Journal of Molecular Structure 1008 (2012) 42–48

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstruc

# Spectroscopic investigations on the binding of persimmon tannin to phospholipase $A_2$ from Chinese cobra (*Naja naja atra*)

Jie Yang [a], Li Zhong [a], Bo Zou [a], Yan Tian [a], Shu-fen Xu [a], Ping Yao [b], Chun-mei Li [a,*]

[a] College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[b] Department of Nutrition and Food Hygiene, School of Public Health, Tongji Medicine College, Huazhong Science and Technology University, Wuhan 430030, China

ARTICLE INFO

*Article history:*
Received 18 July 2011
Received in revised form 9 November 2011
Accepted 17 November 2011
Available online 25 November 2011

*Keywords:*
Persimmon tannin
Phospholipase $A_2$
Fluorescence quenching
Conformational changes

ABSTRACT

To understand the anti-venom mechanism of persimmon tannin, the interaction between persimmon tannin (PT) and phospholipase $A_2$ ($PLA_2$) under physiological conditions was investigated by fluorescence quenching technique in combination with Fourier transform infrared (FT-IR) and circular dichroism (CD) spectra techniques. The results revealed that gradual fluorescence quenching was observed by titration of $PLA_2$ (2.0 μM) with increasing concentrations of PT (from 0 to 2.025 μM), and the type of quenching was found to be a static quenching process. Stern–Volmer plots were not linear but had an intersection at $C_{PT} \approx 1.0$ μM, indicating that PT binded to more than one class of sites on $PLA_2$. The binding sites calculated on basis of Scatchard plots were about 2, supporting this result. The enthalpy change ($\Delta H$) and entropy change ($\Delta S$) of the binding sites were −17.44 kJ/mol and 59.90 kJ/mol·, separately, suggesting that hydrophobic interaction played a main role in the binding. In addition, synchronous fluorescence, FT-IR and CD spectra showed that dramatic conformational changes in $PLA_2$ were induced by its interaction with PT.



## 1. Introduction

Phospholipase $A_2$ ($PLA_2$s, EC 3.1.1.4) are found in abundance in nature especially in many animal venoms. Snake venoms are the richest sources of this enzyme [1]. Snake venom $PLA_2$ exerts many pharmacological activities including neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant and antiplatelet [2,3]. And snake venom $PLA_2$ is believed to be the most toxic component implicated in hemorrhage [4] and a key component of the inflammatory process [5]. There are also several reports of snake venom $PLA_2$ inducing edema is dependent on the ability of the $PLA_2$ to bind to specific membrane proteins [6]. Chinese cobra (*Naja naja atra*) is broadly distributed in Southern China and Taiwan. $PLA_2$ from Chinese Cobra was reported to play an important role on the myotoxicity, hemolyzation, inflammatory and cytotoxicity induced by Chinese cobra venom [7].

Tannins are a group of compounds widely distributed in plant kingdom. Some tannin has been reported to have significant protective effect against snake venoms. Aqueous extract of the dried roots of *Mimosa pudica* was reported to display significant inhibitory effect on the lethality, myotoxicity and enzyme activities of *Naja kaouthia* venom [8]. In our earlier study [9], we found that persimmon tannin has a very unusual structural characterization and exhibited a very strong inhibitory effect on Chinese cobra venom $PLA_2$ and alleviated the myotoxicity, neurotoxicity and lethality induced by the venom, suggesting that it may be useful as an alternative assistant treatment of snakebite victims to serum therapy. However, the modes of the action of these compounds are not clear, and detailed investigations of the interaction of persimmon tannin with snake venom are yet to be conducted.

To understand the mechanism of action of persimmon tannin on isolated $PLA_2$ from Chinese cobra venom, the interaction of persimmon tannin with $PLA_2$ was studied by spectroscopic methods including fluorescence, FT-IR and CD spectra. The binding constants and binding sites were calculated, and the binding mechanism was proposed, meanwhile, the conformational changes of $PLA_2$ were also studied. This work should aid in better understanding the anti-venom mechanism of persimmon tannin.

## 2. Experimental

### 2.1. Materials

Chinese cobra (*Naja naja atra*) phospholipase $A_2$ ($PLA_2$, 9001-84-7) was purchased from ZhongXin DongTai Nano Gene Biotechnology Co. Ltd. (Laiyang, China). The $PLA_2$ presented a single homogeneous strip on the electrophoresis and a single peak on the HPLC, and the purity was above 95%. It was used without further purification and its molecular weight was assumed to be 14,400 Da. Persimmon

* Corresponding author. Tel./fax: +86 27 87282966.
*E-mail address:* lichmyl@126.com (C.-m. Li).

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doi:10.1016/j.molstruc.2011.11.019

