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Sclerotiorin, a Novel Inhibitor of Lipoxygenase from *Penicillium frequentans*

C. CHIDANANDA AND A. P. SATTUR*

Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute, Mysore 570020, India

Foods rich with unsaturated fatty acids are prone to enzymatic and nonenzymatic lipid peroxidation; lipoxygenase, a metalloenzyme and a free radical former, oxidizes polyunsaturated fatty acids and is one of the key enzymes in lipid oxidation. Here, we report sclerotiorin, purified from the fermented broth of *Penicillium frequentans*, as a potent reversible, uncompetitive inhibitor against soybean lipoxygenase-1 (LOX-1) with a half-maximal value (IC_{50}) of 4.2 μM. The inhibitor also showed an antioxidant property by scavenging free radical with an ED_{50} of 0.12 μM; in addition, nonenzymatic lipid peroxidation was inhibited with a PD_{50} value of 64 μM and did not show metal chelation. The observations made in this study suggest that sclerotiorin possibly inhibits LOX in two ways: one, by interacting with the enzyme−substrate complex, and two, as an antioxidant by quenching or trapping the free radical intermediates formed in the enzyme reaction. Sclerotiorin compares well with other known natural and synthetic lipoxygenase inhibitors.

KEYWORDS: Antioxidant; inhibitor; lipoxygenase; *Penicillium frequentans*; sclerotiorin

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) (LOX-1), a dioxygenase and a free radical former, catalyzes the regio- and stereospecific oxygenation of polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene system into their corresponding conjugated hydroperoxydienes (*1*). It is responsible for lipid peroxidation (LPO) of polyunsaturated fatty acids promoting rancidity or off odor in foods of both plant and animal origin, which in turn reduces the nutritive value and functional properties of the food (*2*).

In mammals, the products of lipoxygenase-catalyzed reactions are responsible for a variety of human disorders, such as atherosclerosis, allergy, inflammation, asthma, and hypersensitivity (*3*, *4*). Hence, inhibitors against lipoxygenase may have a dual application in the food industry as antioxidants and in health sectors with pharmacological benefits.

During the course of screening for bioactive metabolites from fungi (*5*, *6*), we screened several cultures for their ability to produce lipoxygenase and aldose reductase inhibitors. Out of the 74 fungal isolates from Western Ghat forest, south India, we identified *Penicillium frequentans* CFTRI A-24 as producing a potent inhibitor against aldose reductase (*7*) and lipoxygenase, which was further purified and characterized as sclerotiorin.

Sclerotiorin was first isolated in 1940 from *Penicillium sclerotiorum* as a chlorine-containing fungal pigment; it belongs to the azaphillone class of compounds containing an isochromane basic ring skeleton (*8*, *9*). Azaphilones are structurally diverse pigments, mostly secondary metabolites of fungal origin, and are highly oxygenated bicyclic quaternary rings showing various biological activities (*10*, *11*). This potent fungal pigment is known for its various biologically beneficiary activities (*12*−*14*). Previously, the radical scavenging property of sclerotiorin was recorded along with some of the xanthones produced from a lichen mycobiont (*15*). With this background, in this paper, we report for the first time sclerotiorin, showing a potent uncompetitive reversible inhibition against soybean LOX-1, along with a potent antioxidant property, which may show potential as a natural antioxidant.

MATERIALS AND METHODS

Organism. *P. frequentans* CFTRI A-24 was maintained on potato dextrose agar (PDA) containing potato starch (200 g/L), dextrose (20 g/L), and agar (20 g/L) and was subcultured every fortnight.

Chemicals. Linoleic acid, 2-thiobarbituric acid (TBA), 1,1-diphenyl-2- picrylhydrazyl (DPPH), butylated hydroperoxy toluene (BHT), resveratrol, ferrozine, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Co. (St. Louis, MO). The solvents chloroform, methanol (MeOH), ethanol (EtOH), and ethyl acetate (EtOAC) were purchased from Sisco Research Laboratories (Mumbai, India). Silica gel 60 F_{254} precoated thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). Other laboratory chemicals like PDA, sucrose, yeast extract, NaCl, and Tween-20 were obtained from Himedia (Mumbai, India).

