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Effects of proanthocyanidins on porcine pancreatic lipase: Conformation, activity, kinetics and thermodynamics

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ABSTRACT

The interactions between proanthocyanidins (PC) and porcine pancreatic lipase (PL) were investigated from variant aspects of lipase conformation, activity, kinetics, and thermodynamics. Results show that 34% inhibitory rate of PC on PL is achieved after about 30-min incubation, and the inhibitory rate increases with the increase of PC concentration and then plateaus at the PC/PL ratio of 200. Circular dichroism and fluorescence spectroscopic analyses demonstrate that PC decreases the α-helix content while increases the β-sheet content of PL, but does not change the microenvironment of Trp, and PC quenches the fluorescence of PL both dynamically and statically through the formation of PL–PC complex. PC induces PL aggregation and then stabilizes the lipase aggregates. Kinetic studies reveal that PC does not change the K_m value while decreases the V_{max} value, implying that PC non-competitively inhibits the PL activity.

1. Introduction

It is well-known that excessive calorie intake results in obesity, subsequently induces many obesity-related diseases, such as cardiovascular disease, diabetes, cancer, and metabolic syndrome [1]. Obesity has rapidly become a worldwide epidemic. Triglyceride is the major energy source with the characteristics of high calories. Hence, inhibition of triglyceride absorption is considered to associate with the prevention of obesity and obesity-related diseases [2]. Pancreatic lipase (PL, EC 3.1.1.3) is the key enzyme involved in triglyceride absorption in the small intestine [3]. Therefore, the suppression of triglyceride absorption by inhibiting the PL activity has become a major approach to prevent obesity. Recently, numerous PL inhibitors have been explored and can be roughly divided into these categories: synthetic chemicals [4], soluble microbial products [5], and natural plant extracts [6].

Proanthocyanidins (PC) are a group of compounds comprising, as constitutional units, condensed type tannin existing in various plants (e.g., apple peel and grape seed), namely flavan-3-ols and flavan-3,4-diols, which are bonded by condensation or polymerization [7]. These polyphenols have been found to function as antioxidants [8], anti-allergy agents [9], anti-tumor agents [10], etc. Among these bioactivities, PC as an antioxidant has been comprehensively studied in literature. For example, McDougall et al. [11] demonstrated that PC from berries can effectively inhibit PL activity in vitro. Yuste et al. [12] reported that PC from beans suppressed chicken PL activity by 32%. Zadernowski et al. [13] found that PC from beans and peas showed great inhibitory effect toward both lipase and lipoxygenase activities. Sugiyama et al. [14] confirmed that PC from apples inhibited PL activity significantly in vitro. On the other hand, it was also found that the inhibitory effect of PC increased as the function of the degree of polymerization from dimer to pentamer. Kimura et al. [15] and Yokota et al. [16] deemed that PC with higher molecular size and more A-type linkages (C—O bonds between monomeric units) was more effective in the inhibition of PL, suggesting that A-type highly polymeric PC was a promising alternative for the attenuation of lipid digestion as dietary supplements. Gonçalves et al. [17] considered that both the inhibition of lipase activity and the respective quenching of intrinsic protein fluorescence increased with the average degree of the tested PC polymerization.

However, no reports have been available on the influences of PC on PL conformation, activity, reaction kinetics, and thermodynamics so far. Therefore, the objects of this work are: (1) to investigate the effects of PC on the PL activity as functions of incubation time and PC concentration using olive oil as a substrate; (2) to elucidate the effects of PC on PL secondary and tertiary structures via circular dichroism (CD) and fluorescence spectroscopes (FS); (3) to determine the hydrodynamic radius (R_h) by dynamic light scattering (DLS), and the transition midpoint temperatures (T_m) by differential scanning calorimetry (DSC); (4) to examine

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the kinetic parameters of K_m and V_{max} from Lineweaver–Burk plots.

