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Lipoxygenase Inhibitory Activity of Anacardic Acids

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6[8′(*Z*)-Pentadecenyl]salicylic acid, otherwise known as anacardic acid ($C_{15:1}$), inhibited the linoleic acid peroxidation catalyzed by soybean lipoxygenase-1 (EC 1.13.11.12, type 1) with an IC_{50} of 6.8 μM. The inhibition of the enzyme by anacardic acid ($C_{15:1}$) is a slow and reversible reaction without residual activity. The inhibition kinetics analyzed by Dixon plots indicates that anacardic acid ($C_{15:1}$) is a competitive inhibitor and the inhibition constant, K_I, was obtained as 2.8 μM. Although anacardic acid ($C_{15:1}$) inhibited the linoleic acid peroxidation without being oxidized, 6[8′(*Z*),11′(*Z*)-pentadecadienyl]salicylic acid, otherwise known as anacardic acid ($C_{15:2}$), was dioxygenated at low concentrations as a substrate. In addition, anacardic acid ($C_{15:2}$) was also found to exhibit time-dependent inhibition of lipoxygenase-1. The alk(en)yl side chain of anacardic acids is essential to elicit the inhibitory activity. However, the hydrophobic interaction alone is not enough because cardanol ($C_{15:1}$), which possesses the same side chain as anacardic acid ($C_{15:1}$), acted neither as a substrate nor as an inhibitor.

KEYWORDS: Lipoxygenase inhibitors; anacardic acids; competitive inhibitor; Dixon plots; time-dependent inhibition

INTRODUCTION

Lipoxygenases (EC 1.13.11.12) are a family of non-heme iron-containing dioxygenases widely distributed in both the animal and the plant kingdoms. They catalyze the regio- and stereospecific oxygenation of polyunsaturated fatty acids, containing (*Z*,*Z*)-1,4-diene moieties, into the corresponding conjugated hydroperoxides. In plants, the main substrates are linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids, and the primary products of lipoxygeneses, 9*S* and 13*S* fatty acid hydroperoxides, are proposed to have regulatory roles in plant and animal metabolism (*1*). Due to their free radical nature, fatty acid hydroperoxides can be quite active by themselves and are capable of producing membrane damage and promoting cell death (*2*).

Lipoxygenases are suggested to be involved in the early event of atherosclerosis by inducing plasma low-density lipoprotein (LDL) oxidation (*3*, *4*). On the other hand, it is well known that lipid peroxidation is one of the major factors in deterioration during the storage and processing of foods, because it can lead to the development of unpleasant rancid or off flavors as well as potentially toxic end products (*5*). Hence, lipoxygenase inhibitors should have broad applications (*6*).

6-Pentadecanylsalicylic acid, otherwise known as anacardic acid ($C_{15:0}$) (**1**) (**Figure 1**), was previously reported to inhibit the linoleic acid peroxidation catalyzed by soybean lipoxygenase-1. However, only the IC_{50} value was reported as 85 μM (*7*). This prompted us to examine anacardic acids and their related compounds for comparison to gain new insights into their inhibitory action on a molecular basis. In our continuing search for biologically active substances from plants, anacardic acids were previously isolated in quantities as prostaglandin synthetase inhibitors from Anacardiaceae plants, *Ozoroa mucronata* (*8*), and antibacterial principles from the cashew *Anacardium occidentale* apple (*9*). In the current experiment, our emphasis was placed on 6[8′(*Z*)-pentadecenyl]salicylic acid, otherwise known as anacardic acid ($C_{15:1}$) (**2**), because it is commonly characterized from *O. mucronata* and *A. occidentale*, but anacardic acid ($C_{15:0}$) is not.

