Chemical Characterization of Lysyl Oxidase Inhibitor from Avocado Seed Oil

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ABSTRACT: The active factor of lysyl oxidase inhibition was separated from unsaponifiables of avocado seed oil and characterized by gas chromatography–mass spectrometry. Results indicated the presence of furan-containing lipids in the active factor mixture and also showed a structural difference compared to previously reported furan-containing lipids of avocado which relates to the length of the hydrocarbon chain substituent. Another structural difference evinced was the availability of the hydroxyl group in the aliphatic moiety of the investigated substances. A purified mixture of furan-containing compounds was tested *in vitro* for inhibitory activity on pure bovine aorta lysyl oxidase. It was shown that mixing furan-containing lipids in Tween 80 reversibly inhibited pure bovine aorta lysyl oxidase activity against tritiated recombinant tropoelastin with the I_{50} value of inhibition of 105 μM. These *in vitro* studies suggested that the mixture of avocado seed oil furan-containing lipids was not a substrate-specific inhibitor of lysyl oxidase, and it might prove to be useful as a potential antifibrotic drug. Moreover, the unique chemistry of the studied compound for lysyl oxidase inhibition should enable the designing of new probes of the active site of this important enzyme.
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KEY WORDS: Avocado seed oil, collagen, connective tissue diseases, furan-containing lipids, lysyl oxidase inhibitor, tropoelastin, unsaponifiables.

Nonsaponifiables of vegetable oil are the subject of cosmetic and medical investigations owing to their biological activity (1–3). They are comprised of such biologically active compounds as sterols, tocopherols and squalene as well as a variety other saturated and unsaturated carbohydrates whose structure has yet to be elucidated (4). Essentially advantageous biological properties have been reported for a mixture of nonsaponifiables from avocado and soybean oil. It was shown that a mixture of avocado and soybean lipid nonsaponifiables is beneficial in the treatment of various disorders of connective tissue, such as scleroderma, parodontopathy as well as in wound healing (5–7). Robert *et al.* (8) reported the pharmacological effects of this mixture on carragenan-induced granuloma and on skin of rats. It was subsequently shown that avocado and soybean nonsaponifiables (Piascledine 300, PIAS; Laboratory Expanscience-Pharmascience, Courberoie, France) stimulated skin fibroblast metabolism and proliferation *in vitro* and also collagen and proteoglycan synthesis in connective tissue cells (9,10). Magloire *et al.* (11) reported that PIAS exerted an influence on the relation between type 3 and type 1 collagen synthesis in cultures of odontoblasts. Independent of this research, Varga *et al.* (12) have shown that type 3 and type 1 collagen synthesis may be regulated by prostaglandin PGE_2 which inhibits type 1 and type 3 procollagen mRNAs in normal human dermal fibroblasts. This last finding stimulated Mauviel *et al.* (13) to study the indirect influence of PIAS on collagen synthesis by its regulation of prostaglandin synthesis. These authors demonstrated partial inhibition of PGE_2 secretion in cultures of synoviocytes, chondrocytes of fibroblasts in the presence of PIAS. A mixture of avocado and soybean nonsaponifiables, when administrated percutaneously, modified dermal connective tissue in hairless rats, producing an increase in the ratio of soluble to insoluble collagen (14). Until recently, the mechanism of this latter effect was not clear, especially since no single purified constituent could be isolated from the mixture of avocado and soybean nonsaponifiables.

Recently, Werman *et al.* (15–17) have reported a significant increase in soluble collagen content in the skin and liver of rats fed diets rich in avocado seed oil unsaponifiables even though the total collagen content was not affected. It also was shown *in vivo* and *in vitro* that avocado seed oil nonsaponifiables decreased the activity of lysyl oxidase. Lysyl oxidase plays a major role in the formation of collagen and elastin cross-links which determine tensile strength of connective tissue. Lysyl oxidase (protein-L-lysine: oxidoreductase, EC 1.4.3.13) deaminates the ε-amino groups of lysine and hydroxylysine residues in collagen and elastin, converting them to peptidyl aldehydes. The reactive aldehydes can then form covalent cross-links by aldol condensation with other aldehyde groups or by interaction with free ε-amino groups of lysine. The level of collagen and elastin cross-links in connective tissue (which correlates with solubility of collagen) is de-

