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Arachidonate 12-lipoxygenases with reference to their selective inhibitors

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Abstract

Lipoxygenase is a dioxygenase recognizing a 1-*cis*,4-*cis*-pentadiene of polyunsaturated fatty acids. The enzyme oxygenates various carbon atoms of arachidonic acid as a substrate and produces 5-, 8-, 12- or 15-hydroperoxyeicosatetraenoic acid with a conjugated diene chromophore. The enzyme is referred to as 5-, 8-, 12- or 15-lipoxygenase, respectively. Earlier we found two isoforms of 12-lipoxygenase, leukocyte- and platelet-type enzymes, which were distinguished by substrate specificity, catalytic activity, primary structure, gene intron size, and antigenicity. Recently, the epidermis-type enzyme was found as the third isoform. Attempts have been made to find isozyme-specific inhibitors of 12-lipoxygenase, and earlier we found hinokitiol, a tropolone, as a potent inhibitor selective for the platelet-type 12-lipoxygenase. More recently, we tested various catechins of tea leaves and found that (−)-gallocatechin gallate was a potent and selective inhibitor of human platelet 12-lipoxygenase with an IC_{50} of 0.14 μM. The compound was much less active with 12-lipoxygenase of leukocyte-type, 15-, 8-, and 5-lipoxygenases, and cyclooxygenases-1 and -2.

Keywords: Lipoxygenase; Enzyme inhibitor; Arachidonic acid; Hinokitiol; Tea leaf catechin; (−)-Gallocatechin gallate

Lipoxygenase is a dioxygenase which recognizes a 1-*cis*,4-*cis*-pentadiene structure of polyunsaturated fatty acids and incorporates one molecule of oxygen producing a hydroperoxy acid. In 1932, André and Hou [1] reported the presence of "lipoxydase" in soybean. Then, the soybean enzyme has been studied extensively by many investigators [2]. Its broad substrate specificity was reported in terms of the carbon-chain length and the number of double bonds [3]. In 1967, furthermore, Hamberg and Samuelsson [4] demonstrated a stereospecific and position-specific removal of hydrogen atom as the initial step of the soybean "lipoxidase" reaction. At that time, as stated by Tappel [2], it was generally believed that there was no lipoxidase in animal tissues.

In 1975, the study on arachidonate metabolism in human platelets led to the discovery of proaggregatory thromboxane A_2 [5]. During this study another metabolite of arachidonic acid was found and identified to be 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12*S*-HETE) (Fig. 1) [6]. In addition, incubation of arachidonic acid with the 600*g* sediment of homogenized human platelets produced 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12*S*-HPETE) (Fig. 1) which was reduced by $SnCl_2$ to 12-hydroxy derivative with an absorption at 237 nm [6]. These findings demonstrated the presence of a novel lipoxygenase in human platelets. Almost concurrently, it was reported that bovine platelets converted 8-*cis*,11-*cis*- and all-*cis*-8,11,14-eicosapolyenoic acids to 12L-hydroxy-8-*cis*,10-*trans*- and 12L-hydroxy-8-*cis*,10-*trans*,14-*cis*-eicosapolyenoic acids [7]. The lipoxygenase activity was found in the high-speed supernatant of the frozen and thawed bovine platelets, and the primary production of hydroperoxy acid from arachidonic acid was shown with a partially purified enzyme [7]. The bovine platelet enzyme was active with arachidonic acid and other eicosapolyenoic acids, but much less active with octadecapolyenoic acids [7].

