# Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins

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**Abstract** **It was determined that flavan-3-ols and procyanidins have an inhibitory effect on angiotensin I converting enzyme (ACE) activity, and the effect was dependent on the number of epicatechin units forming the procyanidin. The inhibition by flavan-3-ols and procyanidins was competitive with the two substrates assayed: *N*-hippuryl-L-histidyl-L-leucine (HHL) and *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG). Tetramer and hexamer fractions were the more potent inhibitors, showing Ki of 5.6 and 4.7 μM, respectively. As ACE is a membrane protein, the interaction of flavanols and procyanidins with the enzyme could be related to the number of hydroxyl groups on the procyanidins, which determine their capacity to be adsorbed on the membrane surface.**


*Key words:* Flavonoid; Hypertension; Cocoa; Oxidative stress

## 1. Introduction

Several epidemiological studies have shown associations between the regular consumption of flavonoid-rich foods and a decreased risk for cardiovascular disease [1–3]. While the inverse association between the risk for vascular disease and dietary flavonoid intake does not prove causality, an increasing amount of experimental data pertaining to certain flavonoids does lend support to this association [4].

Flavonoids and other polyphenols are widely distributed in the human diet, primarily in plant-derived foods and beverages. Procyanidins are a group of polymeric polyphenols composed of the flavan-3-ol units, (−)-epicatechin (epicatechin) and (+)-catechin (catechin). Procyanidins are present in foods such as nuts, cranberries, apples, red wine, tea and cocoa or chocolate [5,6]. Flavan-3-ols and procyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and other protective effects on human health. In vitro, procyanidins can, among other effects, delay the rate of low density lipoprotein oxidation [7], reduce the rate of DNA oxidative damage [8], reduce platelet reactivity [9,10], and modulate the oxidant-responsive transcription factor NF-κB [11].

Angiotensin I converting enzyme (ACE) is a glycoprotein peptidyldipeptide hydrolase, whose main known functions are to cleave histidyl-leucine from angiotensin I forming the potent vasoconstrictor angiotensin II, and to degrade bradykinin to inactive peptides [12]. ACE has two active sites, N- and C-terminal, with different affinities for different substrates. ACE inhibition is considered to be an important therapeutic approach in the treatment of high blood pressure, and the intake of certain synthetic ACE inhibitors provides definitive positive health effects [13]. However, as ACE inhibitors are pharmacological drugs their use in healthy or low-risk populations is not advisable. The finding that certain flavonoid-rich foods can induce reductions in blood pressure and inhibit ACE activity, both in vivo and in vitro [14–19], opens up the possibility that consumption of select flavonoid-rich foods may mimic synthetic ACE inhibitors and provide health benefits but without adverse side effects. In this regard, it was observed that after the regular consumption of a diet rich in flavan-3-ols and procyanidins for 14 days, blood pressure was significantly diminished in aged people [20].

The aim of the present work was to determine if flavan-3-ols and procyanidins have an inhibitory effect on ACE activity, and to characterize such inhibition from a kinetic point of view. We studied ACE inhibition based on the two commonly employed methods for determining ACE activity [21,22]. It was observed that flavan-3-ols and procyanidins isolated from cocoa compete for enzyme-active sites with synthetic ACE substrates. The effects of flavan-3-ols and procyanidins on ACE could contribute to the decreased risk for cardiovascular and other diseases observed in populations that consume high amounts of foods rich in flavonoids.

## 2. Materials and methods

### 2.1. Materials

ACE from rabbit lung (purified ACE), hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL), *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG), epicatechin, and catechin were from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile and acetic acid were from Merck KGaA (Darmstadt, Germany). Purified procyanidin fractions (epicatechin dimers, trimers, tetramers, pentamers, and hexamers) were obtained from Mars Inc. (Hackettstown, NJ, USA). The purity of the procyanidin fractions was 99.0, 94.8, 95.4, 92.0, and 86.2% for dimer, trimer, tetramer, pentamer, and hexamer fractions, respectively.