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pendent on the activity of lysyl oxidase. Enhancement of lysyl oxidase activity has been found in connective tissue diseases, such as fibrosis, which are characterized by connective tissue remodeling (18–20). There are several chemicals which have been considered for use in medicine to diminish cross-linking by inhibiting lysyl oxidase. These are lathyrogens, of which β-aminopropionitril and thiol compounds like D-penicillamine are the more prominent (21,22). The lathyrogenic factor of avocado seed oil was successfully separated by thin-layer chromatography (17) and its quantity was found to be about 0.2% of dried avocado seed oil. Experiments *in vitro* have shown that the active factor in avocado seed oil effectively inhibits of lysyl oxidase with collagen as substrate at a lesser concentration than penicillamine (17). The unusual lipid nature of the inhibitor is quite unlike the other known lysyl oxidase inhibitors.

The present report focuses on the chemical characterization of the lysyl oxidase inhibitor from avocado seed oil and provides details on the nature of the lysyl oxidase inhibition.

EXPERIMENTAL PROCEDURES

Isolation. Dried seeds of avocado pear were extracted for 12 h with boiling hexane. The solvent was then evaporated and the residue chromatographed on silica gel column (2.5 × 30 cm, silica gel Woelm, 100–200 mesh; Woelm Pharma, Eschwege, Germany) by sequencing through hexane/ether mixtures (95:5, 90:10, 85:15, 80:20, 75:25). Active factor was eluted with the latter solvent.

Catalytic hydrogenation of the furan-containing fraction of avocado seed oil. The furan-containing fraction of avocado seed oil (10 mg) in 20 mL ethanol was hydrogenated using PtO_2 (4 mg) under hydrogen gas at an atmospheric pressure for four hours. The resulting mixture was concentrated and then studied *via* gas chromatography–mass spectrometry (GC–MS).

Preparation of trimethylsilyl (TMS) ether derivatives. TMS ethers of furan-containing alcohols were prepared as described by Mangold and Baumann (23). Briefly, 1 mg of furan lipid-containing mixture was combined with 1 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane in a glass-stoppered tube. The solution was shaken and then left at room temperature for 1 h. The mixture was then diluted with 5 mL of hexane, followed by 5 mL of water and then extracted with hexane. Hexane extract was dried under nitrogen, dissolved in chloroform and analyzed by GC–MS.

GC–MS analysis. GC–MS was carried out with a Finnigan (Sunnyvale, CA) ITS-40 on DB-5 fused silica capillary column (30 m × 0.25 cm) programmed from 60 to 250°C at 10°C/min.

Preparation of enzyme and substrates. Lysyl oxidase was isolated from bovine aorta as a co-purified mixture of the four ionic variants of this enzyme (24). The product exhibited homogeneity with a molecular mass 32 kDa as determined by sodium dodecyl sulfate–polyacrylglycerol (25). Plasmids expressing the complete human tropoelastin molecule were constructed and transformed into the lysogenic host, *Escherichia coli* AR 120 as described by Indik *et al.* (26). A tritiated recombinant tropoelastin substrate was prepared from a culture of this bacteria which had been pulsed in the medium with [4,5-^{3}H]L-lysine, according to Bedell-Hogan *et al.* (27).

Assay of lysyl oxidase activity. Enzyme activity was assayed against the recombinant tropoelastin in 0.1 M sodium borate and 0.15 M sodium chloride, pH 8.0, at 37°C for 2 h. Tritiated water formed during the incubation was collected by vacuum distillation, and the radioactivity in 0.5-mL aliquots of the distillates was quantified by liquid scintillation spectrometry. Inhibitor potential was determined by incubating the reaction mixture with various amounts of inhibitor suspension (150 mM in 10%, w/w, Tween 80). Activities were corrected for Tween 80 solution and inhibitor-free controls. Lysyl oxidase activity was calculated as cpm ^{3}H/2 h. Enzyme activity in presence of inhibitor was expressed in percent of initial (without inhibitor) activity of lysyl oxidase.

