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# Inhibition of Secreted Phospholipase $A_2$ by Proanthocyanidins: A Comparative Enzymological and in Silico Modeling Study

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**ABSTRACT:** Secreted phospholipase $A_2$ ($PLA_2$) plays a critical role in mobilizing arachidonic acid in phospholipids. We have previously reported that $PLA_2$ 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 $PLA_2$ using in silico techniques. The B-type trimer and tetramer inhibited $PLA_2$ ($IC_{50}$ = 16 and 10 $\mu$M). The A-type compounds were less potent (18−35% inhibition at 50 $\mu$M). The active site of $PLA_2$ 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-$PLA_2$ activity.

**KEYWORDS:** *proanthocyanidin, phospholipase $A_2$, 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 nonfood plants including cocoa (*Theobroma cacao*), cranberries (*Vaccinium macrocarpon*), apricots (*Prunus armeniaca*), and grapes (*Vitis vinifera*).[1,2] PaCs are broadly classified on the basis of the type of intermonomeric 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 $B_1$ is an example of an A-type tetramer (Figure 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 $C_1$ is an example of a B-type trimer (Figure 1). These differences in linkage types result in significantly different three-dimensional geometries.

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 of 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/day cocoa for 4 weeks reduced markers of inflammation including P-selectin and 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 that 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 have roles 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 $IC_{50}$ of <10 $\mu$M. Kinetic analysis showed that the PaC pentamer and decamer inhibited $PLA_2$ in a noncompetitive manner with respect to substrate concentration.

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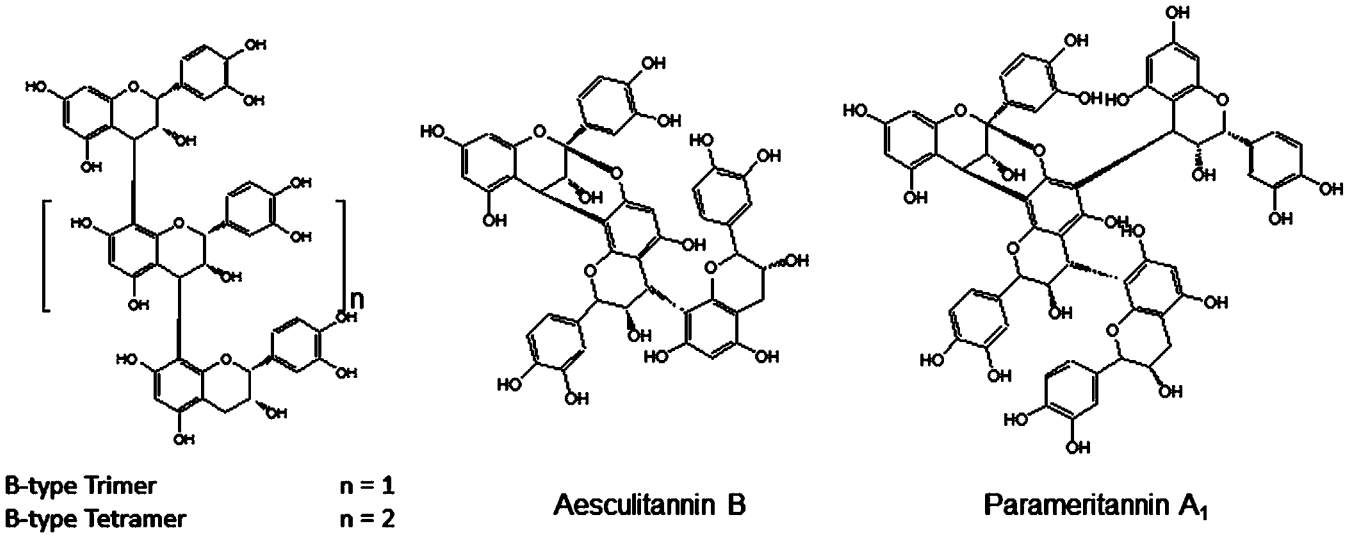


