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Quercetin as an inhibitor of snake venom secretory phospholipase A2

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ABSTRACT

As polyphenolic compounds isolated from plants extracts, flavonoids have been applied to various pharmaceutical uses in recent decades due to their anti-inflammatory, cancer preventive, and cardiovascular protective activities. In this study, we evaluated the effects of the flavonoid quercetin on *Crotalus durissus terrificus* secretory phospholipase A2 (sPLA2), an important protein involved in the release of arachidonic acid from phospholipid membranes. The protein was chemically modified by treatment with quercetin, which resulted in modifications in the secondary structure as evidenced through circular dichroism. In addition, quercetin was able to inhibit the enzymatic activity and some pharmacological activities of sPLA2, including its antibacterial activity, its ability to induce platelet aggregation, and its myotoxicity by approximately 40%, but was not able to reduce the inflammatory and neurotoxic activities of sPLA2. These results suggest the existence of two pharmacological sites in the protein, one that is correlated with the enzymatic site and another that is distinct from it. We also performed molecular docking to better understand the possible interactions between quercetin and sPLA2. Our docking data showed the existence of hydrogen-bonded, polar interactions and hydrophobic interactions, suggesting that other flavonoids with similar structures could bind to sPLA2. Further research is warranted to investigate the potential use of flavonoids as sPLA2 inhibitors.

1. Introduction

Phospholipases A2 (PLA2, EC 3.1.1.4) are small proteins that catalyze the hydrolysis of glycerophospholipids at the sn-2 position in a Ca^{2+}-dependent reaction, releasing lysophospholipids and fatty acids [1–3]. These enzymes are the main component of snake venom and have been investigated not only because they have a wide range of biological effects, but also due to their similarity to mammalian phospholipases [4,5]. However, in contrast to their mammalian counterparts, several snake venom PLA2s are toxins that induce pharmacological effects [6] through arachidonic acid metabolism leading to the production of various lipid pro-inflammatory mediators such as prostaglandins, thromboxanes and leukotrienes [7]. Recent studies have shown that inhibition of cytosolic PLA2 (cPLA2) leads to a decrease in eicosanoid levels and, reduced inflammation [8].

Due to the role of PLA2s in the inflammatory process, there is pharmacological interest in PLA2 inhibitors, and among these, the flavonoids have been successfully studied. Flavonoids are widely produced in plants tissues making them suitable targets for pharmaceutical extraction and chemical synthesis [8,9]. The inhibitory effect of flavonoids on secretory PLA2 (sPLA2) was reported by Gil et al. [10], and Lindahl and Tagesson [11]. Their results showed that inhibition of sPLA2 from different sources following incubation with various flavonoids is dependent on the 5-hydroxyl group as well as the double bond and the double-bonded oxygen in the oxane ring, and that the hydroxyl groups at the 3′- and 4′-

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position are required for selective inhibition of PLA2 [11]. However, the exact mechanism by which flavonoids inhibit PLA2 remains unclear. Iglesias et al. [12] showed that morin modifies the secondary structure of sPLA2 from *Crotalus durissus cascavella* venom, but did not significantly affect its pharmacological activities.

Although there are studies of flavonoids as PLA2 inhibitors, the mode of binding of flavonoids to PLA2 as well as their inhibitory mechanism is still not clear. The aims of this article are to investigate the effect of quercetin, a widely spread flavonoid, on the structure and function of a sPLA2 isolated from *Crotalus durissus terrificus,* to increase our understanding of the mode action of polyphenolic compounds on the snake venoms, and to evaluate therapeutic application against the symptoms caused by snake bites. In the last decades the action of quercetin on the PLA2 has been studied. In 1993 Lindahl and Tagesson [13] showed that quercetin is a potent inhibitor of PLA2 (group II) from *Vipera russuli*. In addition, Lättig et al. [8] showed through computational studies chemical interactions between humans' sPLA2 and quercetin. Due to these characteristics, quercetin has been chosen as flavonoid model. Moreover we also propose, through high-resolution three-dimensional (3D) data (molecular docking), a structural model for understanding the molecular interactions between sPLA2 and quercetin, and how it can influence enzymatic and pharmacological activities of sPLA2.

2. Materials and methods

2.1. Venom, animals and reagents

C. durissus terrificus venom was purchased from Bio-Agents Serpentarium (Batatais, São Paulo, Brazil). Analytical HPLC- and sequencing-grade solutes and solvents were purchased from various suppliers (Bio Rad, Sigma Aldrich, Boehringer Mannheim, and Applied Biosystems). Female Swiss mice (18–20 g) used in the pharmacological assays were obtained from the Multidisciplinary Center of Biological Investigations (CEMIB-UNICAMP) and male chicks were obtained from Itu farm in Campinas City. All animal experiments were approved by the Ethics Committee of State University of Campinas (São Paulo, Brazil) under the number 1916-1.