RESULTS

The lysyl oxidase inhibition fraction was separated from avocado seed oil on silica gel column and then subjected to GC–MS analysis. Gas chromatographic separation of the mixture showed six major peaks (Fig. 1). Analysis of the different parts of the chromatographic peaks 1, 2 and 3 led to the detection of the spectra of the two individual compounds with very similar retention times in each peak. The mass spectra (MS) of each compound is shown in Figure 2. As can be seen, the mass spectral data of the compounds from peaks 1, 2 and 3 are essentially very similar, the main difference residing in the masses of the M^+ and $[M - 17]^+$ fragments. Comparison of the MS of peaks 1, 2 and 3 leads to the conclusion that the compounds are homologous and that the two components in each peak distinguish only the presence of one double bond. Fragments 67 and 81 in the MS of the compounds suggest the presence of a furan ring. This was confirmed by the mass spectra of the hydrogenated compounds (Fig. 3). Three compounds were detected in the hydrogenated mixture instead of the six substances represented by peaks 1, 2 and 3 in the ini-

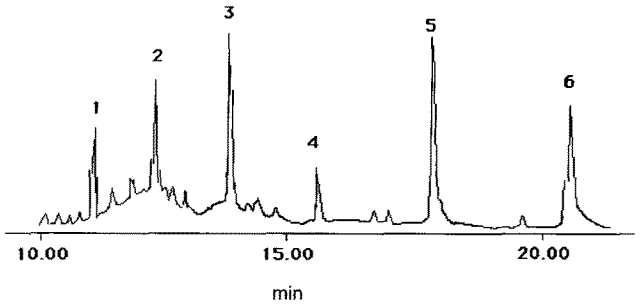

FIG. 1. Capillary gas chromatogram of lysyl oxidase inhibitor fraction from avocado seed oil after preseparation on silica gel column. Retention time (min) for each of the peaks is as follows: peak 1, 11.46–11.50; peak 2, 12.46–12.51; peak 3, 13.45–13.48; peak 4, 15.31; peak 5, 17.37; peak 6, 21.02.