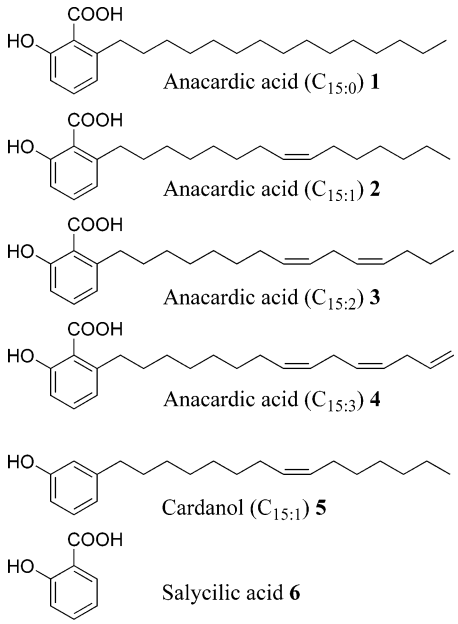

Figure 1. Chemical structures of anacardic acids and related compounds.

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MATERIALS AND METHODS

Chemicals. Anacardic acids ($C_{15:0}$, $C_{15:1}$, $C_{15:2}$, $C_{15:3}$) and cardanol ($C_{15:1}$) were available from our previous work (*9*, *10*). Soybean lipoxygenase-1 (EC 1.13.11.12, type 1), dimethyl sulfoxide (DMSO), salicylic acid, Tween-20, and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Boric acid was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL). 13-Hydroperoxyoctadecadienoic acid (13-HPOD: $\lambda_{max} = 234$ nm, $\epsilon = 25$ mM^{-1} cm^{-1}) was prepared enzymatically by the described procedure (*11*) and stored in ethanol at −18 °C.

Inhibition Experiments on Lipoxygenase-1. These experiments were performed by measurement of the initial rate of soybean lipoxygenase-1 with a Spectra MAX plus spectrophotometer (Molecular device, Sunnyvale, CA) at 25 °C. The enzyme assay was performed as previously reported (*12*) with slight modification. In general, the reaction mixture consisted of 2.97 mL of 0.1 M sodium borate buffer (pH 9.0), 15 μL of 3 mM stock solution of linoleic acid, and 5 μL of an ethanolic inhibitor solution. Next, 10 μL of 0.1 M sodium borate buffer solution (pH 9.0) of lipoxygenase (0.52 μM) was added. The resultant solution was mixed well, and the linear increase of absorbance at 234 nm for 5 min, which express the formation of conjugated diene hydroperoxide (13-HPOD, $\epsilon = 25\,000$ M^{-1} cm^{-1}), was measured continuously. The lag period shown on lipoxygenase reaction (*13*) was excluded for the determination of initial rates. The stock solution of linoleic acid was prepared with Tween-20 and sodium borate buffer at pH 9.0, and then the total Tween-20 content in the final assay was adjusted below 0.01%. For determining reversible inhibition manner, enzyme concentration was changed as 0.094, 0.141, 0.188, 0.235, and 0.282 μg/mL with a constant substrate concentration (30 μM). Two concentrations (15 and 30 μM) of linoleic acid were selected for Dixon plots.

Lipoxygenase-dependent O_2 uptake was performed using a Clark-type oxygen electrode (YSI 53, Yellow Springs Instrument Co., Yellow Springs, OH) at 25 °C as essentially the same procedures in the spectophotomeric experiment. For obtaining IC_{50}, the final assay concentrations of the enzyme and the substrate were adjusted to 43.5 nM and 50 μM, respectively. On the other hand, in the study of Dixon plots, the final concentration of the enzyme was fixed at 43.5 nM, but the substrate concentrations selected were 50 and 80 μM. Because a weak substrate inhibition was induced by the high concentration of linoleic acid (*12*, *13*), 100 μM substrate was used as the maximum concentration.

Preincubation Experiments. Most experiments were performed so that lipoxygenase-1 (1.74 nM) and 2.0−10 μM anacardic acid ($C_{15:2}$) were incubated with 0.1 M sodium borate buffer (pH 9.0) at 25 °C. At timed intervals, reactions were started by addition of 30 μM linoleic acid. Control samples were incubated under identical conditions except for the absence of inhibitor. The increase in the absorbance at 234 nm was monitored for 5 min. A plot of the natural log of the residual enzyme activity versus preincubation time gave a straight line with a slope of $-k_{obs}$. The values of k_3, k_4, and K_i^{app} were calculated from the plot of the k_{obs} versus concentration of inhibitors according to the method of Morrison and Walsh (*14*, *15*).