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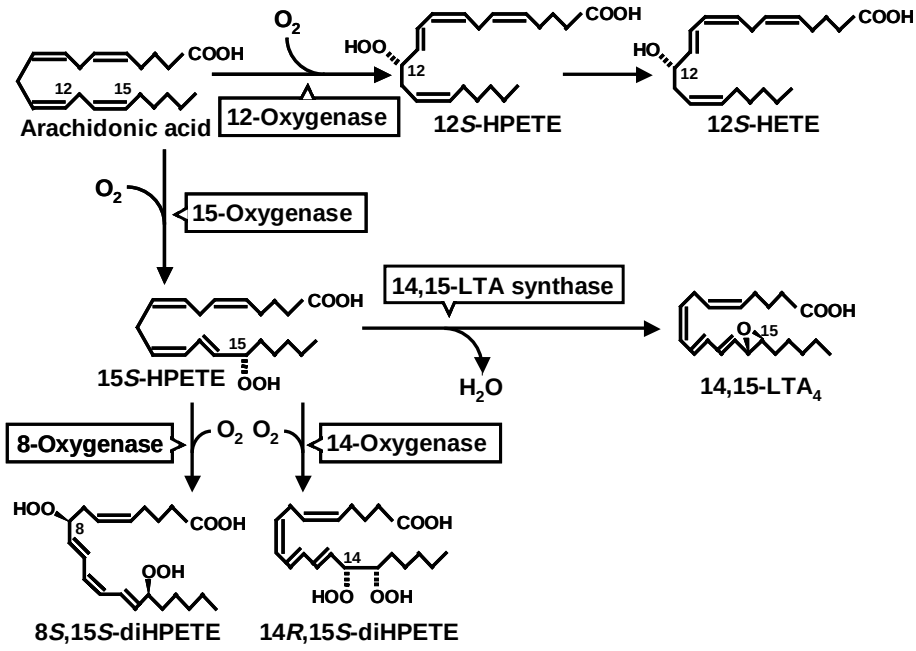


Fig. 1. Reactions catalyzed by arachidonate 12-lipoxygenase.

When we were looking for an enzyme source of 5-lipoxygenase [8], a high activity of 12-lipoxygenase was found in porcine leukocytes, and the enzyme was partially purified [9]. Our attempts to further purify the enzyme were unsuccessful due to the loss of enzyme activity. Then, we attempted to prepare a monoclonal antibody against the enzyme using a crude enzyme preparation as an antigen. We could obtain a hybridoma clone which produced a monoclonal antibody binding to porcine leukocyte 12-lipoxygenase [10]. The antibody was utilized for immunoaffinity chromatography of the enzyme; namely, the antibody was conjugated to Affi-Gel 10. The enzyme in the high-speed supernatant of porcine leukocytes was collected by ammonium sulfate fractionation and then applied to a column of immobilized anti-12-lipoxygenase antibody. A bulk of inactive proteins passed through the column, and the enzyme was adsorbed to and eluted from the column as an almost homogeneous enzyme [11]. The same method was applied to the purification of 12-lipoxygenases from bovine [12] and human [13] platelets. While we were handling these monoclonal anti-12-lipoxygenase antibodies, we found that the antibodies against platelet and leukocyte 12-lipoxygenases did not cross-react with the enzymes purified from leukocytes and platelets, respectively [12,13]. This finding suggested the occurrence of 12-lipoxygenase isozymes, which was established later by various experimental results such as substrate specificity, suicide inactivation, multifunctional catalyses (leukotriene A_4 and dihydroperoxide syntheses as shown in Fig. 1), tissue distribution, primary structure elucidated by cloning of enzyme cDNA, and size of introns of enzyme gene (Table 1). Refs. [14–17] are our review articles including the descriptions of 12-lipoxygenase isozymes. Later, epidermal 12-lipoxygenase was characterized as another isoform [18] (Table 1).

12-Lipoxygenase is involved in the biosynthesis of two groups of bioactive eicosanoids; namely, hepoxilins and lipoxins. Hepoxilins A_3 and B_3 are 8- and 10-hydroxy-11,12-epoxy-derivatives of arachidonic acid. Hepoxilins show various biological activities related to the release of intracellular calcium and the opening of potassium channel [19,20]. In addition, lipoxins A_4 and B_4 are trihydroxy derivatives of arachidonic acid with a conjugated tetraene, and 12-lipoxygenase is involved in the transcellular lipoxin biosynthesis from arachidonic acid via leukotriene A_4. These compounds function as endogenous braking signals in host defense, inflammation, and hypersensitivity [21,22]. Furthermore, many findings have been reported with regard to biological activities of 12*S*-HPETE and 12*S*-HETE, and these reports were reviewed in [14]. Recently, the role of leukocyte-type 12-lipoxygenase in LDL-oxidation leading to atherosclerosis has been intensively investigated [23]. However, as stated by Kühn and Thiele [24] in their review article "The diversity of the lipoxygenase family. Many sequence data but little information on biological significance," "for most lipoxygenase isoforms their biological roles are far from being clear. According to the concept of the arachidonic acid cascade, lipoxygenases are involved in the biosynthesis of lipid hormones. However, certain lipoxygenase subtypes may play a biological role outside the arachidonic cascade."