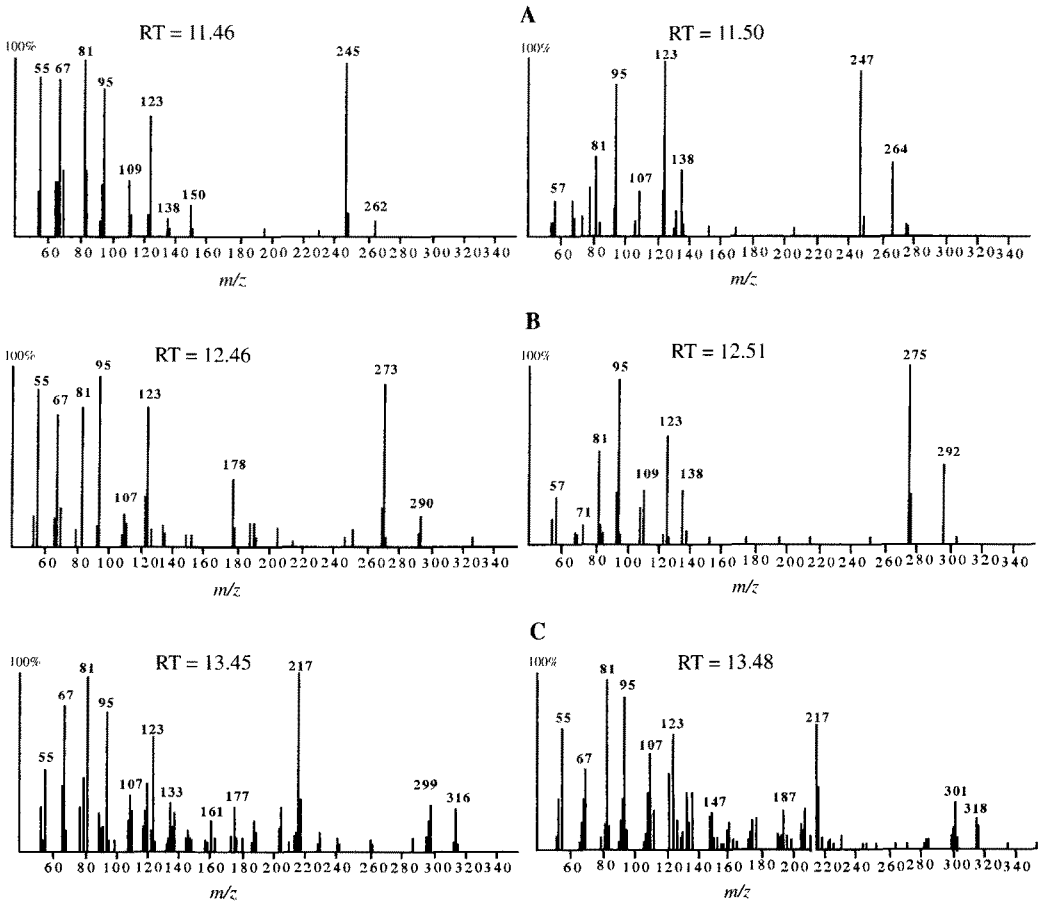

FIG. 2. Mass spectra of individual compounds from lysyl oxidase inhibitor fraction. Here and in Figure 3, (A) compounds from peak 1; (B) from peak 2; (C) from peak 3 (see Fig. 1). RT, retention time.

tial mixture. Further examination of each component yielded valuable information on their structure. The main ion of mass 71 in the spectra of the hydrogenated compounds was shown to be derived by "α-cleavage" of the bond adjacent to the ether-oxygen. A second "α-cleavage" occurred from a molecular ion in which the second oxygen, the OH-group, was ionized. This fragmentation yielded the fragment of mass 129, which loses water to give an ion of mass 111. The production of a fragment having a mass of 253 in the first compound (deriving from the twin compounds of peak 1 after hydrogenation) may be visualized by loss of water from protonated molecular ion, which can also be detected. Similar deduction helps to explain the production of ions in the MS of compounds from peaks 2 and 3 after their hydrogenation.

The spectra of the TMS derivatives of the hydrogenated compounds (Table 1) support the above postulates. Trimethylsilylated OH-groups are more easily ionized at the side-chain oxygen than are the original compounds, and favors "α-cleavage" of C-C bonds adjacent to the $-OSi(CH_3)_3$ group. Thus, key ions of mass 201 and mass 243 are observed for the first component and ions of mass 271 and 299 (instead of mass 243) for the second and third components, respectively. Together, these spectra allow the location of the OH group in the side chain.

Although the general structure of main furan-containing lipids in avocado seed oil fraction has been clarified, there is still need to elucidate the double (triple) bond position in the alkyl chain. It may be that the structure of the unsaturated alkyl chain here is essentially similar to that in the previously described avocado furans (Fig. 4).

Our second group of basic peaks from the initial mixture (Fig. 1, peaks 4, 5 and 6) represented three compounds with similar MS. MS of these compounds are presented by a group of high intensity ion fragments which are typical for unsaturated hydrocarbons: m/z = 57, 69, 83, 97, 111. Molecular ion is not expressed in the spectra of these compounds. This group of substances did not separate on ordinary silica gel column but did precipitate almost completely from a cold ethyl alcohol solution of the mixture. These compounds were not characterized because they lacked pharmacological interest, and were amenable to precipitation from the mixture used for lysyl oxidase inhibition.

