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Inhibition of Secreted Phospholipase A₂ by Proanthocyanidins: A Comparative Enzymological and *In silico* Modeling Study

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Abstract

Secreted phospholipase A₂ (PLA₂) plays a critical role in mobilizing arachidonic acid in phospholipids. We have previously reported that PLA₂ is inhibited by B-type proanthocyanidins (PaCs). To further understand the inhibitory activity of these compounds, we compared the inhibitory potency of B-type PaCs to that of A-type PaCs, and modeled them with PLA₂ using *in silico* techniques. The B-type trimer and tetramer inhibited PLA₂ (IC₅₀ = 16 and 10 μM). The A-type compounds were less potent (18 – 35% inhibition at 50 μM). The active site of PLA₂ lies in a hydrophobic tunnel. Modeling studies revealed that the B-type PaCs occupy this tunnel and are stabilized by a number of van der Waals interactions. The result is reduced substrate access to the active site. The A-type compounds can occupy this tunnel only by shifting the N-terminal loop outward. Our data provide a structural basis to screen additional PaCs for anti-PLA₂ activity.

Keywords

proanthocyanidin; phospholipase A₂; inflammation; *in silico* modeling

Introduction

Proanthocyanidins (PaCs) are polymeric polyphenols composed largely of (–)-epicatechin and (+)-catechin monomers found in a wide variety of food and non-food plants including cocoa (*Theobroma cacao*), cranberries (*Vaccinium macrocarpon*), apricots (*Prunus armeniaca*), and grapes (*Vitis vinifera*) (1, 2). PaCs are broadly classified based on the type of inter-monomeric linkage. A-type PaCs include at least one incidence of two linkages between monomeric units: one is a carbon-carbon bond between C4 on one monomer and C6 or C8 on the adjacent monomer and one is an ether linkage between the C3 on one monomer and 7-OH of the adjacent monomer (3, 4). Aesculitannin B1 is an example of an A-type tetramer (Fig. 1). By contrast, B-type PaCs involve a simpler single linkage between adjacent monomers. Typically, these linkages involve C4 on one monomer and C6 or C8 on the adjacent monomer. PaC C1 is an example of a B-type trimer (Fig. 1). These differences in linkage types result in significantly different 3-D geometries.

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Safety There are no issues related to safety in the present manuscript.

PaCs and PaC-containing plants have been studied for a variety of potential health beneficial effects. Cranberries have been shown to ameliorate urinary tract infections in laboratory studies and some human intervention studies (5). These effects are attributed to A-type PaCs, which are hypothesized to target p-fimbriae on uropathogenic *Escherichia coli* and reduce adhesion to the bladder epithelium (4, 6). Howell et al., have reported that urine from subjects who consumed cranberry juice (equivalent to 36 or 72 mg PaCs) inhibited the adhesion of *E. coli* to cultured bladder epithelial cells for up to 8 h after ingestion (7).

Cocoa, which is rich in B-type PaCs, has been shown to have anti-inflammatory effects *in vivo* (8). For example, Schramm et al., have reported that treatment of healthy volunteers with 37 g of high PaC-containing chocolate reduced plasma leukotrienes by 29% and increased plasma prostacyclins by 32% compared to subjects treated with low PaC-containing chocolate (9). An intervention study by Monagas et al., reported that treatment of 42 subjects at risk for cardiovascular disease with 40 g/d cocoa for 4 wk reduced markers of inflammation including P-selectin, intracellular adhesion molecule-1, as well as expression of VLA-4, CD36 and CD40 on monocytes (10).

Secreted phospholipases (PL) A_2 , are a family of 11 enzymes which hydrolyze the *sn*-2 position of glycerophospholipids to release a fatty acid and lysophospholipids (11). PLA $_2$ s serve a number of important biological functions and may role in various pathological responses including arthritis, heart disease, and obesity. For example, pancreatic PLA $_2$, a group I PLA $_2$, is secreted into the duodenum and is responsible for the release of free fatty acids from dietary phospholipids. It may be important in chronic inflammation, because it makes arachidonic acid, which is the precursor for pro- inflammatory eicosanoids including prostaglandin E $_2$ and leukotriene B $_4$, available for absorption (12). Bee venom contains group III PLA $_2$. These enzymes play a role in the inflammatory responses observed following envenomation, and are homologous to human group III PLA $_2$ enzymes which are linked to atherosclerosis (11).