Data Analysis and Curve Fitting. The assay was conducted in triplicate of separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc., Chicago, IL). The inhibitory concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve by the equation as follows (*15*):

$$\text{activity (\%)} = 100[1/(1 + ([I]/IC_{50}))]$$

Inhibition mode was analyzed by Enzyme Kinetics Module 1.0 (SPSS Inc.) equipped with Sigma Plot 2000. Kinetic parameters for time-dependence inhibitor were analyzed by using the following equations (*15*):

$$v/v_0 = \exp(-k_{obs}t)$$

$$k_{obs} = k_4(1 + [I]/K_i^{app})$$

RESULTS

Soybean lipoxygenase-1 catalyzes the conversion of the fatty acid containing 1(*Z*),4(*Z*)-pentadiene moiety and molecular oxygen into hydroperoxides. In plants, the primary dioxygenation product is 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HPOD) (*5*). The inhibition activity of soybean lipoxygenase-1 was measured by a spectrophotometric and a polarographic method. In the current experiment, linoleic acid was used as a substrate. The buffer used for all experiments was 0.1 M sodium borate at pH 9.0 because soybean lipoxygenase-1 is reported to have its optimum at pH 9.0 (*16*). First, the enzyme assay was performed using a UV spectrophotometer to detect the increase at 234 nm associated with the (2*Z*,4*E*)-conjugated double bonds newly formed in the product but not the substrate. As a result, anacardic acid ($C_{15:1}$) showed a dose-dependent inhibitory effect on this oxidation as shown in **Figure 2**. As anacardic acid ($C_{15:1}$) increased, the enzyme activity was rapidly decreased with completely suppressed. The inhibitor concentration leading to 50% activity lost (IC_{50}) was estimated to be 6.8 μM. Anacardic acid ($C_{15:0}$) also showed a dose-dependent inhibitory effect on this oxidation. The IC_{50} of anacardic acid ($C_{15:0}$) was obtained as 14.3 μM. Second, the bioassay with anacardic acid ($C_{15:1}$) and anacardic acid ($C_{15:0}$) was also monitored by measuring oxygen consumption (polarographic method) for comparison. The IC_{50} obtained was 31.5 and 68.6 μM, respectively.

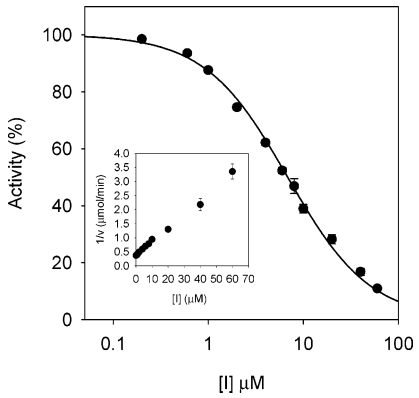

Figure 2. Effects of anacardic acid ($C_{15:1}$) on the activity of soybean lipoxygenase-1 for the catalysis of linoleic acid at 25 °C. (Inset) Replots of data as 1/*v* versus [I].

The linoleic acid peroxidation catalyzed by soybean lipoxygenase-1 follows Michaelis−Menten equations. The plots of the remaining enzyme activity versus the concentrations of enzyme at different concentrations of anacardic acid ($C_{15:1}$) gave a family of straight lines, which passed through the origin as shown in **Figure 3**. Increasing the inhibitor concentration resulted in the descending of the slopes of the lines, indicating that the inhibition of anacardic acid ($C_{15:1}$) on the enzyme was reversible. The presence of anacardic acid ($C_{15:1}$) did not bring down the amount of the efficient enzyme, but resulted in the inhibition and the descending of the activity of the enzyme. In addition, a similar result was also obtained by O_2-electrode monitoring method.