After washing with cold ethyl alcohol, the mixture of furan-containing compounds was tested *in vitro* for inhibitory

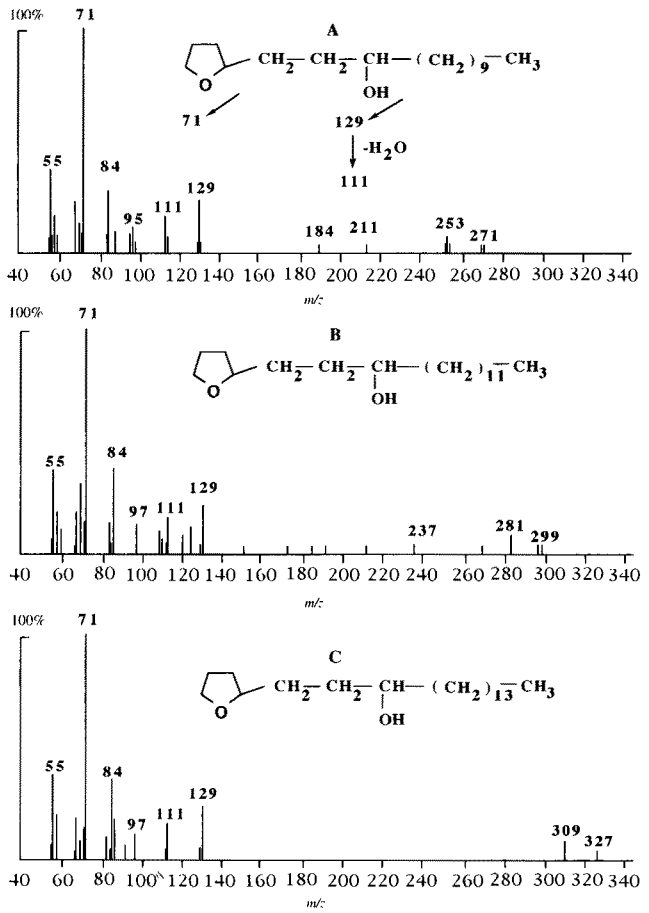

FIG. 3. Mass spectra and structure of hydrogenated compounds from peaks 1, 2 and 3 (see Figs. 1 and 2).

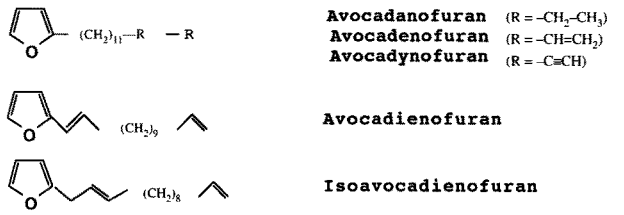

FIG. 4. Structure of known furan-containing lipids from avocado seed oil (Refs. 29, 30).

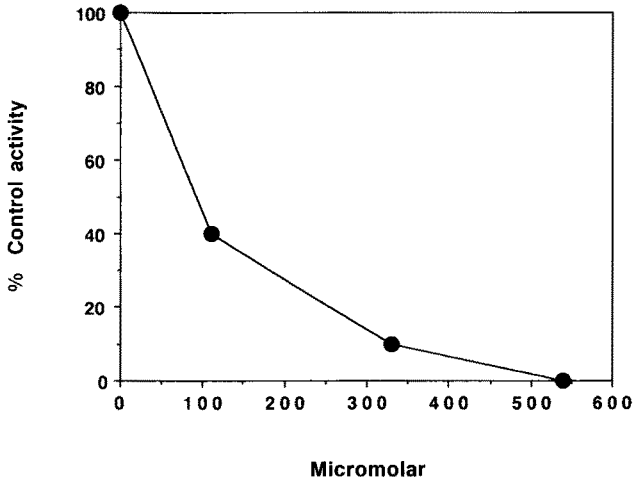

FIG. 5. Lysyl oxidase inhibition by mixture of furan-containing lipids from avocado seed oil. Enzyme activity in presence of inhibitor is expressed in percent of initial activity of lysyl oxidase (without inhibitor).

activity on pure bovine aorta lysyl oxidase. Compared to earlier work (17), this experiment employed recombinant tropoelastin rather than collagen as substrate in the reaction with lysyl oxidase. These modifications allowed interpretation of whether or not lysyl oxidase inhibition by the furan-containing fraction of avocado seed is substrate-specific. In addition, crude skin lysyl oxidase extract was substituted for pure bovine lysyl oxidase to avoid oxidation of furan-containing compounds by contaminating oxidases (28) in enzymatic crude extract. As can be seen from Figure 5, bovine aorta lysyl oxidase was inhibited by the mixture of furan-containing lipids. The inhibition (I_{50}) value was found to be 105 μM. Approximately 85% of the control activity was restored after dialysis of the enzyme against 16 mM potassium phosphate. These findings demonstrated the reversible nature of the lysyl oxidase inhibition by the furan lipid fraction of avocado seed.

