Food Chemistry 159 (2014) 471–476

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Lipoxygenase inhibitory activity of alkyl protocatechuates

Tae Joung Ha, Kuniyoshi Shimizu, Isao Kubo *

Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720-3114, United States

ARTICLE INFO

Article history:
Received 3 January 2013
Received in revised form 7 February 2014
Accepted 10 March 2014
Available online 18 March 2014

Keywords:
Lipoxygenase inhibitors
Alkyl protocatechuates
Slow-binding inhibitor
Competitive inhibitor

ABSTRACT

Alkyl 3,4-dihydroxybenzoates (protocatechuates) inhibited linoleic acid peroxidation catalyzed by soybean lipoxygenase-1 (EC 1.13.11.12, Type 1). Their inhibitory activities displayed a parabolic function of their lipophilicity and maximized with alkyl chain lengths of between C_{11} and C_{14}. Tetradecanyl protocatechuate exhibited the most potent inhibition with an IC_{50} of 0.05 μM, followed by dodecyl (lauryl) protocatechuate with an IC_{50} of 0.06 μM. However, their parent compound, protocatechuic acid, did not show this inhibitory activity up to 200 μM, indicating that the alkyl chain length is significantly related to the inhibition activity. The allosteric (or cooperative) inhibition of soybean lipoxygenase-1 of longer alkyl protocatechuates is reversible but in combination with their iron binding ability to disrupt the active site competitively and to interact with the hydrophobic portion surrounding near the active site (sequential action). In the case of dodecyl protocatechuate, the enzyme quickly binds this protocatechuate and then its dodecyl group undergoes a slow interaction with the hydrophobic domain in close proximity to the active site in the enzyme. The inhibition kinetics analyzed by Lineweaver–Burk plots indicates that octyl protocatechuate is a competitive inhibitor and the inhibition constant (*Ki*) was obtained as 0.23 μM but dodecyl protocatechuate is a slow binding inhibitor.

Published by Elsevier Ltd.

1. Introduction

Lipoxygenases are suggested to be involved in the early event of atherosclerosis by inducing plasma low-density lipoprotein (LDL) oxidation (Cornicelli & Trivedi, 1999; Kris-Etherton & Keen, 2002). 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 flavours as well as potentially toxic end products (Grechkin, 1998). Hence, lipoxygenase inhibitors should have broad applications (Richard-Forget et al., 1995).

Lipoxygenases are known to act mainly in two different ways; by chelating the iron of the active site of the enzyme (Clapp, Banerjee, & Rotenberg, 1985; Nelson, 1988) and/or by reducing the ferric form of the enzyme to an inactive ferrous form (Kemal, Louis-Flamberg, Krupinski-Olsen, & Shorter, 1987; Mansuy, Cucurou, Biatry, & Battioni, 1988). In continuing search for lipoxygenase inhibitors, anacardic acids were previously reported to chelate metal ions such as Fe^{2+} and Fe^{3+} (Tsujimoto, Hayashi, Ha, & Kubo, 2007). Their parent compound, salicylic acid also chelated the same metal ions but did not show any lipoxygenase inhibitory activity up to 200 μM, indicating that their alkyl tail portion is an essential element in eliciting the activity. Their head and tail structure suggests that optimization of the inhibitory activity is possible through a synthetic approach. Based on a preliminary study of their structures and lipoxygenase inhibitory activity relationship, 3,4-dihydroxybenzoic acid (protocatechuic acid) was found to be a superior hydrophilic head portion. Effective lipoxygenase inhibitors can be designed by selecting specific head portions and appropriate hydrophobic tail portions and their mode of action should be investigated.