An enzyme inhibitor is a pharmacological tool to elucidate the biological significance of the enzyme; for example, the use of aspirin and indomethacin as cyclooxygenase inhibitors in in vivo experiments suggests the involvement of prostaglandin or thromboxane in a certain biological function. Earlier, gossypol was reported to inhibit 5- and 12-lipoxygenases [25], and panaxynol was shown to inhibit leukocyte-type 12-lipoxygenase and 5-lipoxygenase [26]. As

Table 1
Three isoforms of 12-lipoxygenase

Properties	Porcine leukocyte[a,b]	Human platelet[a,b]	Murine epidermis[c]
Substrate specificity			
Arachidonic acid	Active	Active	Active
Linoleic acid	Active	Almost inactive	Inactive
Docosahexaenoic acid	Active	Less active	Almost inactive
Phospholipids	Active	Almost inactive	Inactive
Reaction products from arachidonic acid			
12-HPETE/15-HPETE	9:1	99:1	6:1
Other products	14,15-Leukotriene A_4 8,15- and 14,15-DiHPETE	Almost none	—
Suicide inactivation	Marked	Almost none	—
Immunogenicity			
Anti-leukocyte 12-lipoxygenase	Reactive	Unreactive	—
Anti-platelet 12-lipoxygenase	Unreactive	Reactive	—
Amino acid identity with 15-lipoxygenase (%)[e,d]	79.3	60.9	64.6
Exon–intron structure compared with 15-lipoxygenase [e]	Similar	Different	Different

[a] Ref. [14].
[b] Ref. [16].
[c] Ref. [18].
[d] Ref. [24].
[e] Rabbit reticulocyte 15-lipoxygenase.

an isoform-specific inhibitor of 12-lipoxygenase, 4-(2-oxapentadeca-4-yne)phenylpropanoic acid was developed. The compound was more inhibitory by about three orders of magnitude to leukocyte-type 12-lipoxygenase than to platelet-type 12-lipoxygenase [27], and no inhibition of human platelet 12-lipoxygenase was observed at concentrations as high as 10 μM [28]. As an inhibitor of platelet-type 12-lipoxygenase, 3-methoxytropolone isolated from *Streptoverticillium hadanonense* KY11449 inhibited the enzyme of bovine platelet cytosol with an IC_{50} of 1.8 μM. The compound was inhibitory to 5-lipoxygenase of RBL-1 cells with an IC_{50} of 280 μM. Soybean lipoxygenase and cyclooxygenase of bovine platelet microsomes were hardly affected [29]. Our further studies with tropolone derivatives [30] demonstrated a potent inhibition of human platelet 12-lipoxygenase by hinokitiol (4-isopropyltropolone) with an IC_{50} of 0.1 μM. The compound was much less effective on porcine leukocyte 12-lipoxygenase (IC_{50}: 50 μM), rabbit reticulocyte 15-lipoxygenase (>100 μM), human leukocyte 5-lipoxygenase (17 μM), sheep seminal vesicle cyclooxygenase-1 (>100 μM), and mouse cyclooxygenase-2 (>100 μM).

Catecholic antioxidants are known to inhibit lipoxygenases; for example, nordihydroguaiaretic acid has been used widely as a non-specific lipoxygenase inhibitor. Tea leaf catechins are currently noted as antioxidants and utilized as food supplements. We were interested in tea leaf catechins as possible lipoxygenase inhibitors and tested various catechins with several lipoxygenases available in our research group.