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*Abbreviations:* ACE, angiotensin I converting enzyme; HHL, *N*-hippuryl-L-histidyl-L-leucine; HA, hippuric acid; FAPGG, *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine

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### 2.2. Inhibition of purified ACE activity

A mixture consisting of 100 μl of 6.3 nM purified ACE and 100 μl of flavan-3-ols or procyanidins (0–1500 μM) was preincubated for 30 min at 37°C. For ACE activity determination, the 200-μl mixture was added to 100 μl of HHL (1.5–12 mM) in 50 mM HCl–Tris, 300 mM NaCl (pH 8.3) and incubated from 20 to 90 min at 37°C [21]. The reaction was stopped by addition of 100 μl of 12% (w/v) phosphoric acid. The HA was separated and quantified by HPLC with UV detection [23]. The system consisted of a Supelcosil LC-18-DB column (15 cm×4.6 mm×5 μm) using a mobile phase composed of 5 mM phosphoric acid:acetonitrile (80:20 v/v), pH 2.5 (flow rate, 1 ml/min; retention time, 3.65 min). The UV detection was carried out at 228 nm (Jasco UV 1575, Intelligent UV/VIS Detector, Japan Spectroscopic Co. Ltd.). Commercial HA was used as standard.

For FAPGG hydrolysis determination, a 50-μl aliquot of the 200 μl mixture was added to 280 μl of FAPGG (50–400 μM) dissolved in 0.1 M potassium phosphate buffer, 0.5 M NaCl, 0.1 mM $ZnCl_2$, pH 7.5. After incubating for 10 min at 37°C, hydrolysis of FAPGG by ACE was quantified by recording the decrease of absorbance at 340 nm [22].

### 2.3. Kinetic calculations

The kinetic parameters were calculated by adjusting curves to the Michaelis–Menten equation: $V_i = (V_{maxA} \times S)/(K_{MA}+S)$ where $V_i$ = initial rate; $V_{maxA}$ = apparent maximum rate; $K_{MA}$ = apparent Michaelis constant, and $S$ = substrate concentration. $K_{MA}$ and $V_{maxA}$ were plotted vs. concentration of inhibitor ($I$). $K_i$ values were calculated adjusting the curves to the equation: $K_{MA} = K_M(1+I/K_i)$.

The $IC_{50}$ were calculated by adjusting inhibition curves to the equation for a two-parameter hyperbolic decay: $f = (V_i \times IC_{50})/(IC_{50}+I)$, in which $V_i$ = initial rate with 1 mM HHL. The $K_i$ values were calculated from $IC_{50}$ considering a competitive inhibition with the following equation: $K_i = IC_{50}/(1+(S/K_M))$. Gibbs free energy ($\Delta G$) was calculated as $\Delta G = RT\ln K_i$.

### 2.4. Data analysis

Curves were adjusted using routines available in Sigma Plot 5.0. Data were analyzed for statistical significance using ANOVA (StatView 5, Mountain View, CA, USA).

## 3. Results

### 3.1. Kinetics of the inhibition of purified ACE activity by epicatechin, dimer and hexamer fractions

For the kinetics studies epicatechin, dimer, and hexamer fractions were used as ACE inhibitors. Inhibition was evaluated by means of two different ACE activity assays, i.e. HHL and FAPGG hydrolysis. Fig. 1 shows the inhibition on ACE activity (reduction of $V_i$) by the hexamer fraction using the HHL hydrolysis assay (Fig. 1A), and by the dimer fractions using the FAPGG hydrolysis (Fig. 1B). Similar curves were obtained when epicatechin or dimer were assayed as inhibitors of FAPGG hydrolysis, and epicatechin or hexamer were assayed inhibiting HHL hydrolysis.