**Figure 1.** Structures of test proanthocyandins.

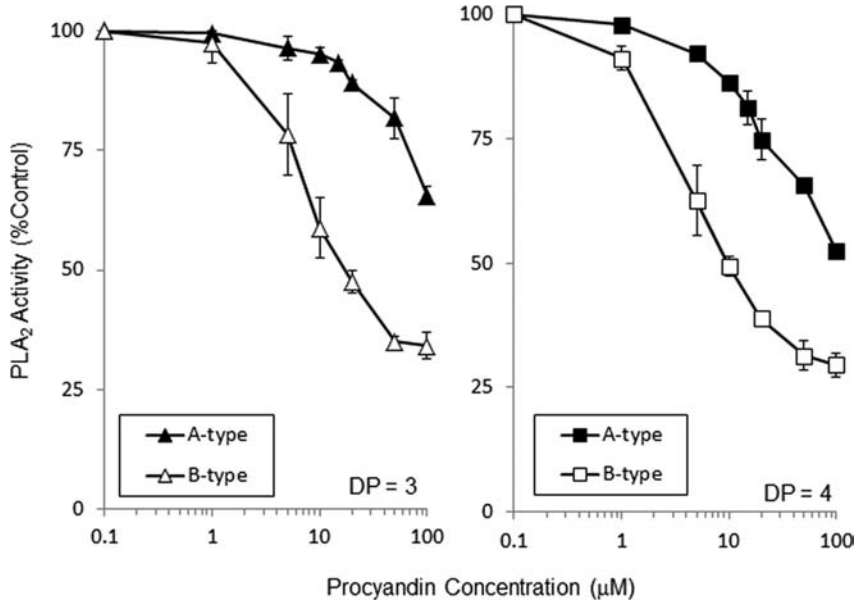


**Figure 2.** Inhibition of group III (honey bee venom) $PLA_2$ by test proanthocyanidins. A-type and B-type trimers (DP = 3) and tetramers (DP = 4) were compared. Activity was normalized to the vehicle-treated controls and expressed as the mean ± standard deviation of at least three independent experiments.

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 $A_1$ 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, USA) and were >90% pure. Stock solutions (20 mM) were prepared in dimethyl sulfoxide and stored at −80 °C. B-type PaCs with DP3 and DP4 were selected for study because our previous work indicated that they had intermediate 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 $A_2$ Assay Kit (containing honey bee venom group III $PLA_2$) was purchased from Invitrogen (Carlsbad, CA, USA).

**Phospholipase $A_2$ Assay.** Inhibition of group III $PLA_2$ was studied using a commercially available fluorometric assay (Invitrogen). Briefly, test PaCs (final concentration = 1−100 $\mu$M) was combined with $PLA_2$ (1 unit/mL, in 250 mM Tris-HCl, 500 mM NaCl, 5 mM $CaCl_2$, pH 8.9). After addition of the fluorogenic substrate (Red/Green BODIPY PC-$A_2$, 1.67 $\mu$M), the reaction was incubated at room temperature for 10 min, and the fluorescence was determined at $\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 538 nm. All reactions were normalized to vehicle-treated controls.

**In Silico Modeling.** An in silico model of $PLA_2$ was developed by analysis of the crystal structure of bee venom $PLA_2$ (PDB code 1poc).[14] The enzyme crystal structure was reported with a transition state analogue bound in proximity to His34 in the active site (Figure 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 has a maximum diameter of 11 Å.