Ultraviolet (UV/vis) absorption spectra were recorded on a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). ^{1}H NMR spectra were recorded at 500 MHz on a Bruker DRX-500 MHz spectrometer operating at 27 °C. Gas chromatography−mass spectrometry (GC-MS) analysis was carried out on Va autospec M mass spectrometer equipped with HP 5980 series II gas chromatograph under GC−eletron impact (EI) MS conditions.

* To whom correspondence should be addressed. Tel: +91-821-2515792. Fax: +91-821517233. E-mail: ftbe@cftri.res.in.

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Fermentation of *P. frequentans* CFTRI A 24. From a 5 day old culture slant, a conidial spore suspension was prepared by adding 15 mL of sterile 0.1% Tween-20 and gentle scraping of the surface of the aerial mycelium with a sterile loop to liberate spores. One milliliter of this spore suspension (1.8×10^6 cfu/mL) was inoculated to a 500 mL Erlenmeyer flask containing 100 mL of production medium (20 g/L sucrose, 10 g/L yeast extract, and 10 g/L NaCl, pH 5.2), which was previously autoclaved at 121 °C at 15 lbs. for 15 min. The flasks were kept for fermentation at 30 °C for 5 days on a rotary shaker operating at 200 rpm.

Extraction and Purification of Sclerotiorin. At the end of the fermentation, to 1 L of the broth, EtOAC (1:1; v/v) was added, and upon vacuum distillation, the crude compound was obtained as a reddish residue (2.5 g). This was dissolved in 100 mL of MeOH and 200 mL of distilled water. The resultant precipitate (1 g) containing the inhibitor was filtered through Whatman #1 and air-dried. The solid residue was confirmed and purified on silica gel 60 F_{254} precoated TLC plates (5 cm × 7.5 cm) and developed on benzene:diethyl ether (95:5), where it showed a single spot under UV (361 nm) with an R_f value of 0.62, which was 96% GC pure. ^{1}H NMR spectra were recorded at 500 MHz (500.13 MHz proton and 125 MHz ^{13}C). A 30 mg amount of the solid sample dissolved in $CDCl_3$ was used for recording the NMR spectra at 27 °C. For GC/EIMS conditions, a BP-1 capillary column was used with the temperature program as follows: 120 °C at 10 °C/min to 180 °C at 4 °C/min to 280 °C (15 min); injection temperatures at 250 °C; detector (flame ionization detection) temperature at 280 °C; and 2 mL/min flow rate (N_2).

Soybean Lipoxygenase (LOX-1) Assay. The assay was carried out by monitoring the appearance of *cis*,*trans*-hydroperoxide derivative at 234 nm. LOX-1 was partially purified from soybeans (Hardy variety) with a specific activity of 135 units/mg protein; purification of soybean LOX-1 was carried out as per ref *16*. The 3 mL assay mixture contained 20 μL of enzyme, 10−20 μL of sclerotiorin (2 mg/mL DMSO stock), and 2.955 mL of 0.2 M sodium borate buffer (pH 9.0). The reaction was initiated by the addition of 15 μL of substrate (10 mM linoleic acid). The control sample received equal volumes of DMSO without the inhibitor. The relative activity was expressed as the percentage of the enzyme activity in relation to the control without sclerotiorin used in the assay.

Total Antioxidant Activity by Phosphomolybdate Method. Various aliquots of 10−100 μL of sclerotiorin from 3.9 mg/mL DMSO and 10 mM stock were mixed in an Eppendrof tube with 1 mL of phosphomolybdate reagent (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated at 95 °C for 90 min. The control contained 1 mL of reagent solution and equal volumes of DMSO. After the samples had cooled to room temperature, the absorbance of the samples was recorded at 695 nm against control. BHT was used as a standard. The activity was expressed in terms of molar absorption equivalent range (*17*).