Purified ACE showed a Michaelis–Menten mechanism using HHL or FAPGG as substrates. The apparent maximum rate of substrate hydrolysis ($V_{maxA}$) and the apparent Michaelis constant ($K_{MA}$) were determined to characterize the kind of inhibition exerted by flavan-3-ols and procyanidins. $K_{MA}$ for both HHL and FAPGG hydrolysis were plotted against epicatechin, dimer, or hexamer concentrations (Fig. 1C,D). $K_{MA}$ values were dependent on the inhibitor and its concentration. By contrast, the $V_{maxA}$ were independent of the inhibitor concentration ($3.0 \pm 0.3$, $3.4 \pm 0.4$, and $3.6 \pm 0.2$ μM $min^{-1}$ for FAPGG and $0.8 \pm 0.2$, $1.2 \pm 0.3$ and $1.5 \pm 0.3$ μM $min^{-1}$ for HHL for epicatechin, dimer, and hexamer, respectively). These data indicate a competitive enzyme inhibition.

The dissociation constant for the binding of inhibitor to the free enzyme ($K_i$) was calculated for the ACE inhibition by epicatechin, dimer, and hexamer, using both substrates, HHL and FAPGG. $K_i$ values showed significant differences between both substrates for epicatechin (1302 μM for HHL and 39 μM for FAPGG) and dimer (227 μM for HHL and 35

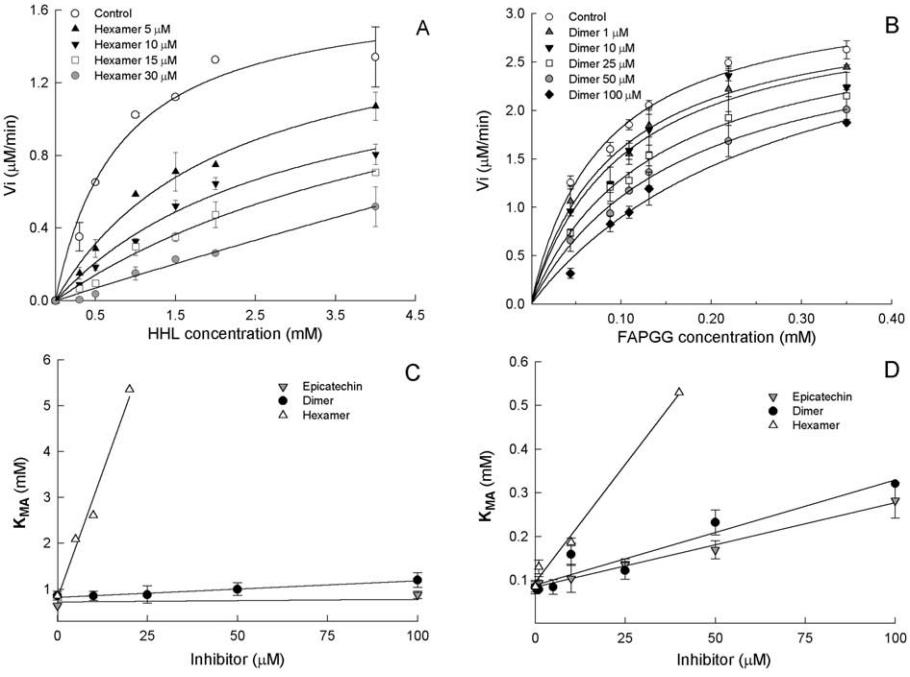


Fig. 1. Initial velocities for the inhibition of ACE activity by epicatechin, dimer and hexamer vs. substrate concentration (A,B) and $K_{MA}$ vs. epicatechin, dimer, and hexamer concentrations for HHL (C) or FAPGG (D). Purified lung ACE was preincubated at 37 °C for 30 min in the absence (control), or the presence of different amounts of epicatechin, dimer or hexamer, and then incubated from 20 to 90 min in the presence of different concentrations of HHL or FAPGG. Values are mean ± S.E.M. of at least three independent experiments.