Materials and methods

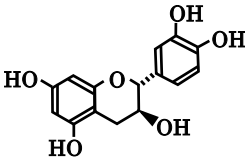
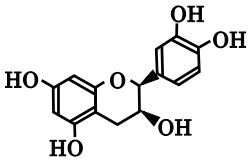
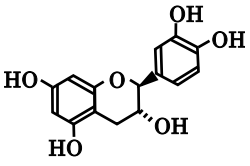
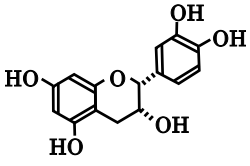
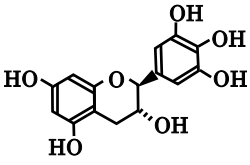
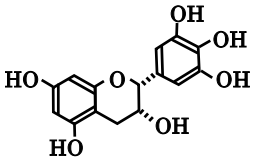
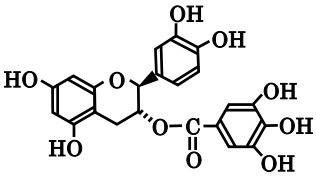
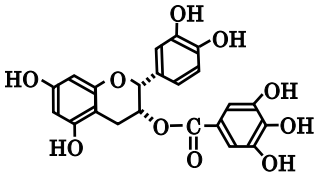
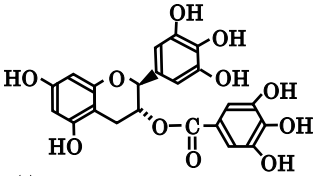
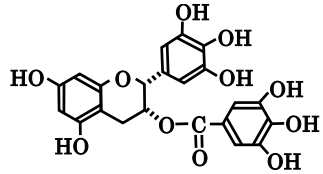
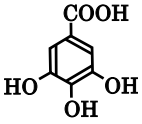
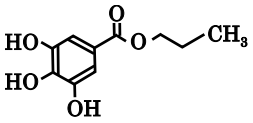
Recombinant human 12-lipoxygenase of platelet-type [31] and recombinant porcine 12-lipoxygenase of leukocyte-type [32] were expressed in *Escherichia coli*. The enzymes in the supernatant of *E. coli* homogenates were collected by ammonium sulfate fractionation. Recombinant murine 8-lipoxygenase with a hexahistidine tag was expressed in *E. coli*, and the enzyme was purified by the use of Ni-NTA agarose [33]. 15-Lipoxygenase was prepared from rabbit reticulocytes, and the ammonium sulfate fraction was used [34]. Recombinant human 5-lipoxygenase, cyclooxygenase-1 from ovine seminal vesicle and cyclooxygenase-2 from ovine placenta were purchased from Cayman Chemical (Ann Arbor, MI). Soybean lipoxygenase (159,300 U/mg) was supplied by Serva (Heidelberg). Catechins were the products by Nagara Science (Gifu, Japan).

Platelet and leukocyte 12-lipoxygenases, and reticulocyte and soybean 15-lipoxygenases were allowed to react with 250 μM arachidonic acid, and murine 8-lipoxygenase with 10 μM arachidonic acid. Absorption at 240 nm due to a conjugated diene of the product was followed spectrophotometrically. These lipoxygenases are the so-called suicide enzymes and their reaction time courses are not strictly linear [16]. Platelet 12-lipoxygenase shows the suicide to much less extent than other lipoxygenases, but its time course is not strictly linear. Therefore, an approximate increase in absorption for 1 min was determined, and the amount of product was calculated on the basis of a molar absorption coefficient of 22,000 [35]. The amount of enzyme was adjusted to that amount increasing absorption of 0.3–0.6 for 1 min for 12- and 15-lipoxygenases, and 0.04–0.05 for 8-lipoxygenase. 5-Lipoxygenase was incubated with 20 μM [1-^{14}C]arachidonic acid (Amersham Pharmacia Biotech, Buckinghamshire) in the presence of $CaCl_2$ and ATP [36]. Cyclooxygenases-1 and -2 were incubated with 20 μM [1-^{14}C]arachidonic acid in the presence of hematin and tryptophan [37]. The reaction products were separated by silica gel thin-layer chromatography, and the amounts of radioactive products were determined by a Fujix BAS 1500 imaging analyzer [37]. Catechins and arachidonic acid were dissolved in ethanol or dimethyl sulfoxide, and the amount of the vehicle (1% final concentration) did not significantly affect the enzyme activities. Each enzyme was preincubated with an inhibitor at room temperature for 5 min.

Results and discussion

As summarized in Table 2, inhibitory effects of various catechins and related compounds were tested with various lipoxygenases. At 10 μM concentrations all the 10 catechin compounds inhibited platelet 12-lipoxygenase much more potently than the other lipoxygenases. Catechins esterified

Table 2
Effects of various tea leaf catechins and related compounds on soybean lipoxygenase (S15-LOX), reticulocyte 15-lipoxygenase (R15-LOX), leukocyte 12-lipoxygenase (L12-LOX), and platelet 12-lipoxygenase (P12-LOX)