2.2. Purification of sPLA2

Whole *C. durissus terrificus* venom was first fractioned as previously described by Toyama et al. [14]. Dried venom (45 mg) was dissolved in ammonium bicarbonate buffer (1.0 M; pH 8.0) and clarified by centrifugation (4500 × *g* for 1 min). The sPLA2 from *C. durissus terrificus* was eluted using a non-linear gradient of acetonitrile 66% in 0.1% of trifluoroacetic acid (TFA) by reverse-phase chromatography using a Supelco C5 column (0.10 cm × 25 cm) with flow rate of 1 mL/min with absorbance monitoring at 280 nm. The resulting PLA2 was termed sPLA2 and its purity was evaluated by SDS-PAGE.

2.3. Incubation of sPLA2 with quercetin and purification of modified sPLA2

The incubation of sPLA2 with quercetin (mol:mol) was according to the procedure described by Zhao et al. [15]. Quercetin was dissolved in dimethyl sulfoxide (DMSO), and its concentration never exceeded 1% during incubation. Quercetin (400 μL of a 0.1 mM solution) was added to 400 μL of a homogenized, purified sPLA2 solution (1 mg/mL). The mixture was incubated for 90 min at room temperature, and 200-μL samples of this mixture were loaded onto a preparative reverse-phase column to separate the treated enzyme (sPLA2:Q) from quercetin. After column equilibration with HPLC buffer A (aqueous 0.1% TFA), samples were eluted using a discontinuous gradient of HPLC buffer B (66.6% of acetonitrile in 0.1% TFA) at a constant flow rate of 1.0 mL/min. The chromatographic run was monitored at 280 nm.

2.4. Electrophoresis

Electrophoresis was carried out following the Laemmli method [16]. The degree of purity of fractions was assessed by discontinuous electrophoresis using a final acrylamide concentration of 12.5% in the resolving gels (1.0 M Tris–HCl, pH 8.8) and 5% in the stacking gel (0.5 M Tris–HCl, pH 6.8). Electrophoretic separation was carried out in a 250 Mighty Small (Hoefer Scientific Instruments) for SDS-PAGE. All samples and the molecular marker were treated with SDS and 1.0 M dithiothreitol (DTT), and the run was conducted at 60 mA for stacking gel and 90 mA for running gel. After electrophoresis, samples were stained with Coomassie brilliant blue R-250.

2.5. Circular dichroism spectroscopy

sPLA2 and sPLA2:Q (sPLA2 + quercetin) were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and final protein concentrations were adjusted to 8.7 mM. After centrifugation at 4000 × *g* for 5 min, samples were transferred to a 1-mm path length quartz cuvette. Circular dichroism spectra in the wavelength range 185–300 nm were acquired in-house with a J720 spectropolarimeter (Jasco Corp., Japan) using a bandwidth of 1 nm and a response time of 1 s. Data collection was performed at room temperature, with a scanning speed of 100 nm/min. Nine scans were obtained for each sample, and all spectra were corrected by subtracting buffer blanks.

2.6. Intrinsic fluorescence.

The relative intrinsic fluorescence intensities of sPLA2 and sPLA2:Q were monitored with a Varian Cary Eclipse. The proteins were solubilized in water at room temperature. The measurements were performed in a 1.5-mL 1-cm path length quartz cuvette. Fluorescence was measured between 300 and 450 nm after excitation at 280 nm.

2.7. Mass spectrometry

The molecular mass of sPLA2 and sPLA2:Q were determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). One microliter of samples (sPLA2 and sPLA2:Q) in 0.1% TFA was mixed with 2 μL of the matrix α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% TFA (v/v). The matrix was prepared with 30% acetonitrile and 0.1% TFA (v/v). Ion masses were determined with an acceleration voltage of 25 kV, the laser operated at 2890 μJ/com2, a 300-ns delay, and the linear analysis mode.

2.8. Measurement of sPLA2 activity

sPLA2 activity was measured following the protocol described by Lee et al. [17] and modified by Toyama et al. [14] in 96-well plates, using 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA, BIOMOL, USA) as substrate. Enzyme activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance after 20 min. All assays were performed with absorbance at 425 nm using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA). After the addition of native or treated sPLA2 (20 μg), the reaction mixture was incubated for up to 40 min at 37 °C and the absorbance read at 5-min intervals.