μM for FAPGG). However, hexamer presented $K_i$ in the same range with both substrates (4 μM for HHL and 12 μM for FAPGG). The inhibition of HHL hydrolysis was more clearly related to the number of monomer units than FAPGG hydrolysis.

### 3.2. Inhibition of purified ACE activity by epicatechin and procyanidin fractions

Based on the results obtained above, procyanidins were tested to investigate whether their inhibitory effect was related to polymer size. The preincubation of purified ACE in the presence of flavan-3-ols or procyanidins inhibited the transformation of HHL into HA in a dose-dependent manner (Fig. 2). The inhibition was also dependent on the procyanidin molecular weight. $IC_{50}$ was $1781 \pm 381$, $1593 \pm 443$, $267 \pm 24$, $126 \pm 11$, $12 \pm 1$, $25 \pm 3$, and $10 \pm 1$ μM, for epicatechin, catechin, dimer, trimer, tetramer, pentamer, and hexamer, respectively.

The dissociation constant for the binding of inhibitor to the free enzyme ($K_i$) was calculated taking into account that enzyme inhibition was competitive. The $K_i$ were 828, 124, 59, 5.6, 11.6, and 4.7 μM for epicatechin, dimer, trimer, tetramer, pentamer and hexamer, respectively. These values were on the same order as those calculated previously by the kinetic assay for epicatechin (1302 and 828 μM), dimer (227 and 124 μM), and hexamer (4 and 4.7 μM).

Using the $K_i$ values, changes in Gibbs free energy ($\Delta G$) were calculated and $IC_{50}$ and $\Delta G$ were plotted versus units of monomers present in procyanidins (Fig. 3). The coefficient for linear regression ($R^2$) was 0.87 for $\Delta G$, and the average energy increase per unit of monomers was $2.6 \pm 0.5$ kJ/mol ($P < 0.01$).

## 4. Discussion

We postulate that the observed effect of the consumption flavonoid-rich foods reducing blood pressure in humans [20,24] and in rats [25] can be related to the inhibition of ACE by flavan-3-ols and procyanidins. Flavonoid-containing plant extracts have been described as possible ACE inhibitors [14,15,26]. In this study, epicatechin, as well as procyanidins, inhibited the activity of rabbit lung ACE.

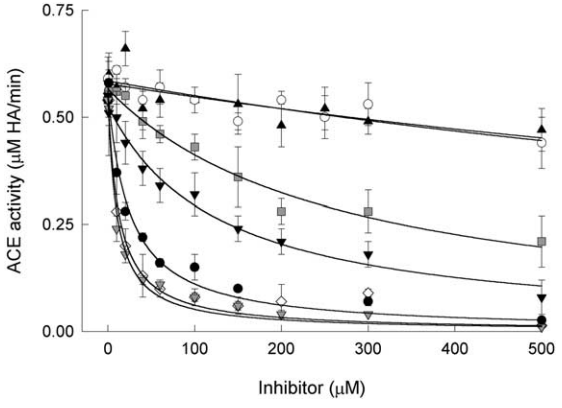


Fig. 2. Effect of different concentrations of flavan-3-ols and procyanidins on ACE activity. Purified lung ACE was preincubated at 37°C for 30 min in the absence (control), or the presence of different amounts of flavan-3-ols or procyanidins, and then during 60 min in the presence of HHL (5.2 mM). Inhibitors assayed were: catechin (black triangle up), epicatechin (empty circles), dimer (gray square), trimer (black triangle down), tetramer (empty diamond), pentamer (black circle), and hexamer (gray triangle down). Values are mean ± S.E.M. of at least three independent experiments.