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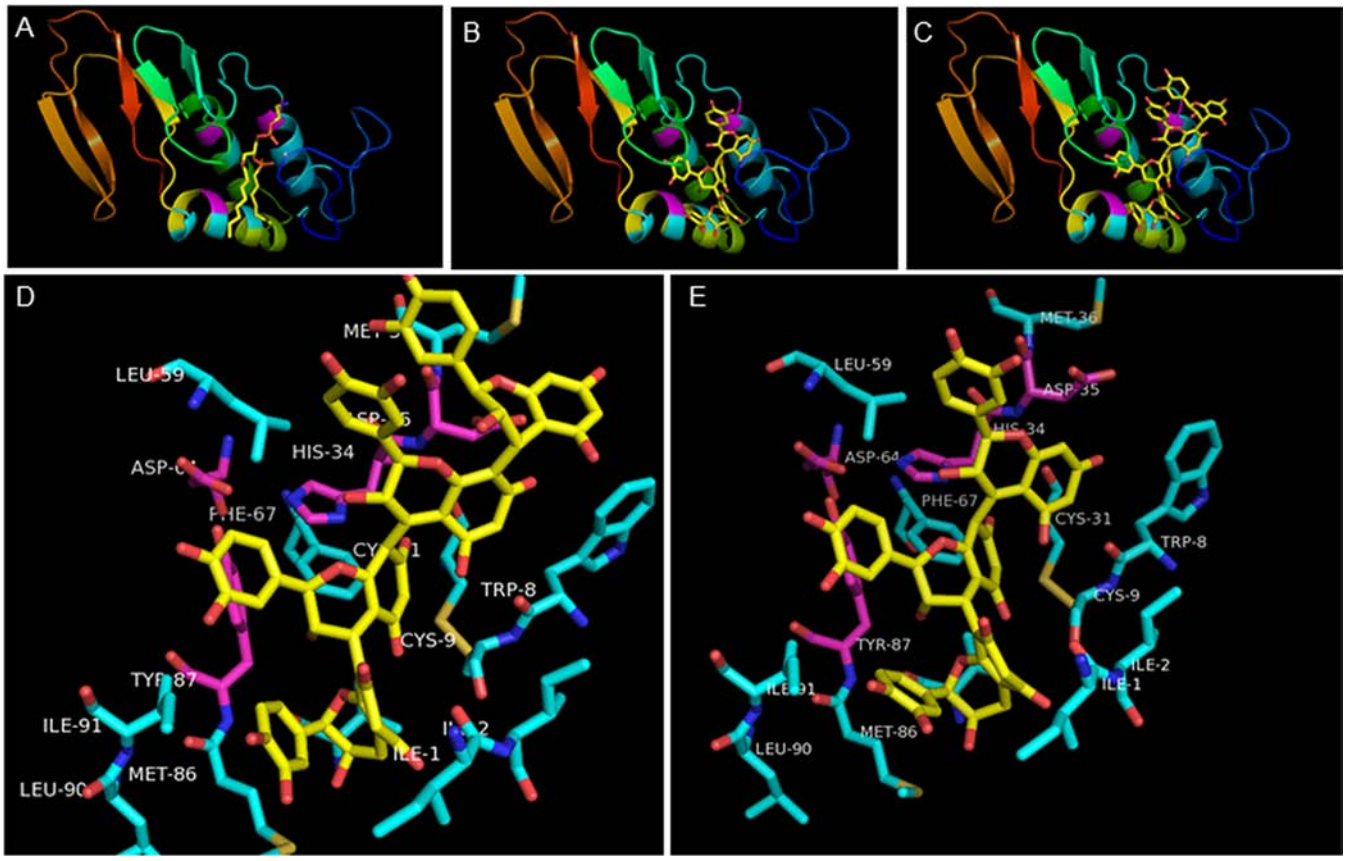


**Figure 3.** Model of $PLA_2$ with B-type PaCs. Based on the transition state analogue crystal structure (A), both the B-type trimer (B) and tetramer (C) were modeled with group III (honey bee venom) $PLA_2$. Key amino acids involved in binding are indicated for both trimer (D) and tetramer (E). Active site residues are indicated in magenta and key nonactive site hydrophobic residues in cyan.