Free Radical Scavenging Activity by DPPH Assay. The assay consisted of 2 mL of ethanolic solution of 10−50 μL of sclerotiorin (1 mM stock in DMSO) acidified with 2 mL of 0.1 M acetate buffer (pH 5.5), to which 1 mL of 0.5 mM ethanolic DPPH solution was added. The mixture was vigorously shaken and incubated at 28 °C for 30 min. The absorbance was measured at 517 nm. The free radical quenching ability of the inhibitor was determined as a percentage decrease in the absorbance with respect to control. The control received the appropriate quantity of the solvent without any inhibitor. The ED_{50} value was determined as the concentration of the inhibitor required to reduce 50% of the absorbance with respect to the control (*18*).

Antioxidant Ability in a Liposome Model System. *Preparation of Egg Liposome.* Fifteen milliliters of freshly beaten egg yolk was dispersed in 100 mL of cold acetone to obtain a precipitate (2 g), which was washed with 3 × 100 mL cold acetone till the precipitate turned white. A 300 mg amount of this precipitate was dispersed in 100 mL of 10 mM phosphate buffer (pH 7.4) and sonicated in an ultrasonic cleaner for 15 min/4 °C. This liposome mixture stock (3 mg protein/mL) was stored at 4 °C.

LPO Assay. The assay was performed as described by ref *19* with slight modification. Various aliquots of 10−50 μL of sclerotiorin (1.95 mg/mL DMSO and 0.5 mM stock) were added to 1 mL of liposome mixture (3 mg protein/mL), and the corresponding control samples contained the appropriate volumes of DMSO followed by addition of 200 μL of 400 mM ferric chloride and 200 μL of 400 mM ascorbic acid. The final assay volume was made to 2 mL with 20 mM phosphate buffer (pH 7.4) and incubated for 1 h at 37 °C. The reaction was stopped by adding 1 mL of 1% TBA and 1 mL of 2% of trichloroacetic acid (TCA) and mixed thoroughly. The reaction mixture was boiled for 15 min and cooled to room temperature. The precipitate was removed by centrifuging the samples at 1000*g*/15 min. The absorbance of the samples was measured at 535 nm against blank sample, which contained all of the reagents except lipid sample. The PD_{50} value was determined as the concentration of the sclerotiorin required to reduce 50% of the absorbance with respect to the DMSO control.

Metal Binding Assay. To various aliquots of sclerotiorin from 100 to 500 μL (3.9 mg/mL DMSO and 10 mM stock), 20 μL of freshly prepared 2 mM $FeCl_2$ was added followed by addition of 400 μL of ferrozine reagent (5 mM stock). The total volume was adjusted to 1 mL with MeOH. The samples were incubated at room temperature for 10 min, and absorbance of a colored Fe^{2+}−ferrozine complex formation was recorded at 562 nm. Equal aliquots of DMSO served as the control, while EDTA, which completely blocked the formation of Fe^{2+}−ferrozine complex, was used as a positive control (*20*).