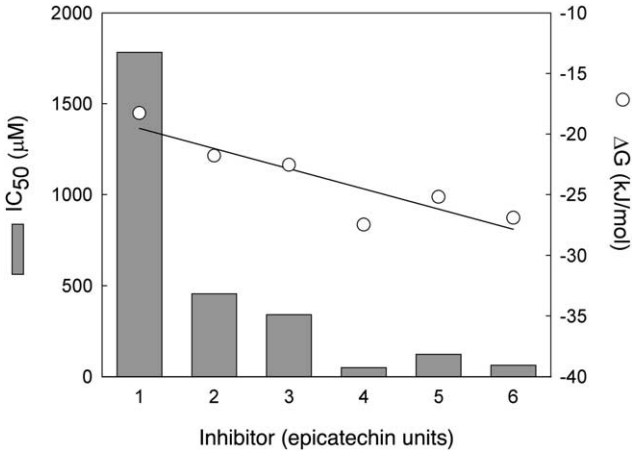


Fig. 3. Effect of the number of epicatechin units in procyanidins on $IC_{50}$ and $\Delta G$ values. Values of $IC_{50}$ and $\Delta G$ were calculated from the data shown in this figure, and analyzed mathematically as indicated in Section 2. Inhibitors are epicatechin and procyanidins.

The kinetic analysis of the results suggests that flavan-3-ols and procyanidins inhibit the enzyme activity by competing with the substrate for the active sites. The calculated $K_i$ were different depending on the type of substrate used (HHL or FAPGG) for epicatechin and dimer, but were similar for the hexamer. It can be suggested that the larger molecules can inhibit both the C- and the N-active sites to a similar extent; meanwhile, monomers and dimers seem to inhibit preferentially the N-active site.

From thermodynamic considerations, the linear relationship between changes in Gibb's free energy ($\Delta G$) and procyanidin size, and the calculated binding stabilization enhancement of about 2.6 kJ/mol/monomer unit, suggest an increase in hydrogen bond formation between ACE and procyanidin hydroxyl groups [27]. The calculated $\Delta G$ values are on the same order as those estimated for the interaction between hydroxyl groups and some macromolecules [28,29], but are higher than those calculated for the interaction between flavonoids and certain proline-rich proteins [27].

To characterize the relation between the size of procyanidins and their ability to inhibit ACE activity, we assayed the ACE activity using the assay for the hydrolysis of HHL. We observed that catechin and epicatechin were the weaker inhibitors, showing inhibitory effects at concentrations higher than 100 μM. Increasing the number of epicatechin units in the procyanidin yielded an increasing inhibitory effect. Thus, three levels of $IC_{50}$ could be established: $IC_{50}$ in the mM range for monomeric flavan-3-ols, in the 100 μM range for dimer and trimer, and in the 10 μM range for the larger procyanidins. The relevance of these concentrations should be interpreted with caution since they were estimated using non-physiological in vitro conditions (isolated enzyme, synthetic substrates, and high substrate concentration).

With regard to the physiological relevance for the inhibition of ACE by flavan-3-ols and procyanidins it is important to note the actual concentrations of these substances in vivo. The presence of catechin, epicatechin, and dimers has been determined in human plasma at low micromolar concentrations

[30], but there are few reports on tissue concentrations of monomer and dimer, and no studies addressing the presence of larger procyanidins in tissues. As ACE is a membrane-bound enzyme, and flavan-3-ols and procyanidins, especially the larger molecules, can adsorb to lipid–water interphases [31], it is feasible that a local enrichment of these compounds on the membrane surface could lead to an enhanced interaction with the enzyme. This accumulation could take place on the membranes of vascular endothelial cells, which are thought to be responsible for regulating blood pressure [32].

Supporting the concept that ACE inhibition could partially explain the health effects of flavonoids in general, we have shown that ACE inhibitors can modulate antioxidant defenses and regulate mitochondrial NO production [33]. Thus, it is possible to speculate that flavonoids could regulate antioxidant defenses through mechanisms that involve ACE, underscoring the role of flavan-3-ols and procyanidins in the modulation of oxidative stress, vascular function and cardiovascular disease.

In summary, the results presented here afford a biochemical base to consider the inhibition of ACE by flavan-3-ols and procyanidins as a potential mechanism to occur in vivo, explaining the reduction in blood pressure associated with the intake of flavanol-rich foods.

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