Subsequently, the inhibition kinetics of soybean lipoxygenase-1 by anacardic acid ($C_{15:1}$) was investigated. The kinetic behavior of soybean lipoxygenase-1 during the linoleic acid peroxidation was studied first. Under the condition employed in the present investigation, the oxidation of linoleic acid catalyzed by soybean lipoxygenase-1 follows Michaelis−Menten kinetics. The kinetic parameters for this oxidase obtained from a Dixon plot show that K_m is equal to 11.7 μM and V_m is equal to 4.8 μM/min. The estimated value of K_m obtained with a spectrophotometric method is in good agreement with the previously reported value (*17*, *18*). The kinetic and inhibition

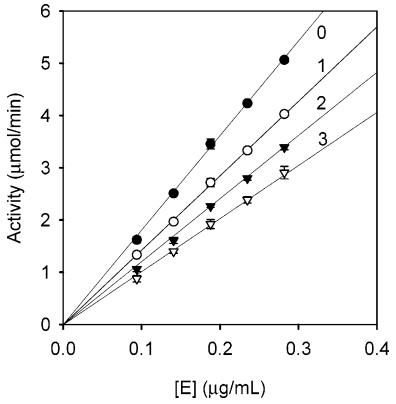

Figure 3. Relationship of catalytic activity of soybean lipoxygenase-1 with the enzyme concentrations at different concentrations of anacardic acid ($C_{15:1}$). Concentrations of inhibitor for curves 0–3 were 0, 2, 4, and 6 μM, respectively.

Table 1. Kinetics and Inhibition Constants of Anacardic Acid ($C_{15:1}$)

	detector	
	UV_{234}	O_2
IC_{50}	6.8 μM	31.5 μM
K_m	11.7 μM	36.5 μM
V_m	4.8 μmol/min	6.5 μmol/min
inhibition	reversible	reversible
inhibition type	competitive	competitive
K_I	2.8 μM	14.7 μM

constants obtained are listed in **Table 1**. As illustrated in **Figure 4**, the inhibition kinetics analyzed by Dixon plots shows that anacardic acid ($C_{15:1}$) is a competitive inhibitor because increasing substrates resulted in a family of lines with a common intercept above the [I] axis but with different slopes. The equilibrium constant for inhibitor binding, K_I, was obtained from −[I] value at the intersection of two lines. The inhibition kinetics analyzed by Lineweaver−Burk plots also confirmed that the anacardic acid ($C_{15:1}$) is a competitive inhibitor (data not illustrated). In addition, a similar result was also obtained by O_2-electrode monitoring method, and the results are listed in **Table 1**. The estimated value of K_m is approximately 3.6-fold higher than that obtained with a spectrophotometric method. This is in good agreement with the previously reported observations (*18*).

Because anacardic acids are salicylic acid derivatives with a pentadec(en)yl side chain, their inhibitory activity was also compared to that of salicylic acid. Salycilic acid (**6**) did not inhibit soybean lipoxygenase-1 up to 200 μM, indicating that a pentadec(en)yl side chain is essential to elicit the activity. On the other hand, anacardic acids were described to show high selectivity toward transition metal ions, especially iron and copper (*19*). It appears therefore that anacardic acids can be expected to inhibit the lipoxygenases activity as iron chelators. If this is so, the UV absorption of anacardic acid ($C_{15:1}$) should be shifted by adding excess Fe^{2+}. However, this shift was not viewed in the case of anacardic acids because the absorption overlapped with that of the enzyme itself. The iron chelation mechanism can be indirectly supported by the observation that cardanol ($C_{15:1}$), 3[8(*Z*)-pentadecatrienyl]phenol (**5**), an artifact of the corresponding anacardic acid ($C_{15:1}$) (**2**) by heating treatment, did not show any inhibitory activity up to 3 mM.