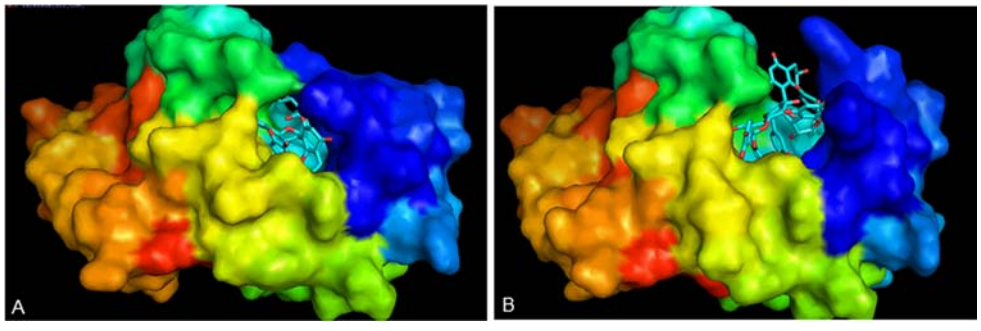


**Figure 4.** Comparison of the effects of binding of B-type or A-type PaCs on the three-dimensional structure of group III (honey bee venom) $PLA_2$s: (A) the B-type tetramer (represented as sticks in cyan) occupies a closed hydrophobic tunnel in the crystal structure of bee venom $PLA_2$ without significantly altering it; (B) on binding of the A-type (nonlinear) tetramer, the tunnel is rendered open.

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 $PLA_2$ structure using the program COOT.[14,15] The initial model of the complex of PaCs and $PLA_2$ from COOT was energy minimized using the online YASARA server.[16] The minimized model was analyzed, and figures were generated using the program PYMOL (The PyMOL Molecular Graphics System, version 1.4.1, Schrödinger, LLC).

## RESULTS AND DISCUSSION

Both A-type compounds (aesculitannin B and parameritannin $A_1$) inhibited the activity of $PLA_2$ in a dose-dependent manner (Figure 2). Parameritannin $A_1$, the tetramer, was more potent than aesculitannin B, the trimer (35% inhibition vs 18% inhibition at 50 $\mu$M). Both compounds were significantly less potent than the B-type analogues, which showed $IC_{50}$ values of 16 and 10 $\mu$M, respectively (Figure 2). The increase in inhibitory potency with DP, observed for both A- and B-type PaCs, is in agreement with our previous studies on $PLA_2$ using B-type PaCs isolated from cocoa.[13]

On the basis of the transition state analogue crystal structure (Figure 3A), we modeled the B-type trimer and tetramer PaCs with bee venom $PLA_2$ ([3] Figure 3B,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


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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 in Figure 3D,E). The hydrophobic residues Ile1, Ile2, Trp8, Cys9, Cys31, Met36, Leu59, Leu90, Ile91, Phe67, Val83, and Met86 (shown in cyan in Figure 3C,D) stabilize the binding of the PaCs to bee venom $PLA_2$ through van der Waals interactions. Binding of the PaCs in the hydrophobic tunnel is expected to result in noncompetitive inhibitory kinetics with regard to substrate concentration. Indeed, we have previously reported that B-type PaCs inhibit $PLA_2$ activity in a noncompetitive manner with regard to substrate.[13] Nonlinear A-type PaCs also bind in the channel leading to the active site of $PLA_2$, and binding is stabilized by similar van der Waals interactions.

Surface representations of bee venom $PLA_2$ show a closed hydrophobic tunnel where substrate/inhibitor binds (Figure 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 (Figure 4A). By contrast, binding of the nonlinear 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 (Figure 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 (Figure 2).

The results of our enzyme inhibition studies, as well as the in silico modeling studies, would suggest that extracts from food and nonfood plants containing high levels of B-type PaCs would have strong $PLA_2$ 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 $PLA_2$ inhibitors than extracts that contained largely monomeric (−)-epicatechin, (+)-catechin, and PaC dimers. Such increased potency results in the increased number of interactions between the higher DP PaCs and the hydrophobic residues that line the active site channel of $PLA_2$ compared to lower DP PaCs. The experimental data in our previous report on cocoa PaCs and $PLA_2$ support this prediction, although further work is needed with other food and nonfood plants.[13]

In summary, we have used in silico models of group III secreted $PLA_2$ to develop an understanding of the chemical interactions that govern the inhibitory activity of PaCs. Furthermore, we have developed some understanding of the structural characteristics of PaCs that potently inhibit $PLA_2$ (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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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

DP, degree of polymerization; PaC, proanthocyanidin; $PLA_2$, phospholipase $A_2$.

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