Previously, we have reported that cocoa extract and purified cocoa B-type PaCs dose-dependently inhibited the activity of group III PLA $_2$ *in vitro* (13). The inhibitory potencies of the compounds were directly proportional to the degree of polymerization (DP), with the PaCs larger than DP4 having IC $_{50}$ less than 10 μ M. Kinetic analysis showed that the PaC pentamer and decamer inhibited PLA $_2$ in a non-competitive manner with respect to substrate concentration.

In order to more fully understand the mechanism by which PaCs inhibit PLA $_2$ and the structural requirements of active inhibitors, we combine enzymology studies of two A-type PaCs (parameritannin A1 and aesculitannin B) and corresponding B-type PaCs with *in silico* modeling studies of interactions with group III PLA $_2$. Herein we report the results of our studies.

Materials and Methods

Reagents

PaCs were generously provided by the Hershey Chocolate Co. (Hershey, PA) and were greater than 90% pure. Stock solutions (20 mM) were prepared in dimethylsulfoxide and stored at -80°C . B-type PaCs with DP3 and DP4 were selected for study because work in our previous study indicated that they had middling inhibitory potency and were significantly different from one another in terms of potency (13) Aesculitannin B and parameritannin A $_1$ were selected as A-type PaCs because they are relevant to the diet (from Lychee and cinnamon, respectively), commercially-available, and matched the B-type PaC

selected. The EnzChek Phospholipase A₂ Assay Kit (containing honey bee venom group III PLA₂) was purchased from Invitrogen (Carlsbad, CA).

Phospholipase A₂ Assay

Inhibition of group III PLA₂ was studied using a commercially-available fluorometric assay (Invitrogen). Briefly, test PaCs (final concentration 1 – 100 μ M) was combined with PLA₂ (1 U/mL, in 250 mM Tris-HCl, 500 mM NaCl, 5 mM CaCl₂, pH 8.9). After addition of the fluorogenic substrate (Red/Green BODIPY PC-A₂, 1.67 μ M), the reaction was incubated at room temperature for 10 min and the fluorescence was determined at λ_{ex} = 485 nm and λ_{em} = 538 nm. All reactions were normalized to vehicle-treated controls.

In silico Modeling

An *in silico* model of PLA₂ was developed by analysis of the crystal structure of bee venom PLA₂ (PDB code 1poc, (14)). The enzyme crystal structure was reported with a transition state analog bound in proximity to His34 in the active site (Fig. 3A). The active site is contained within a tunnel formed by three helices (shown in lime green and blue) and the calcium binding loop near the N-terminus. The tunnel is approximately 20 Å in length and having a maximum diameter of 11 Å.

Initial atomic coordinates for the PaCs were built using the online PRODRG server (<http://davapc1.bioch.dundee.ac.uk/prodrg/>). The PaCs thus generated were manually moved and rotated into the active site tunnel of the PLA₂ structure using the program COOT (14, 15). The initial model of the complex of PaCs and PLA₂ from COOT was energy minimized using the online YASARA server (16). The minimized model was analyzed and figures generated using the program PYMOL (The PyMOL Molecular Graphics System, Version 1.4.1, Schrödinger, LLC.).

Results and Discussion

Both the A-type compounds (aesculitannin B and parameritannin A₁) inhibited the activity of PLA₂ in a dose-dependent manner (Fig. 2). The Parameritannin A₁, the tetramer, was more potent than aesculitannin B, the trimer (35% inhibition vs 18% inhibition at 50 μ M). Both compounds were significantly less potent than the B-type analogs, which showed IC₅₀ values of 16 and 10 μ M, respectively (Fig. 2). The increase in inhibitory potency with DP, observed for both A- and B-type PaCs, is in agreement with our previous studies on PLA₂ using B-type PaCs isolated from cocoa (13).