On the other hand, we became aware that anacardic acid ($C_{15:3}$) (**4**) and anacardic acid ($C_{15:2}$) (**3**) were oxidized as substrates at lower concentrations (<40 μM), because both possess a

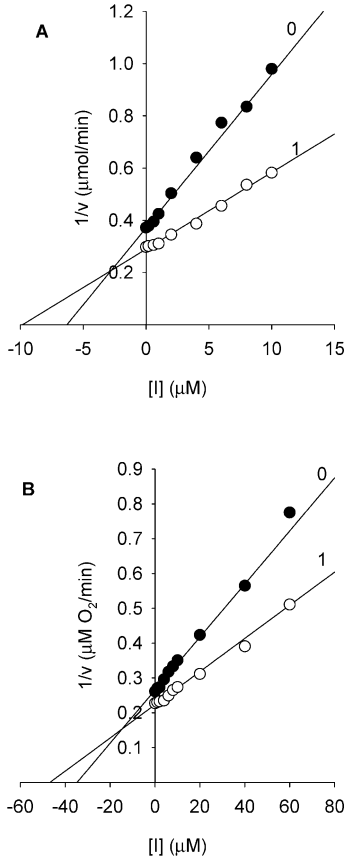

Figure 4. Dixon plots of 13-HPOD generation (**A**) and oxygen consumption (**B**) by soybean lipoxygenase-1 in the presence of anacardic acid ($C_{15:1}$) in borate buffer (pH 9.0) at 25 °C. (**A**) Concentrations of substrates for curves 0 and 1 were 15 and 30 μM, respectively. (**B**) Concentrations of substrates for curves 0 and 1 were 50 and 80 μM, respectively.

Table 2. Kinetic Parameters for Time-Dependent Inhibition of Lipoxygenase-1 by Anacardic Acid ($C_{15:2}$)

compound	k_3 (μM^{-1} min^{-1})	k_4 (min^{-1})	K_i^{app} (μM)
3	0.00504 ± 0.0002	0.0186 ± 0.0012	3.69 ± 0.11

1(*Z*),4(*Z*)-pentadiene system in their C_{15}-alkenyl side chain. However, both exhibited inhibitory activity at higher concentrations (>40 μM). Hence, the inhibition kinetics of anacardic acid ($C_{15:2}$) was also studied in detail for comparison (**Table 2**). Preincubation of the enzyme with anacardic acid ($C_{15:2}$) but without linoleic acid was tested. **Figure 5** shows a plot of the anacardic acid ($C_{15:2}$) concentration-dependent initial rates of the enzyme-catalyzed reaction. Note that as the concentration of anacardic acid ($C_{15:2}$) increases up to the 40 mM, the initial rates of the enzyme-catalyzed reaction first increase, and then start decreasing at still higher concentrations of anacardic acid ($C_{15:2}$).

As far as the present cell-free experiment using soybean lipoxygenase-1 is concerned, the inhibition kinetics observed is not exceeding 5 min. However, from a practical point of view, much longer observation is needed. The time course of oxidation of linoleic acid catalyzed by soybean lipoxygenase-1 in the presence of different anacardic acid ($C_{15:1}$) and anacardic acid ($C_{15:2}$) concentrations is also shown in **Figure 6**. As anacardic acid ($C_{15:1}$) increased, the rate of formation of 13-HPOD is slowly decreased, whereas anacardic acid ($C_{15:2}$) showed the typical progress curves of slow binding behavior. These slow binding inhibition mechanisms can be investigated by preincu-

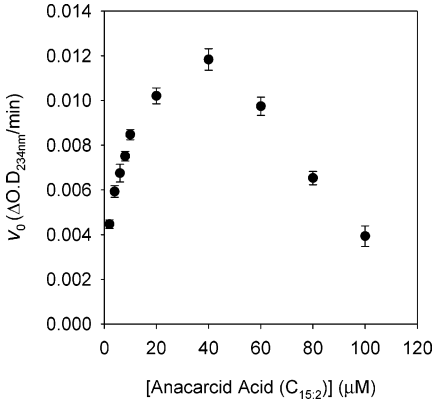

Figure 5. Concentration dependence of the initial rate of the oxidation of anacardic acid ($C_{15:2}$) at 25 °C, 0.1 M borate buffer, pH 9.0.