tannin extract (PTE) was prepared as we previously reported [10]. The obtained PTE was purified on Toyopearl TSK HW-50F as follows: Lyophilized PTE (0.2 g) was dissolved in about 2 mL of methanol and fractionated by gravity chromatography (30 cm × 500 mm i.d.) on Toyopearl TSK HW-50 (F) using 1000 mL of methanol to remove the lower molecular weight phenolic compounds and 1000 mL of 40% aqueous acetone to yield the target fraction. The obtained fraction was evaporated under reduced pressure at 30 °C, lyophilized, and stored at −20 °C for further analysis. The purified persimmon tannin (PT) was characterized by MALDI-TOF, Thiolysis–HPLC–ESI-MS and NMR as we previously reported [9]. Total polyphenol content of PT was determined to be 98.7% by Folin–Denis method [11] using gallic acid as a standard on a mass basis. The mean degree of polymerization of PT was estimated as 24.86 by thiol degradation combined HPLC–MS–MS analysis. The extension units were determined to be epicatechin, epigallocatechin, (epi) gallocatechin-3-*O*-gallate, and (epi) catechin-3-*O*-gallate with the relative moles of 1.207, 0.305, 13.335 and 9.01, respectively, and the terminal units were determined to be catechin, (epi) gallocatechin-3-*O*-gallate, and myricetin with the relative moles of 0.26, 0.52 and 0.22, separately. The proposed structure of persimmon tannin was shown in Fig. 1. All working solutions were prepared in 0.01 M Tris–HCl buffer solution (pH7.4). All chemicals were of analytical reagent grade, and doubly distilled water was used throughout all the experiments.

### 2.2. Apparatus and methods

#### 2.2.1. Fluorescence spectra

Fluorescence spectra were obtained on a RF-5301PC (Shimadzu, Japan) spectrofluorimeter using a quartz cell with 10 mm path length. The fluorescence quenching spectra were measured at three temperatures (288, 298 and 310 K) in the range of 300–500 nm with excitation wavelength at 280 nm. The excitation and emission slits were 3 nm and 5 nm, separately. In performing the fluorescence titration experiments, 5.0 mL of Tris–HCl buffer solution (pH7.4) containing 10.0 μM of $PLA_2$ was titrated manually by the successive addition of various concentrations of PT (0–2.025 μM) with trace syringes, and the final concentration of $PLA_2$ was fixed at 2.0 μM. Appropriate blanks corresponding to the buffer solution were subtracted to correct background fluorescence. The protein could not precipitate with PT at the concentrations we used in the experiment throughout this paper.

The synchronous fluorescence spectra were recorded from 280 nm to 400 nm at $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm, respectively.

#### 2.2.2. UV/vis absorption

The absorbance of PT were recorded from 200 to 600 nm with 1 nm increments on a UV-1700 spectrophotometer (Shimadzu, Japan) with 1 nm bandwidth and a path length of 1 cm.

#### 2.2.3. FT-IR spectra

FT-IR measurements were carried out at room temperature on a Nicolet Nexus 670 FT-IR Spectrometer (USA) equipped with a germanium attenuated total reflection (ATR) accessory. All spectra were taken via the attenuated total reflection (ATR) method with a resolution of 4 $cm^{-1}$ and 60 scans. The infrared spectra of $PLA_2$ and PT–$PLA_2$ complex (the molar ratio of $PLA_2$ to PT was 2.5:1) were obtained in the featured region of 1800–1500 $cm^{-1}$. The FT-IR spectrum of free $PLA_2$ was acquired by subtracting the absorption of the Tris–HCl buffer solution from the spectrum of the protein solution, and the difference spectrum of $PLA_2$ was obtained by subtracting the spectrum of PT-free form from that of PT–$PLA_2$ form with the same concentration. The subtraction criterion was that the original spectrum of the protein solution between 2200 and 1800 $cm^{-1}$ was featureless.

#### 2.2.4. CD spectra

CD measurements were carried out on a J-810 automatic recording spectrophotometer (Jasco Corp., Japan), using 0.1 cm

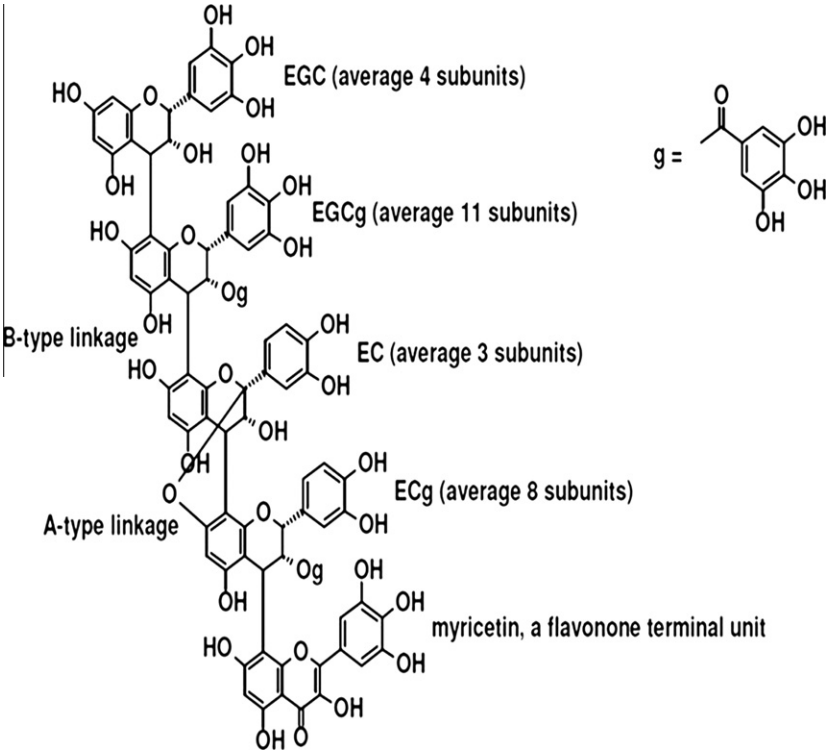


**Fig. 1.** Proposed structure for persimmon proanthocyanidin. Sequence of subunits and position of A-tye linkages is not known. Terminal units include myricetin, CAT or EGCg.