Based on the transition state analog crystal structure (Fig. 3A), we modeled the B-type trimer and tetramer PaCs with bee venom PLA₂ ([3], Fig. 3B and C). The model indicated that these PaCs would sterically occlude the active site from substrate binding. The higher the DP, the greater the van der Waals interactions observed with the residues lining the tunnel. This aspect of the model seems to account for the increase in potency of B-type PaCs as a function of DP. The compounds located close to active site residues His34, Asp35, Asp64 and Tyr87 (shown in magenta, Fig. 3D and 3E). The hydrophobic residues Ile1, Ile2, Trp8, Cys9, Cys31, Met36, Leu59, Leu90, Ile91, Phe67, Val83 and Met86 (shown in cyan (Fig. 3C and D) stabilize the binding of the PaCs to bee venom PLA₂ through van der Waals interactions. Binding of the PaCs in the hydrophobic tunnel is expected to result in non-competitive inhibitory kinetics with regard to substrate concentration. Indeed, we have previously reported that B-type PaCs inhibit PLA₂ activity in a non-competitive manner with regard to substrate (13). Non-linear A-type PaCs also bind in the channel leading to the active site of PLA₂, and binding is stabilized by similar van der Waals interactions.

Surface representations of bee venom PLA₂ show a closed hydrophobic tunnel where substrate/inhibitor binds (Fig. 4A, represented as sticks in cyan). Our models on the linear B-type trimer and tetramer PaCs tightly occupy this region and preserve the integrity of the tunnel (Fig. 4A). By contrast, binding of the non-linear A-type PaCs induces a shift of the calcium-binding loop (X-Cys-Gly-X-Gly), which is close to the N-terminus and is flexible (Fig. 4B). This shift of the loop results in an open hydrophobic channel. Such an open structure would allow easier release of the bound inhibitor, and would be predicted to result in a concomitant decrease in inhibitory potency. This prediction is borne out by our enzyme inhibition curves (Fig.2).

The results of our enzyme inhibition studies, as well as the *in silico* modeling studies, would suggest that extracts from food and non-food plants containing high levels of B-type PaCs would have strong PLA₂ inhibitory activity and perhaps strong anti-inflammatory activity. By contrast, those containing mainly A-type PaCs, for example cranberries, would be less potent. Moreover, extracts with a higher content of B-type PaCs with higher DP would be expected to be more potent PLA₂ inhibitors than extracts which contained largely monomeric EC, (+)-catechin, and PaC dimers. Such increased potency results in the increased number of interactions between the higher DP PaCs and the hydrophobic residues which line the active site channel of PLA₂ compared to lower DP PaCs. The experimental data in our previous report on cocoa PaCs and PLA₂ supports this prediction, although further work is needed with other food and non-food plants (13).

In summary, we have used *in silico* models of group III secreted PLA₂ to develop an understanding of the chemical interactions that govern the inhibitory activity of PaCs. Further, we have developed some understanding of the structural characteristics of PaCs which potently inhibit PLA₂ (*i.e.* B-type PaCs) and those that do not (*i.e.* A-type PaCs). This model is supported by experimental inhibition studies, but further X-ray crystallographic studies are needed to confirm the predicted inhibitor-enzyme complex.

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Abbreviations

DP	degree of polymerization
PaC	proanthocyanidin
PLA ₂	phospholipase A ₂

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Figure 1.
Structure of test proanthocyanidins.

Figure 2.

Inhibition of group III (honey bee venom) PLA₂ by test proanthocyanidins. A-Type and B-type trimers (DP =3) and tetramers (DP = 4) were compared. Activity was normalized to the vehicle-treated controls and expressed as the mean $\pm$ standard deviation of at least three independent experiments.

Figure 3. Model of PLA₂ with B-type PaCs. Based on the transition state analog crystal structure (A), both the B-type trimer (B) and tetramer (C) were modeled with group III (honey bee venom) PLA₂. Key amino acids involved in binding are indicated for both trimer (D) and tetramer (E). Active site residues are indicated in magenta and key non-active site hydrophobic residues are indicated in cyan.

Figure 4.

Comparison on the effects of binding of B-type or A-type PaCs on the three dimensional structure of group III (honey bee venom) PLA₂. (A) The B-type tetramer (represented as sticks in cyan) occupy a closed hydrophobic tunnel in the crystal structure of bee venom PLA₂ without significantly altering it. (B) On binding of the A-type (non-linear) tetramer, the tunnel is rendered open.