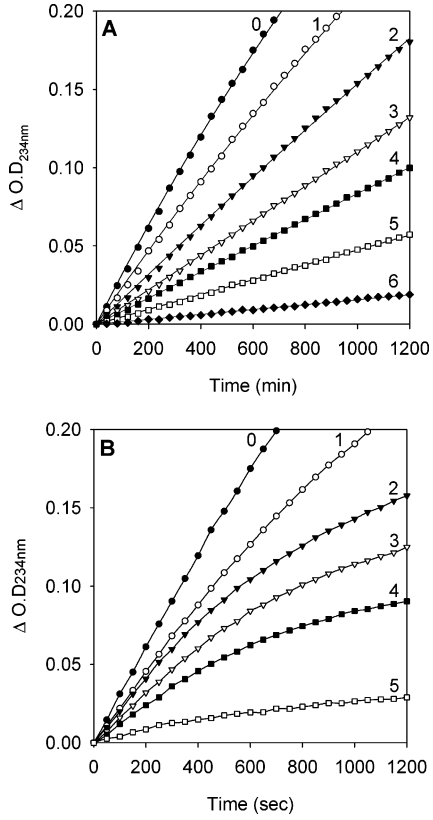

Figure 6. Time-dependent inhibition of soybean lipoxygenase-1 in the presence of anacardic acid ($C_{15:1}$, **A**) and anacardic acid ($C_{15:2}$, **B**). (**A**) Concentrations of inhibitor for curves 0–6 were 0, 2, 4, 8, 10, 20, and 40 μM. (**B**) Concentrations of inhibitor for curves 0–5 were 0, 40, 60, 80, 100, and 300 μM. Conditions were as follows: 20 μM linoleic acid, 0.1 M borate buffer, pH 9.0 at 25 °C.

bation of enzyme with inhibitor followed by the measurement of initial velocities for substrate peroxidation as a function of preincubation time. In this case, the inhibition can be described by eq 1,

$$v/v_0 = \exp(-k_{obs}t) \quad (1)$$

$$k_{obs} = k_4[I]/K_i^{app} + k_4 \quad (2)$$

In the concentration range of 2.0−10.0 mM, loss of the enzyme activity followed a simple exponential as shown in **Figure 7**. Anacardic acid ($C_{15:2}$) exerted a potent and slow inactivation of soybean lipoxygenase-1, with more than 70%

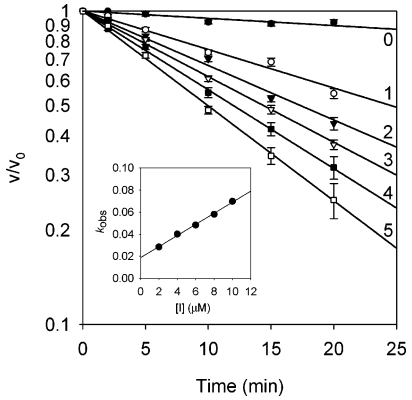

Figure 7. Time course of the inactivation of soybean lipoxygenase-1 by anacardic acid ($C_{15:2}$). Concentrations of anacardic acid ($C_{15:2}$) for curves 0–5 were as follows: 0, 2, 4, 6, 8, and 10 μM. (Inset) Dependence of the values for k_{obs} on the concentration of anacardic acid ($C_{15:2}$).

loss of enzyme activity occurring in 20 min at 10 mM. The k_{obs} values for the anacardic acid ($C_{15:2}$) inhibition of lipoxygenase-1 at different concentrations of anacardic acid ($C_{15:2}$) were determined by fitting data to the slow-binding equation (eq 1). The k_{obs} values were plotted as a function of anacardic acid ($C_{15:2}$) concentration. The results indicated that anacardic acid ($C_{15:2}$) inhibits soybean lipoxygenase-1 by simple reversible slow binding. This was evidenced by the observation that the k_{obs} values exhibited a liner dependence on the inhibitor concentration as shown in **Figure 7** inset. Thus, analysis of data according to eqs 1 and 2 yielded the following values: $k_3 = 0.00504 \pm 0.0002$ mM^{-1} min^{-1}, $k_4 = 0.0186 \pm 0.0012$ min^{-1}, $K_i^{app} = 3.69 \pm 0.11$ mM.