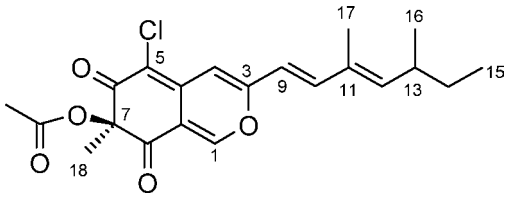

Figure 1. Nuclear magnetic resonance spectroscopy. ^{1}H NMR (500 MH_Z, $CDCl_3$): 7.94 (1H, s, H-1), 6.60 (1H, s, H-4), 6.08 (1H, d, J = 15.7 Hz, H-9), 7.06 (1H, d, J = 15.6 Hz, H-10), 5.71 (1H, d, J = 9.7 Hz, H-12), 2.49 (1H, m, H-13), 1.34 (1H, m, H_a-14), 1.44 (1H,m, H_b –14), 0.87 (3H, t, J = 7.3 Hz, H-15), 1.01 (3H, d, J = 6.6 Hz, H-16), 1.85 (3H, s, H-17), 1.57 (3H, s, H-18), 2.17 (3H, s, H-20). ^{13}C NMR (125 MHz, $CDCl_3$): 153.3 (C1), 158.8 (C2), 107.0 (C4), 139.3 (C5), 115.2 (C5), 192.4 (C6), 85.2 (C7), 186.6 (C8), 111.4 (C8a), 116.3 (C9), 143.5 (C10), 132.6 (C11), 149.5 (C12), 35.8 (C13), 30.7 (C14), 12.6 (C15), 20.8 (C16), 13.0 (C17), 23.2 (C18), 170.7 ($COCH_3$), 20.7 ($CO\mathit{CH}_3$). From NMR spectra and GC-MS, the structure of the compound was determined as sclerotiorin.

RESULTS AND DISCUSSION

Sclerotiorin and other structurally similar compounds isolated mostly from *Penicillium* species exhibit various biologically beneficiary properties such as inhibition of cholestryl ester transfer protein (*21*), monoamine oxidase (*22*), gp120-CD4 binding (*23*), acyl-CoA:cholesterol acyltrasferase (*24*), endothelin receptor binding antagonistic activity (*25*), and aldose reductase inhibition (*7*). As a part of the screening program from our laboratory for lipoxygenase inhibitors from microbial sources, sclerotiorin was purified and characterized from the fermented broth of *P. frequentans* A-24.

The following characters were determined for the isolated compound. The molecular formula of the inhibitor was elucidated as $C_{21}H_{23}O_5Cl$. Molecular weight, 390; mp, 202 °C. UV spectrometry: λ_{max} (MeOH) nm (ϵ) 361 (23, 732), 284 (9672). Solubility: highly soluble in chloroform, EtOAC, diethyl ether, acetone, DMSO, EtOH, and MeOH and insoluble in water. EI-MS: *m*/*z* 390 [M^+], 392 [$M^+ + 2$]. The structure of sclerotiorin was confirmed by NMR and GC-MS (**Figure 1**), and the assignments were confirmed by comparing them with the literature (*9*, *25*).

Oxidation of lipids is a significant factor in foods rich with fats, to bring about biochemical changes leading to loss of flavor, color, and nutritive values (*2*). The factors inducing such a

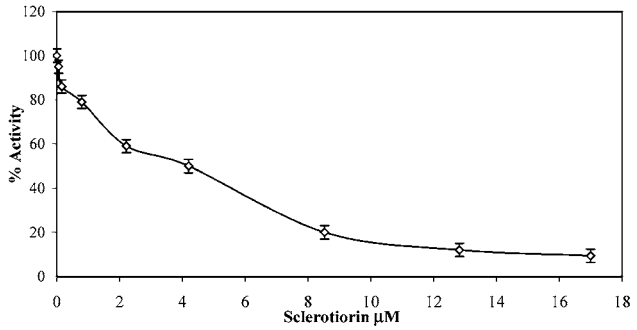

Figure 2. Sclerotiorin showed a dose-dependent inhibition to LOX-1 at 27 °C with an IC_{50} of 4.2 μM. All of the concentrations were carried out in triplicate, and the mean value is reported with a percentage error of ±5%.