2. Materials and methods

2.1. Chemicals

A homologous series of alkyl protocatechuates (Nihei, Nihei, & Kubo, 2003) and dodecyl gallate (Kubo et al., 2002) were available from our previous study. Nordihydroguaiaretic acid (NDGA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Soybean lipoxygenase-1 (EC 1.13.11.12, Type 1), dimethyl sulphoxide (DMSO), Tween-20, and linoleic acid (purity > 99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris buffer was obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL, USA). 13(*S*)-Hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HPOD: λ_{max} = 234 nm, ε = 25 mM^{-1} cm^{-1}) was prepared enzymatically by

* Corresponding author. Tel.: +1 510 643 6303; fax: +1 510 643 0215.
E-mail address: ikubo@berkeley.edu (I. Kubo).

http://dx.doi.org/10.1016/j.foodchem.2014.03.037
0308-8146/Published by Elsevier Ltd.

described procedure (Gibian & Galaway, 1976) and stored in ethanol at −18 °C.

2.2. Enzyme assay

Linoleic acid (**5**) was used as a substrate. Soybean lipoxygenase-1 (EC 1.13.11.12, Type 1) is known to catalyze the dioxygenation of the (1*Z*, 4*Z*)-diene moiety of linoleic acid. In plants, the primary dioxygenation product is 13(*S*)-hydroperoxy-9*Z*,11*E*-octadienoic acid (13-HPOD) (Grechkin, 1998). The inhibitory concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data from spectrophotometric experiment to the logistic curve by the equation as follows (Copeland, 2000)

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

where [I] is the inhibitor concentration.

The oxygenase activity of the soybean lipoxygenase was monitored at 25 °C by Spectra MAX plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The enzyme assay was performed as previously reported (Rickert & Klinman, 1999) with slight modification. In general, 5 μL of an ethanolic inhibitor solution were mixed with 54 μL of 1 mM stock solution of linoleic acid and 2.936 mL of 0.1 M Tris-HCl buffer (pH 8.0) in a quartz cuvette. Then, 5 μL of a 0.1 M Tris-HCl buffer solution (pH 8.0) of lipoxygenase (1.02 μM) was added. The resultant solution was mixed, and the linear increase of absorbance at 234 nm, which expresses the formation of conjugated diene hydroperoxide (13-HPOD, $\varepsilon = 25{,}000\ M^{-1}\ cm^{-1}$), was measured continuously. A lag period shown in the lipoxygenase reaction (Ruddat, Whitman, Holman, & Bernasconi, 2003) was excluded for the determination of initial rates. The stock solution of linoleic acid was prepared in methanol and Tris-HCl buffer at pH 8.0, and, then, total methanol content in the final assay was adjusted below 1.5%. Lipoxygenase-dependent O_2 uptake was performed using a Clark-type oxygen electrode (YSI 53, Yellow Springs Instrument Co., Yellow Springs, OH, USA) at 25 °C as essentially the same procedures in the spectrophotometric experiment. For obtaining IC_{50}, the final assay concentrations of the enzyme and the substrate were adjusted to 4.25 nM and 46 μM, respectively. All assays were conducted in separate triplicate experiments.

2.3. Progress curve determinations

All reactions were carried out using linoleic acid as a substrate in 0.1 M Tris-HCl buffer (pH8.0) at 25 °C. Enzyme activities were measured for 10 min continuously using UV spectrophotometer. To determine the kinetic parameters associated with slow-binding inhibition of soybean lipoxygenase-1, progress curves with 25 or more data points, typically at 2 second intervals, were obtained at several inhibitor concentrations and fixed concentration of substrate. The data were analyzed using the nonlinear regression program of Sigma Plot (SPSS Inc., Chicago, IL) to give the individual parameters for each progress curve; v_i (initial velocity), v_s (steady-state velocity), k_{obs} (apparent first-order rate constant for the transition from v_i to v_s), A (absorbance at 234 nm), A_0 (included to correct any possible deviation of the baseline), and K_i^{app} (apparent K_i) according to Eqs. (2) and (3) (Frieden, 1970):

$$A = v_s t + (v_i - v_s)[1 - \exp(-k_{obs} t)]/k_{obs} + A_0 \quad (2)$$

$$K_{obs} = k_6 + [(k_5 \times [I])/(K_i^{app} + [I])]. \quad (3)$$

To classify the type of inhibition of a time-dependent inhibitor, analysis of various linoleic acid concentrations on k_{obs} at a fixed dodecyl protocatechuate and decahydro-2-naphthyl protocatechuate concentration were performed. Kinetic parameters were also calculated by nonlinear regression fitting the data to Eq. (2).