DISCUSSION

The human body produces free radicals during the course of its normal metabolism. Free radicals are even required for several normal biochemical processes. For example, the phagocyte cells involved in the body's natural immune defenses generate free radicals in the process of destroying microbial pathogens. If free radicals are produced during the normal cellular metabolism in sufficient amounts to overcome the normally efficient protective mechanisms, metabolic and cellular disturbances will occur in a variety of ways. Evidence is accumulating that several cell types other than phagocytes also produce extracellular free radicals in vivo. For example, lipids are oxidized by lipoxygenases and cyclooxygenases, which generate peroxide intermediates. More specifically, lipoxygenases catalyze the oxygenation of polyenoic fatty acids containing a 1(*Z*),4(*Z*)-pentadiene system such as linoleic acid and arachidonic acid to their 1-hydroperoxy-2(*E*),4(*Z*)-pentadiene product. In this connection, lipoxygenases are of importance because they may generate peroxides in human low-density lipoproteins (LDL) in vivo and facilitate the development of atherosclerosis, a process in which lipid peroxidation appears to be intimately involved (*3*, *4*).

Anacardic acids are known to inhibit various enzymes such as urease (*20*), glycerol-3-phosphate dehydrogenase (*21*), lipoxygenases (*7*), β-lactamase (*22*, *23*), aldose reductase (*24*), prostaglandin endoperoxide synthase (*25*), and prostaglandin synthase (*8*, *26*). Because salicylic acid has little or no effects on these enzymes, the hydrophobic alkyl side chain in anacardic acids is undoubtedly associated with the enzyme inhibitory activity. In addition, the number of double bonds in the side chain is not directly associated with the enzyme inhibitory

activity (*7*, *10*), suggesting that interaction of the double bond with a specific amino acid residue in the enzymes is unlikely. Anacardic acids are amphipathic molecules, so that their hydrophobic properties dominate the properties of the molecule. The precise explanation still remains unclear, but it seems that these enzymes commonly possess a relatively nonspecific and hydrophobic domain. The hydrophobic pentadec(en)yl side chain in anacardic acids likely interacts with this hydrophobic domain and disrupts enzymes' quaternary structure (*27*), because native proteins form a sort of intramolecular micelle in which their nonpolar side chains are largely out of contact with the water-based test solution. The hydrophilic head part of anacardic acids first binds with the salicylic acid portion like a "hook" in attaching itself to the hydrophilic portion of the molecule, and then the hydrophobic tail portion begins interacting with the hydrophobic domain. This concept can be more broadly conceivable because the hydrophobic alkyl side chain should allow for conformational flexibility and interacts with many types of the hydrophobic domains in different enzymes. Anacardic acids' nonspecificity of enzyme inhibition supports this assumption.

In summary, safety is a primary consideration for antioxidants in food products, which may be utilized in unregulated quantities. That anacardic acids are found in many edible plants that have been consumed for many years should be a considerable advantage. Metal chelation capacity of anacardic acids is their additional advantage because it reduces the concentration of the catalyzing transition metal in lipid peroxidation. It is known that chelating agents, which form bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. Despite the advantage of the isolation from regularly consumed edible plants, biological significance of anacardic acid as lipoxygenase inhibitors in living systems is still largely unknown. Thus, it is not clear if ingested anacardic acids are absorbed into the system through the intestinal tract and delivered to the places where lipoxygenase inhibitors are needed. The relevance of the in vitro experiments in simplified systems to in vivo protection from oxidative damage should be carefully considered. Their further evaluation is needed from not only one aspect, but from a whole and dynamic perspective.

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