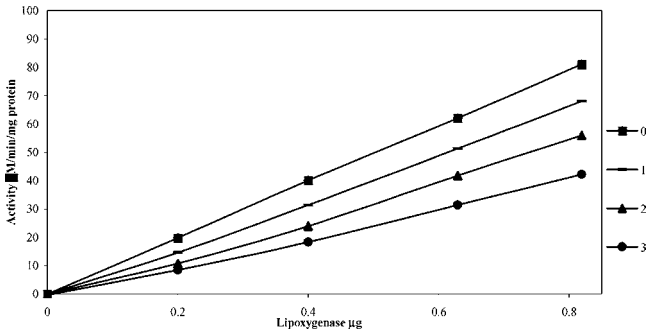

Figure 3. Relationship of catalytic activity of LOX-1 with enzyme concentrations at different concentrations of sclerotiorin. The concentrations of sclerotiorin for curves 0–4 were 0, 10, 5, and 2 μM, respectively.

process can be both enzymatic and nonenzymatic. It is against this background that LOX inhibition by sclerotiorin was investigated. The results showed that the inhibitor demonstrated a dose−response inhibition against soybean LOX-1 with a half-maximal inhibitory value or IC_{50} of 4.2 μM (**Figure 2**). One unit of the enzyme activity is defined as the amount of enzyme required for the oxidation of 1 μmol of linoleic acid per min at 30 °C at 234 nm. The enzyme-catalyzed linoleic acid peroxidation kinetic parameters obeyed the Michaelis−Menton order of reaction. The plots of residual enzyme activity vs concentration of enzyme at different concentrations of sclerotiorin gave a family of straight line, which passed through the origin and indicated that the inhibition of enzyme by sclerotiorin was reversible (**Figure 3**). The double reciprocal Lineweaver−Burke plot showed linear lines intercepting on $1/V$ axis in a parallel manner, with the increasing concentration of the inhibitor [I], decreasing values in maximum velocity (V_{max}), and Michaelis−Menton constant (K_M) recorded. The data demonstrated that the inhibitory effect of sclerotiorin was an uncompetitive type (**Figure 4**). The inhibition kinetics and constants values are as shown in **Table 1**. The equilibrium constant for inhibitor binding, K_i, value was determined as 9 μM by plotting a secondary plot K_i = Michaelis−Menton constant (K_M values obtained from the LB plot) vs concentration of inhibitor [I], which is as illustrated in **Figure 5**.

As lipoxygenase enzyme generates peroxide free radicals, one of the modes of action of a lipoxygenase inhibitor could be due to the antioxidant nature of the compound, and many of the natural antioxidants are known to inhibit lipoxygenase by interacting with the radicals generated in the LPO process (*26*). Hence, the total antioxidant potential of sclerotiorin was studied using the phosphomolybdate method, which is based on the reduction of Mo(VI) to Mo(V). The formation of green

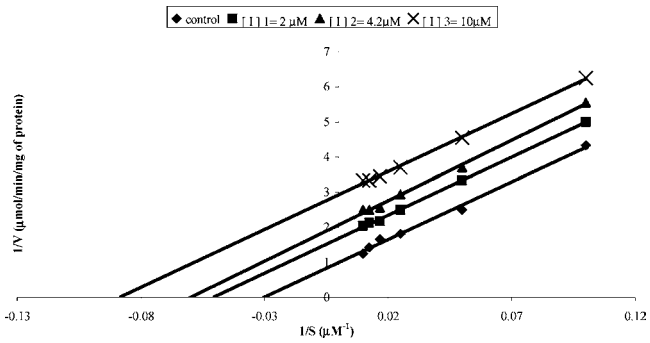

Figure 4. Lineweaver–Burk plot of soybean LOX-1 inhibition by sclerotiorin. The assay was carried out with enzyme (0.25 μg of 135 units/mg protein) in the absence (control) and in the presence of sclerotiorin concentrations [I]1 = 2 μM, [I]2 = 4.2 μM, and [I]3 = 10 μM, respectively. A K_M value of 33 μM and a V_{max} value of 1.0 μmol/min were observed in the absence of the inhibitor (control), whereas with the increasing concentration of the inhibitor [I] at 2, 4.2, and 10 μM, the K_M values were 19.6, 16.7, and 11.3 μM and the V_{max} values were 0.5, 0.4, and 0.3, respectively.

