Partial Isolation and Characterization of a New Natural Inhibitor of Lysyl Oxidase from Avocado Seed Oil

M. J. Werman, S. Mokady, and I. Neeman*

Department of Food Engineering and Biotechnology, Technion—Israel Institute of Technology, Haifa 32000, Israel

A new natural inhibitor of lysyl oxidase was separated by thin-layer chromatography from avocado seed oil. This compound, called component C, is unique to the avocado seed. It has a molecular weight of 248 and is extracted from the unsaponifiable matter. Of all seed oil unsaponifiables, only component C was found to inhibit rat skin and chick tibia lysyl oxidase activity. The in vitro inhibitory effects of component C on rat skin lysyl oxidase activity was similar to that of seed oil and revealed an I_{50} of 1 mM.

INTRODUCTION

Many of the unique properties of collagenic connective tissue, such as its high tensile strength and low solubility, are due to the formation of covalent cross-links in collagen and elastin (Bailey et al., 1974). The reaction that initiates cross-link formation is catalyzed by lysyl oxidase [protein-L-lysine:oxygen 6-oxidoreductase (deaminating), EC 1.4.3.13]. This was first demonstrated by Pinnell and Martin (1968). Lysyl oxidase oxidatively deaminates the ϵ-amino groups of lysine and hydroxylsine residues in collagen, converting them to peptidyl aldehydes. The reactive aldehydes then can form covalent cross-links by aldol condensation of two neighboring allysine aldehydes or by the Shiff base reaction between allysine (or hydroxyallysine) and a lysine (or hydroxylysine) residue on an adjacent molecule (Rucker and Murray, 1978). The only enzyme activity required for the initiation of collagen cross-linking is that ascribed to lysyl oxidase. In addition to its copper cofactor, lysyl oxidase also contains a carbonyl cofactor identified as pyrroloquinoline quinone, found in bovine aortic (Williamson et al., 1986) and human placental (van der Meer and Duine, 1986) enzymes. However, various studies have pointed toward the possibility that pyridoxal 5'-phosphate is the carbonyl cofactor of lysyl oxidase (Bird and Levene, 1982; Carrington et al., 1984). It has been shown that in cases of tissue remodeling such as burn scars (Hayakawa et al., 1976), wound healing (Riley and Martin, 1970), hepatic fibrosis (Siegel et al., 1978), granuloma (Chapvil et al., 1974), and lung fibrosis (Counts et al., 1981) increased lysyl oxidase activity accompanied collagen accumulation. Few chemicals are able to influence so selectively the metabolism of connective tissues. Feeding the lathyrogen β-aminopropionitrile (BAPN) produces osteolathyrism, since the primary effect of this drug is inhibition of lysyl oxidase activity (Rucker and Murray, 1978). Thiol compounds such as penicillamine alter collagen cross-linking, but in a manner that differs from that of BAPN (Nimni, 1983). The influence of lipids on the metabolism of collagen has received only a little attention. Thiers et al. (1961) showed that a mixture of avocado and soybean lipidic nonsaponifiables administered per os has been active in the treatment of various disorders of connective tissue, such as scleroderma, as well as in wound healing. Robert et al. (1974a,b, 1975) reported the pharmacological effects of this mixture on carrageenan-induced granuloma and on skin of rats, but, in fact, no single purified constituent was isolated. A previous study (Werman et al., 1990) has shown that rats fed lipid components from the avocado exhibited decreased activity of skin lysyl oxidase. It has been suggested that these lathyrogenic factors probably originate in the unsaponifiable material of the avocado seed oil. In the present study, the influence (in vitro) of various components of avocado unsaponifiables on lysyl oxidase activity was examined to isolate the active factor and compare its activity to other known inhibitors.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Avocado Oils. Unsaponifiables were prepared from refined avocado oil (RAO), produced by centrifugal separation from cored fruit, Hass variety (Avochem Santa Paula, CA), from unrefined avocado oil (URAO) produced by hexane extraction of intact fruit, Fuerte variety, (Miluot, Haifa, Israel), and from avocado seed oil, Fuerte variety, extracted by chloroform/methanol (2:1) as suggested by Gutfinger et al. (1972).

Unsaponifiable Material. Unsaponifiable material was prepared according to the Ca 6b-53 method of AOCS (1974).

