Q ultrapure water purification system (Millipore, Billerica, USA) was used throughout the experiments.

All the experiments were performed in phosphate buffer (0.1 M) of pH 7.0 because this pH is the one that occurs in duodenum, where lysozyme is located, after the neutralization of gastric fluids (Gonçalves, Mateus, & de Freitas, 2011a). Stock solutions of lysozyme (300 μM), procyanidin B3 (300 μM), gum arabic, and sucrose were prepared in this buffer. Different volumes of procyanidin B3 solution (to final concentrations of 0 μM to 36 μM) were diluted with phosphate buffer in a series of 10 ml volumetric flasks. Then, 0.1 ml lysozyme solution was added into each flask, and the mixtures were incubated at 37 °C for 2 h in a shaking water bath to equilibrate the system (Julabo, Germany). The effect of carbohydrates on lysozyme/B3 interaction was also studied. Mixtures of procyanidin B3 (30 μM) with different concentrations of gum arabic and sucrose were prepared (both of the carbohydrates concentration are 0 mg/ml to 0.6 mg/ml), and allowed to stand for 30 min at 37 °C. Then, 0.1 ml of lysozyme solution and a calculated amount of phosphate buffer were added to a final volume of 10 ml. Incubation was maintained for 1.5 h to equilibrate the system.

2.2. UV–Vis absorption spectroscopy

The interaction between lysozyme and procyanidin B3 with different concentrations was investigated using UV–Vis absorption spectroscopy. Phosphate buffer or B3 solution was subtracted as the reference solution. The measurements were carried out using a TU-1810 spectrophotometer (Persee, China) in a 10 mm quartz cuvette. The UV–Vis absorption spectra were recorded from 190 nm to 450 nm at 37 °C.

2.3. Fluorescence measurements

The fluorescence quenching effect between lysozyme and different concentrations of procyanidin B3 was recorded on a FluoroLog fluorescence spectrophotometer (Horiba Ltd., Japan). The influence of gum arabic and sucrose on the lysozyme/B3 interaction was also analyzed using fluorescence spectroscopy. The excitation wavelength was set to 290 nm and the emission spectrum was recorded from 300 nm to 500 nm. The synchronous fluorescence spectra of lysozyme with and without B3 were measured ($\Delta\lambda$ = 15 nm, λ_{ex} = 275 nm to 360 nm; and $\Delta\lambda$ = 60 nm, λ_{ex} = 320 nm to 400 nm, respectively). The excitation and emission slit widths were both set at 2 nm and the recording speed was 120 nm/min.

2.4. Circular dichroism

The far-UV CD spectra were recorded using a Jasco 810 spectrophotometer (Jasco Inc., Tokyo, Japan) from 250 nm to 190 nm with a 2 mm path length quartz cuvette. Baseline corrections were performed by subtracting the spectra of phosphate buffer from the sample spectra. The spectra were recorded using 0.5 nm resolution and 100 nm/min scanning speed, with 1 s response times and a bandwidth of 2 nm. Each spectrum presented is the average of three consecutive measurements.

2.5. Nephelometry measurements

In the nephelometry experiments, a FluoroLog fluorescence spectrophotometer was used as a 90° light scattering photometer. Both excitation and emission wavelengths were set to 400 nm, which enables the measurement of light scattered by particles in the lysozyme/B3 system. As protein and procyanidin B3 do not absorb the incident light at this wavelength, the response of this technique was improved (Carvalho, Mateus, Plet, Pianet, Dufourc, & De Freitas, 2006). A calculated relative aggregation value (%), which represents the ratio between the scattered intensity of the measured sample and that of the most turbid sample, was used to study the aggregation behaviour of lysozyme/B3 experiment.

2.6. Dynamic light scattering

DLS measurements were performed using a Brookhaven Zeta-Plus Nanoparticle Size Analyzer (Brookhaven) instrument equipped with a 633 nm laser. The intensity of light scattered was monitored at a 90° angle. The size of the lysozyme/B3