2. Materials and methods

2.1. Materials

PL from porcine pancreas type II was purchased from Sigma (St. Louis, MO) with the hydrolysis activity of 351 U/mg-protein (using olive oil as substrate and incubating for 30 min as reported by the manufacturer). PL form was lyophilized powder and protein content is about 5%. Therefore, PL was purified by a 48-h dialysis before usage to insure the purity of PL (The SDS-page analysis of PL before and after dialysis is shown in Fig. 1). PC with a purity of 98% was bought from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). Olive oil was purchased from Terra Delyssa (Shanghai, China). Other chemicals and agents were all of the analytic grades and bought from local markets.

2.2. PL protein content and r parameter determination

PL protein content was determined by Bradford protein assay with the value of 5.21 ± 0.26% (m/m) [18]. Each experiment was repeated five times and the average value with standard error was reported.

To facilitate the comparison of experimental data under various conditions, r representing the molar ratio of PC to PL was introduced in this work, which was calculated as follows:

$$r = \frac{[PC]}{[PL]} = \frac{[PC] \times M_{PL}}{m_{PL} \times w\%} \quad (1)$$

where [PC] and [PL] are the final concentrations of PC and PL after mixing (mmol/L), respectively. m_{PL} is the mass concentration of PL protein (mg/mL), M_{PL} is the molecular weight of PL (ca. 52 kD) [2], and $w\%$ is the PL protein content.

2.3. PL activity assays

PL activity assay was modified according to the method reported in our previous work [18]. PL activities affected by different PC concentrations were titrated with 0.5 mol/L sodium hydroxide using 25% homogenized olive oil (v/v) as the hydrolytic substrate and phenolphthalein as the indicator. The reaction mixture without the enzyme was titrated as the blank control. One 'lipase unit' was defined as the amount of the enzyme that released 1 μmol free fatty acid per minute under standard assay conditions. The inhibitory rate of PC was expressed as Eq. (2):

$$I = \left(1 - \frac{A}{A_0}\right) \times 100\% \quad (2)$$

where I is the inhibitory rate of PC on the PL activity, A_0 and A are the PL activities with or without PC, respectively.

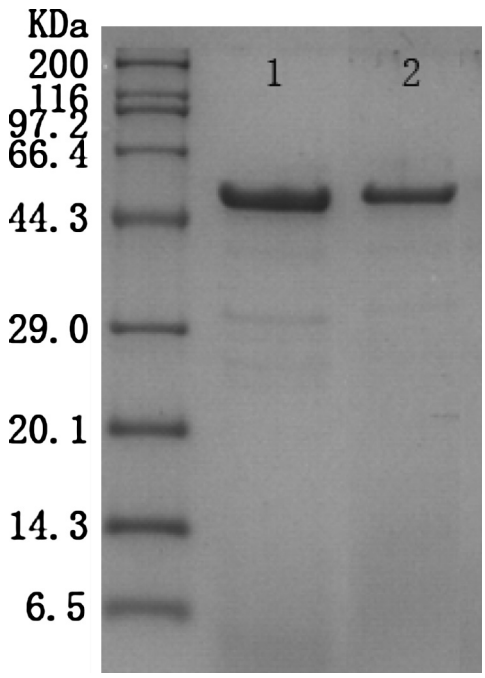

Fig. 1. SDS-PAGE analysis of PL before (lane 1) and after dialysis (lane 2). The molecular weight cut of the dialysis membrane is 35 kD.

2.4. Far-UV circular dichroism

The determination of PL secondary structure was modified according to the method reported by Wang et al. [19] in a Jasco 810 circular dichroism (CD) spectrophotometer (Jasco Inc., Tokyo, Japan). Briefly, aliquot of 0.1 mg/mL PL solution with a certain PC concentration (0–20 μmol/L) was centrifuged at 5000 rpm for 15 min and the supernatant was injected into a 1-mm path length quartz cuvette. A background CD spectrum of buffer solution was subtracted from the sample spectrum for baseline correction. Spectra were recorded under the conditions: a resolution of 0.5 nm, scanning rate of 100 nm/min, response time of 1 s, bandwidth of 2 nm, room temperature and the wavelength ranges from 250 to 190 nm. The PL secondary element percentages of α-helix, β-sheet, turn, and unordered coil were calculated by spectra data using SELCON3 website online [20].