Identification and Quantitation of Unsaponifiable Components. Unsaponifiables were separated to their fractions by thin-layer chromatography (TLC) on 20 × 20 cm plates, covered with a 0.25-mm layer of silica gel (Merck, Darmstadt, W. Germany) as described by Gutfinger et al. (1972). Forty milligrams of the unsaponifiables in chloroform was applied to the plate as a strip 15 cm long. To characterize the fractions, a control solution in chloroform of a mixture of β-sitosterol, α-tocopherol, squalene, β-carotene, and β-amyrin was spotted at one edge of the strip. To identify fraction location, a spot of the unsaponifiable solution was applied on the second edge. The developing solvent was petroleum ether (60–80 °C)/ethyl ether (1:1). After developing, the edges of the plates were removed with a glass cutter and were sprayed with 50% (w/w) sulfuric acid for color development at 115 °C for 10 min. Each of the silica gel fraction stripes was scraped off with a sharp spatula. The scraped silica gel was placed in a 15-mL centrifuge tube that contained 10 mL of the developing solvent. The tube was shaken for 20 s in a vibrator, centrifuged (10 min at 3000g), and decantated. The solvent was evaporated at 50 °C with a stream of nitrogen to near dryness, and the residue was rechromatographed as described above until it appeared as a single spot. The concentration of the rechromatographed fractions was determined gravimetrically.

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Instrumentation. Mass spectrometry (MS) was performed on a Varian MAT Model 711 magnetic section double-focusing mass spectrometer. Infrared (IR) spectrometry was performed on a Perkin-Elmer 298 infrared spectrophotometer using NaCl cell and chloroform as solvent. Nuclear magnetic resonance (NMR) was performed on a Bruker AC-E 200 NMR spectrometer using $CDCl_3$ as solvent.

Lysyl Oxidase Assay. The effect of avocado lipids on lysyl oxidase activity was determined in vitro on the enzyme extracted from the skin of female rats weighing 150 g and from the tibia of 17-day-old chick embryos. Skin samples were cut finely with scissors and homogenized with 19 volumes of 4 M urea and 0.16 M NaCl in 0.05 M phosphate buffer, pH 7.4 at 4 °C, using a Virtis homogenizer. The resultant supernatant after centrifuging at 135000*g* for 45 min and dialyzing for 24 h against 0.16 M NaCl in 0.05 M phosphate buffer, pH 7.4, to remove the urea served as the enzyme extract. Tibia enzyme was prepared as suggested by Prockop and Tuderman 1982). Protein content in the hydrolysate extracts was determined according to the method of Lowry et al. (1951). For preparation of collagen substrate, calvarial parietal bones from 18 17-day-old chick embryos were incubated at 37 °C in a 50-mL Erlenmeyer flask with 10 mL of Eagle's minimum essential medium without lysine and supplemented with 4 mg of proline, 5 mg of glycine, 5 mg of BAPN, 5 mg of ascorbic acid, and 20 000 units of penicillin G per 100 mL of medium.