	% inhibition at 10 μM			% inhibition at 10 μM	
 (+)-catechin	S15-LOX	21.6 ± 3.5	 (+)-epicatechin	S15-LOX	4.8 ± 3.0
	R15-LOX	10.4 ± 10.3		R15-LOX	23.1 ± 9.4
	L12-LOX	−1.7 ± 5.9		L12-LOX	−3.6 ± 1.9
	P12-LOX	92.5 ± 2.5		P12-LOX	89.0 ± 0.6
	IC_{50} 1.13 μM			IC_{50} 3.46 μM	
 (-)-catechin	S15-LOX	25.3 ± 7.0	 (-)-epicatechin	S15-LOX	13.6 ± 2.4
	R15-LOX	3.9 ± 3.7		R15-LOX	19.8 ± 6.7
	L12-LOX	7.8 ± 6.4		L12-LOX	1.9 ± 2.3
	P12-LOX	43.7 ± 8.9		P12-LOX	86.9 ± 2.1
	IC_{50} 5.84 μM			IC_{50} 2.12 μM	
 (-)-gallocatechin	S15-LOX	12.6 ± 0.9	 (-)-epigallocatechin	S15-LOX	6.6 ± 6.0
	R15-LOX	22.3 ± 6.2		R15-LOX	−4.7 ± 2.1
	L12-LOX	4.8 ± 1.9		L12-LOX	0.0 ± 5.3
	P12-LOX	95.1 ± 0.7		P12-LOX	88.2 ± 1.9
	IC_{50} 1.88 μM			IC_{50} 1.52 μM	
 (-)-catechin gallate	S15-LOX	27.2 ± 11.3	 (-)-epicatechin gallate	S15-LOX	1.6 ± 3.9
	R15-LOX	0.3 ± 1.4		R15-LOX	3.5 ± 6.4
	L12-LOX	31.0 ± 5.9		L12-LOX	4.4 ± 4.9
	P12-LOX	98.0 ± 1.0		P12-LOX	103.5 ± 9.2
	IC_{50} 0.31 μM			IC_{50} 0.25 μM	
 (-)-gallocatechin gallate	S15-LOX	19.8 ± 6.5	 (-)-epigallocatechin gallate	S15-LOX	3.4 ± 4.2
	R15-LOX	20.6 ± 7.5		R15-LOX	7.9 ± 10.1
	L12-LOX	25.1 ± 3.7		L12-LOX	34.0 ± 8.7
	P12-LOX	96.9 ± 2.8		P12-LOX	101.0 ± 1.8
	IC_{50} 0.14 μM			IC_{50} 0.37 μM	
 gallic acid	S15-LOX	8.5 ± 5.4	 n-propyl gallate	S15-LOX	−2.3 ± 6.2
	R15-LOX	4.4 ± 9.3		R15-LOX	35.0 ± 4.4
	L12-LOX	7.2 ± 8.4		L12-LOX	30.8 ± 4.4
	P12-LOX	17.7 ± 3.8		P12-LOX	23.9 ± 0.2
	IC_{50} 98.9 μM			IC_{50} 76.9 μM	

% inhibition by each compound at 10 μM and IC_{50} values for P12-LOX are listed.

by gallic acid showed lower IC_{50} values by one order of magnitude as compared with free catechins. Gallic acid and its *n*-propyl ester were much less inhibitory. (−)-Gallocatechin gallate was most active in terms of IC_{50} (0.14 μM), and, as shown in Fig. 2, it was a selective inhibitor of platelet 12-lipoxygenase. The compound was much less active with leukocyte 12-lipoxygenase, soybean and reticulocyte 15-lipoxygenases, and epidermis 8-lipoxygenase with IC_{50} values of 24.8, >100, >100, and >100 μM, respectively. 5-Lipoxygenase and cyclooxygenases-1 and 2 were assayed with radioactive arachidonic acid, and the substrate and the products were separated by thin-layer chromatography. The assays at 0.1–100 μM (−)-gallocatechin gallate gave IC_{50} values of 2.0, 8.8, and 80.8 μM, respectively.

As a generally proposed mechanism of the lipoxygenase reaction, the non-heme iron contained in the enzyme