2.5. Fluorescence spectroscopy

Fluorescence spectra were conducted according to the method described in our previous work [21] on a Varian Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, California, USA). Briefly, aliquot of 2.5 mg/mL PL solution mixed with a certain PC concentration (0–500 μmol/L) was centrifuged at 5000 rpm for 15 min and the supernatant was injected into a 1 cm-path length quartz cuvette. The excitation wavelength was 295 nm, and the intrinsic fluorescence was recorded from 300 to 500 nm. The excitation and emission slits were 5 and 10 nm, respectively. The scanning rate was 600 nm/min and the resolution was 1.0 nm.

The fluorescence quenching parameters were calculated according to the method by Gonçalves et al. [17]. The fluorescence quenching is described by the linear Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher (herein refers to PC), respectively. k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorescence in the absence of the quencher, and [Q] is the concentration of the quencher. K_{SV}, the Stern–Volmer quenching constant, is calculated by $k_q \tau_0$, in which τ_0 is equal to 1.59 ns [22].

A linear Stern–Volmer plot is generally an indicative of a single class of fluorophore, all equally accessible to the quencher, which indicates that only one mechanism (dynamic or static) of quenching occurs. Positive deviations from the Stern–Volmer equation may occur if the extent of quenching is large. The positive deviations suggest that the fluorophore is being quenched by both mechanisms (dynamic and static) simultaneously or the presence of a sphere of action [23]. As a result, the Stern–Volmer equation should be modified to Eq. (4):

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{[Q]} \times \frac{1}{f_a K_A} \quad (4)$$

where f_a is the fraction of fluorophore accessible to the quencher, and K_A is the modified Stern–Volmer quenching constant, which is very close to the binding constant. f_a and K_A values can be calculated from the intercept and slope of the $F_0/(F_0 - F)$ versus 1/[Q] plot.

2.6. Measurement of hydrodynamic radius (R_h)

Zetasizer Nano series of DLS (Malvern Instruments Ltd., Worcestershire, UK) was employed to measure the hydrodynamic radii of PL in the absence and presence of PC at different concentrations at 25 °C. The path length of the cuvette was 10 mm. Triplicate measurements were done and the average value of hydrodynamic radius was presented [24].

2.7. Kinetic parameters (K_m and V_{max}) assessment

In order to probe kinetic parameters K_m and V_{max}, the effect of substrate concentration on the initial reaction velocity was studied using olive oil as a substrate through the hydrolysis reaction by PL. The PL solutions with and without PC were incubated with various concentrations of emulsified olive oil with the final concentrations of 0.03, 0.06, 0.08, 0.09, and 0.10 mg/mL, respectively. In all cases, the enzymatic activity in phosphate buffer (pH 7.4) was assayed at 40 °C and detailed in Section 2.3 [18]. From the Lineweaver–Burk plots, the Michaelis constant (K_m) and maximum velocity (V_{max}) were determined [25].

2.8. Thermodynamic parameter (T_m) assessment

Thermodynamic parameter (T_m) was performed using a VP-DSC differential scanning calorimeter (MicroCal, Northampton, MA). Temperature scans were from 25 to 95 °C at a scan rate of 1 °C/min. The sample cell was loaded with 2.5 mg/mL PL with a certain PC concentration (0–500 μmol/L), and the same concentration phosphate buffer was loaded into the cell as the blank control. The samples and control were degassed for 15 min immediately before DSC scanning. A buffer-buffer reference scan was subtracted from each sample scan prior to concentration normalization. DSC data were analyzed by MicroCal Origin Version 7.0.