TABLE 1
Mass Spectral (MS) Fragments of Trimethylsilyl Derivatives of the Hydrogenated Furan-Containing Lipids from Avocado Seed[a]

Fragment	Major MS (*m/z*)		
	Compound 1	Compound 2	Compound 3
M – 1	341(2)	369(2)	397(2)
M – 15	327(30)	355(38)	383(40)
M – [$C_5H_{10}O$]	256(14)	284(14)	312(12)
M – [C_4H_7O-C_2H_4]	243(35)	271(35)	299(32)
[C_4H_7O-C_2H_4-CH=OSi(CH_3)$_3$]$^+$	201(100)	201(100)	201(100)
a	173(12)	173(12)	173(12)
b	130(15)	130(15)	130(15)
c	143(15)	143(15)	143(15)
[C_4H_7O-C_3H_4]$^+$	111(10)	111(10)	111(10)
d	83(19)	83(19)	83(18)
[(CH_3)$_3$-Si]$^+$	73(65)	73(65)	73(65)

[a]a, b, c and d are nonidentified fragments.

DISCUSSION

Three novel natural 2-alkyl (C-17) furan derivatives in avocado (Fig. 4) have been reported by Kashman *et al.* (29). The carbohydrate moiety in one of the furan-containing compounds was represented by a C-13 aliphatic chain, while the two other compounds contained a double or triple bond at the end of their aliphatic chain. Magalhaes Alves *et al.* (30) have reported two other avocado furan-containing lipids with an additional double bond in the carbohydrate chain (avocadienofuran and isoavocadienofuran, Fig. 4). Results of the

present study revealed the occurrence of additional furan-containing lipids in avocado seed oil. These compounds were distinguished by the length of the hydrocarbon chain and the availability of a hydroxyl group in the aliphatic moiety. Conceivably, structural differences in the furan-containing lipids may depend on the strain of avocado tree involved and/or on the particular crop or storage conditions of the avocado fruit.

These *in vitro* studies indicate the furan-containing fraction of avocado oil can inhibit (reversibly) lysyl-oxidase catalyzed oxidation of the ε-amino group of collagen lysyl (or hydroxylysyl) residues, as well as tropoelastin (elastin). Therefore, the furan lipid-containing fraction from avocado seed might potentially serve as an antifibrotic drug in the treatment of diseases entailing excess collagen and elastin deposition (31). Moreover, the unique chemistry of these compounds should enable design of new probes for the active site of this important enzyme and thereby enhance understanding of its structure and mechanism. The presence of lysyl oxidase inhibitor in avocado nonsaponifiables explains many biological effects of avocado/soybean nonsaponifiable mixtures on connective tissue, and may account for its pharmacological value in the treatment of scleroderma-related afflictions and parodontopathy stemming from the inhibition of intra- and intermolecular cross-linking and possibly from enhancing the cleavage of newly-formed cross-links (32). Although the mechanism of inhibition is not yet clear, the furan-containing lipids may inhibit ε-amine group oxidation by interacting with the substrate (collagen or tropoelastin) rather that with the enzyme. Such interactions could neutralize positive charges on the substrate's surface which are essential for their oxidation by lysyl oxidase. A similar mechanism of lysyl oxidase regulation has been described by Kagan *et al.* (33) for certain anionic amphiphiles, wherein the amphiphiles inhibited elastin oxidation but had no effect on the oxidation of low molecular alkyl amine *n*-butyl amine by lysyl oxidase. The latter findings are consistent with a charge-based, substrate-directed mechanism of modulation of lysyl oxidase activity by substrate-bound ligand. Regrettably the inhibitory mechanism of our mixture could not be elucidated by the same procedures. Nevertheless, further work is now in progress on the furan lipid-containing fraction of avocado seed oil and its inhibitory effect on lysyl oxidase.

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