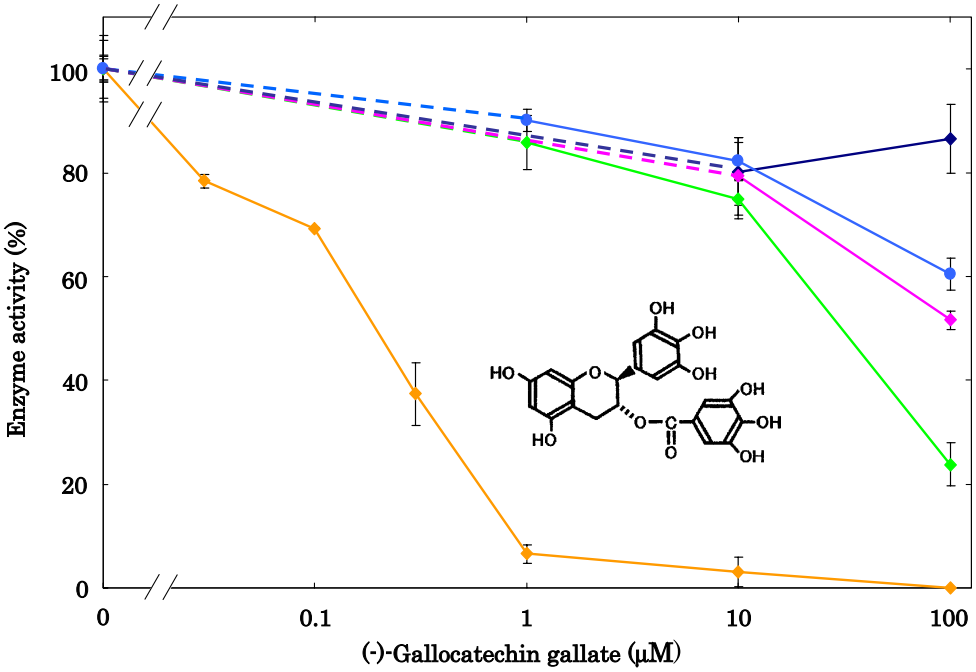


Fig. 2. Inhibition of various lipoxygenases by (−)-gallocatechin gallate. Each lipoxygenase was preincubated for 5 min with various concentrations of (−)-gallocatechin gallate dissolved in ethanol and then allowed to react with arachidonic acid as described under Materials and methods. Each point is an average of three determinations. Yellow line, platelet 12-lipoxygenase (74.2 μg protein); green line, leukocyte 12-lipoxygenase (62.2 μg protein); red line, reticulocyte 15-lipoxygenase (16.9 μg protein); purple line, soybean lipoxygenase (1.4 μg protein); blue line, 8-lipoxygenase (9.4 μg protein).

protein must be oxidized to the ferric state so that the enzyme can be activated to start the reaction with arachidonic acid. Peroxy compounds are used for the oxidation of the enzyme-bound iron from the ferrous state to the ferric state [38]. The peroxy product of lipoxygenase is proposed to be utilized as the oxidant and to abolish a lag phase before the initiation of lipoxygenase reaction [14]. Although under our assay conditions a prominent lag phase was not observed with each lipoxygenase, it is possible that catechins scavenge the peroxy activators thereby inhibiting the initiation of the lipoxygenase reaction. Thus, we tested whether or not the addition of peroxides to the reaction mixture of enzyme and (−)-gallocatechin gallate reversed the inhibition by the catechin. However, the platelet 12-lipoxygenase activity inhibited by the catechin at 0.1 or 1 μM was not reversed by the addition of 1 μM hydrogen peroxide, cumene hydroperoxide or tertiary butyl hydroperoxide.

When the platelet 12-lipoxygenase was incubated with 1 μM (−)-gallocatechin gallate and an aliquot of the mixture was diluted by 40-fold, the enzyme was as active as in the presence of the inhibitor at 25 nM. Thus, the enzyme inhibition was reversible. We tried to examine the type of inhibition by Lineweaver–Burk plots, but we could not conclude whether the inhibition was of a competitive or non-competitive nature because it was difficult to precisely determine the initial velocity of the enzyme reaction due to the suicide nature of the enzyme [16]. It should be noted that 12-lipoxygenase of platelet-type is "selectively" inhibited. Thus, the inhibitory effect of the compound may be attributed to its specific chemical structure and cannot be explained only by a general function as an antioxidant.

A variety of catechins are contained in green tea leaves. Since we tested only 10 compounds, we cannot discuss much of the structure–activity relationship. It should be noted that catechins esterified by gallic acid are more potent inhibitors of platelet 12-lipoxygenase than free catechins although gallic acid is much less inhibitory. There are reports for the inhibition of lipoxygenases by catechins; for example, reference [39] reported inhibition of soybean lipoxygenase-3 by (−)-epigallocatechin gallate, and reference 40 described 15-, 5-, and 12-lipoxygenases inhibited by several catechins. Human platelet 12-lipoxygenase was inhibited by (−)-epicatechin with an IC_{50} of about 15 μM and rabbit 15-lipoxygenase was inhibited by epicatechin gallate and epigallocatechin gallate with IC_{50} values of 5 and 4 μM, respectively [40]. However, our paper is the first report of the inhibitory activity of catechins relatively selective for 12-lipoxygenase of platelet-type.

Acknowledgments

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