2.4. Measurement of pseudoperoxidase activity

The pseudoperoxidase activity was determined from the inhibitor dependent consumption of 13-HPOD catalyzed by the soybean lipoxygenase-1 using the variation in A_{234} as previously described (Riendeau, Falgueyret, Guay, Ueda, & Yamamoto, 1991). The assay mixture contained 10 μM 13-HPOD, various concentrations of inhibitors, and soybean lipoxygenase-1(329 nM) in 0.1 M Tris-HCl, pH 8.0 at 25 °C.

3. Results

The synthesized series of alkyl 3,4-dihydroxybenzoates (protocatechuates) (**1–13**) was tested for effects on the soybean lipoxygenase-1 (EC 1.13.11.12, Type 1) catalyzed oxidation of linoleic acid (see Fig. 1 for structures). Results are listed in Table 1. It appears that the inhibitory activity of alkyl protocatechuates displayed a parabolic function of their lipophilicity and maximized with alkyl chain length between C_{11} and C_{14}. Among the compounds tested, tetradecanyl protocatechuate (**8**) showed the most potent inhibition with an IC_{50} of 0.05 μM, followed by dodecyl (lauryl) protocatechuate (**7**) with an IC_{50} of 0.06 μM. Interestingly, their parent compound, protocatechuic acid did not show any activity up to 200 μM, indicating that the alkyl chain length was an essential element in eliciting the inhibitory activity and is associated with increasing the activity. It thus appears that hydrophilic ligands with a longer alkyl side chain length up to C_{14} tend to the more potent inhibitors. Since their head portions are the same, the data are interpreted to mean that changes in the hydrophobic tail portions correlated to the activity. The activity of alkyl 3,4-dihydroxybenzoates was compared with those of alkyl 2,3-dihydroxybenzoates

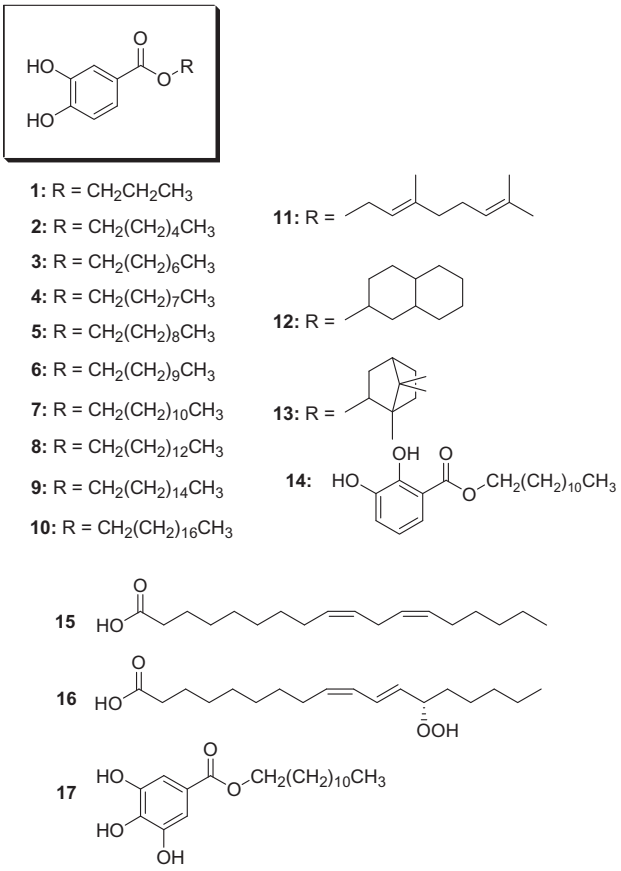

Fig. 1. Chemical structures of alkyl protocathechuates and related compounds.