Table 1. Kinetics and Inhibition Constants of Soybean LOX-1 by Sclerotiorin

kinetic constants	concentration
IC_{50}	4.2 μM
K_M	33.0 μM
K_{cat}	50 μmol/min/mg protein
inhibition type	uncompetitive
K_i	9 μM

phosphate complex Mo(V) at acidic pH is indicative of antioxidant activity, which is measured in molar equivalent linearity intervals (*17*). Sclerotiorin showed the formation of Mo(V) complex at A_{695} with the increasing concentration showing a molar equivalent linearity range at 100−1000 μM (**Table 3**). This was compared to a standard synthetic antioxidant, BHT, which showed molar equivalent linearity at A_{695} 200−2000 μM. Sclerotiorin showed redox potential, which is twice as high as molar equivalent with respect to standard antioxidant BHT.

The DPPH assay is a well-known chemical model system to evaluate the antioxidant property of compounds. After establishing the redox antioxidant potential of sclerotiorin, our next studies were on its free radical quenching ability, which was determined by the DPPH method (*27*). Sclerotiorin showed decolorization of DPPH in a dose-dependent manner and the half-maximal ED_{50} (effective dose) value of the inhibitor required to reduce the absorbance by 50% was found to be 0.12

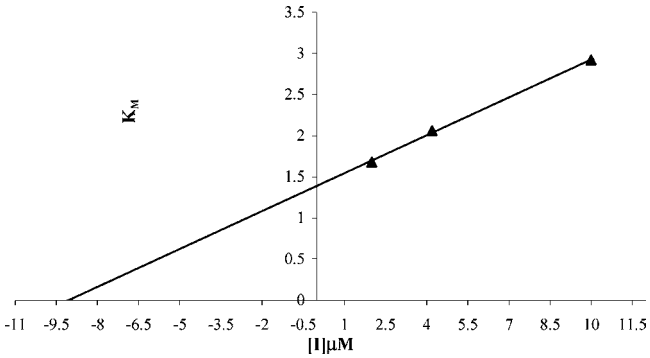

Figure 5. Inhibition constant (K_i) was determined by plotting values of slope obtained from (**Figure 3**) vs sclerotiorin [I] concentration, 2, 4.2, and 10 μM, respectively, and the K_i value corresponds to 9 μM.

Table 2. Comparison of Sclerotiorin with Other Soybean LOX-1 Inhibitors

inhibitors	inhibitor source	soybean LOX-1 IC_{50} (μM)	inhibition	antioxidant ED_{50} (μM)
sclerotiorin	*P. frequentans* CFTRI A-24	4.2	uncompetitive	0.12
resverotol (*33*)	red wine	15	competitive	25
curcumin (*34*)	*Curcuma longa*	8.6	competitive	37
nigerloxin (*5*)	*Aspergillus niger* CFR-W-105	79	mixed	66
asperenone (*6*)	*A. niger* CFTRI 1105	300	mixed	100

Table 3. Comparison of Antioxidant Potential of Sclerotiorin to Standard BHT

assays	half-maximal value	sclerotiorin (μM)	BHT (μM)
DPPH	ED_{50}	0.12	35.0
Fe^{2+}–ascorbate-induced LPO	PD_{50}	64.0	6.0
phosphomolybdate total antioxidant potential	molar equivalent	100–1000	200–2000
soybean LOX-1 inhibition	IC_{50}	4.2	270

μM (**Figure 6**). These results showed that sclerotiorin has a potent scavenging ability in comparison with the known synthetic and natural antioxidants with LOX inhibition (**Table 2**).

Free radicals such as superoxide anions ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) trigger the process of LPO, a well-known phenomenon occurring in both plant and animal systems. MDA is a major end product and a key index molecule of the LPO process (*28*). A liposome emulsion model system was used to evaluate the antioxidant ability of sclerotiorin in preventing LPO. LPO was induced by ferrous ascorbate system and the MDA formed from the peroxidation is reacted with TBA to give a red-colored species, which is read at 535 nm (*19*). The results indicated that sclerotiorin showed protection against LPO in a dose-dependent manner with the half-maximal protection dose (PD_{50}) value of 64 μM, as against synthetic BHT, which showed 6 μM (**Table 3**).

Biological membranes are rich with unsaturated fatty acids and are surrounded by metal-containing fluids. These inherent metal ions, like Fe^{2+}/Cu^{+}, are known to initiate LPO (*29*). In continuation with the previous experiments on LPO and in order to assess the ability of sclerotiorin to prevent LPO initiation, the inhibitor was tested for its metal-binding ability. The assay uses ferrozine reagent to react with metal ions such as ferrous

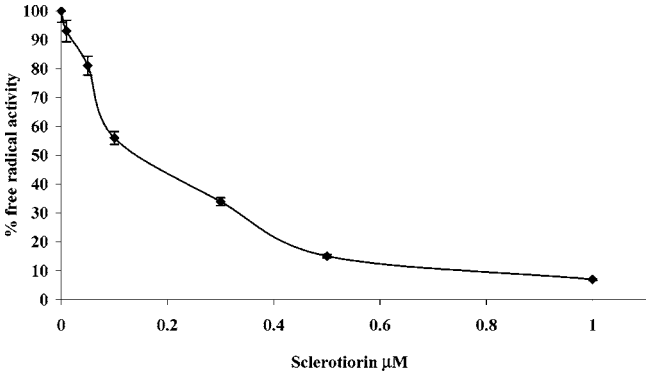

Figure 6. Dose-dependent radical scavenging activity of sclerotiorin carried out at 30 °C, using a DPPH radical. Sclerotiorin showed an ED_{50} value of 0.12 μM (concentration of the compound required to reduce radical scavenging activity by 50%). All of the concentrations were carried out in triplicate, and the mean value is reported with a percentage error of ±5%.

species by competing for the formation of a ferrozine−Fe^{2+}-colored complex at an absorbance maxima of 562 nm. The results showed the formation of ferrozine−Fe^{2+} complex even in the presence of an increasing concentration of sclerotiorin from 100 to 500 μM. This suggested that sclerotiorin does not possess metal-chelating property, unlike the control EDTA, which blocked the ferrozine−Fe^{2+} complex completely even at very low concentrations of <20 μM.

There are three modes of lipoxygenase inhibition: (i) inhibition by chelating iron metal ion in a catalytic site (*30*), (ii) removal of oxygen from the system (*31*), and (iii) preventing interaction with free radical formation (*32*).

The experimental results suggest that sclerotiorin might be inhibiting soybean LOX-1 activity by trapping radical intermediates that are formed by enzyme substrate catalysis. This hypothesis is further supported by its uncompetitive nature of inhibition, because lipid radicals can be generated at the active site only when there is an enzyme−substrate complex (ES). All uncompetitive inhibitors bind exclusively to the ES complex with or no affinity to the free enzyme itself. Furthermore, the radical scavenging ability and protection shown in a nonenzymatic LPO model suggest that lipoxygenase inhibition of sclerotiorin is due to its antioxidant nature by trapping or quenching the fatty acid radical species and not by Fe metal ion chelation.

Much work on lipoxygenase inhibitors is from plant and synthetic sources with a focus of application for therapeutic purposes. The results presented in this paper highlight the potential of a natural lipoxygenase inhibitor for a possible application in food processing as a natural lipoxygenase inhibitor and free radical scavenger. Also, as a metabolite from a natural microbial source, it may have an attractive commercial possibility. It will be interesting to further see the structural relation activity of sclerotiorin and its analogues on lipoxygenase.

ABBREVIATIONS USED

LOX-1, soybean lipoxygenase; LPO, lipid peroxidation; TBA, thiobarbituric acid; MDA, malondialdehyde; TCA, trichloroacetic acid; BHT, butylated hydroperoxy toluene; ES, enzyme−substrate complex; DMSO, dimethyl sulfoxide.

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