After preincubation for 60 min to reduce the free lysine pool, the medium was changed, 250 μCi of DL-[4,5-^{3}H]lysine was added to each flask, and the flasks were gassed for 1 min with a mixture of 5% CO_2/95% O_2. After incubation for 24 h, the calvaria were washed twice with 10 mL of 0.15 M NaCl in 0.1 M phosphate buffer (PBS), pH 7.8, and four flasks were pooled and homogenized in 40 mL of 1 M NaCl in 0.05 M Tris-HCl, pH 7.4 at 4 °C, for 10 min with a Ystral homogenizer and then centrifuged at 20000*g* for 10 min. The collagen in the supernatant was precipitated by the addition of solid NaCl to a final concentration of 20%. The precipitate was collected by centrifugation at 20000*g* for 10 min, resuspended in 15 mL of PBS, and then dialyzed for 72 h against the same buffer at 4 °C. The dialysate was clarified by centrifugation at 30000*g* for 15 min. To inactivate endogenous lysyl oxidase, the supernatant was incubated with 5 mM BAPN for 2 h at 37 °C. During this treatment, the collagen formed fibrils which were recovered by centrifugation. The pellet was redissolved in PBS and dialyzed against the same buffer for 24 h at 4 °C, and the dialysate was used as the substrate. Lysyl oxidase activity was determined by measuring the tritiated water formed during incubation of the enzyme extracts and radioactive substrate with avocado lipids. The lipid components were added to the reaction as an emulsion by using Tween 80 (Fisher Scientific Co.) as the emulsifier. Standard reaction mixtures contained 1.2 mL of enzyme preparation, containing 2–3 mg of protein per assay (Counts et al., 1981); 0.2 mL of the substrate (2–4 × 10^5 dpm); 0.1 mL of 1 M NaCl in 0.05 M phosphate buffer, pH 7.4, with L-lysine at a final concentration of 0.01 M per assay (Siegel, 1979); 0.4 mL of emulsion of 20% (w/v) of Tween 80/lipid component (1:1 w/w) in water; 0.1 mL of water; and 1 drop of toluene to prevent microbial growth. When less emulsion was added to determine the influence of various lipid contents, a complementary volume of 10% (w/v) Tween 80 in water was added so that the Tween 80 content remained constant. The control contained 0.1 mL of BAPN fumarate solution (4 mg/mL) instead of water. After incubation for 20 h at 37 °C, the tubes were rapidly chilled in ice, and the reaction was terminated by the addition of 0.25 mL of 50% trichloroacetic acid (TCA). The resulting protein precipitate was removed by centrifugation in a table centrifuge. Lysyl oxidase activity was determined by measuring the tritiated water formed during incubation of enzyme extracts and radioactive substrate. The tritiated water formed during the incubation was collected by ion-exchange columns according to the method described by Melet et al. (1977). Columns were prepared by pipetting a slurry of Dowex 50W-X8, 200–400 mesh (Serva feinbiochemica, Heidelberg, W. Germany) in distilled water into plugged disposable Pasteur pipets. Immediately before use, the columns were flushed with 3 mL of 5% TCA, and then 0.5 mL of the TCA supernatants was passed through. The samples were eluted with 5% TCA directly into 2-mL volumetric flasks. Volumes of 1 mL of the effluent were counted in a Beckman LS-9800 liquid scintillation spectrometer. Lysyl oxidase activity was expressed as disintegrations per minute of ^3H_2O per milligrams of protein in assay.

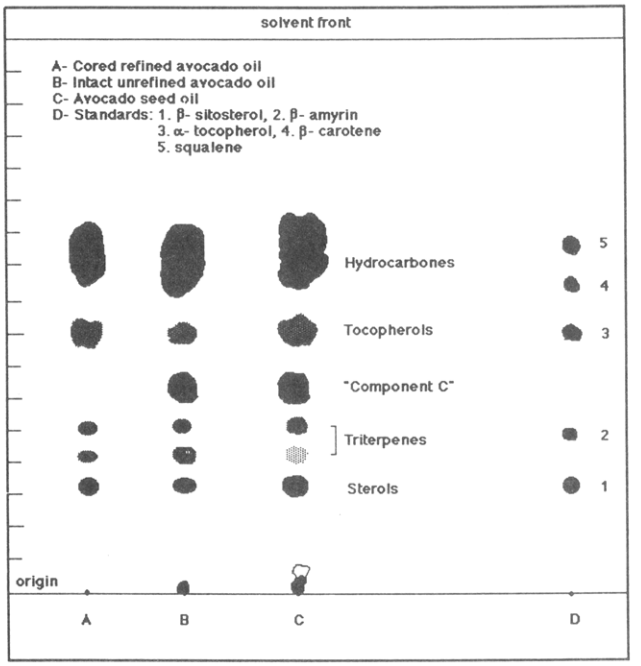

Figure 1. Thin-layer chromatography of unsaponifiable matter from avocado oils. Developing solvent: petroleum ether (60–80 °C)/ethyl ether (1:1). Spraying reagent: 50% (w/w) H_2SO_4.

Table I. Content of the Unsaponifiable Fraction of Refined Avocado, Unrefined Avocado, and Avocado Seed Oils, Obtained by Thin-Layer Chromatography (TLC)[a]

fractions, mg/g of unsaponifiables	R_f[b]	RAO	URAO	seed
hydrocarbons	0.53–0.65	557 ± 29	487 ± 21	284 ± 27
tocopherols	0.47	83 ± 7	75 ± 12	108 ± 18
unknown	0.38		23 ± 4	58 ± 8
triterpenes	0.24–0.29	114 ± 19	106 ± 14	215 ± 25
sterols	0.18	220 ± 33	196 ± 27	47 ± 5
origin		43 ± 6	123 ± 11	280 ± 32
total unsaponifiables, mg/g of oil		15.3 ± 0.8	19.6 ± 1.9	534 ● 68

[a] Values are means ± SD for three determinations of each oil. [b] R_f = [fraction migration (cm)/solvent migration (cm)].

RESULTS

TLC separation of unsaponifiable matter from various avocado oils is presented in Figure 1. Refined avocado oil was separated into five fractions. These fractions were identified as hydrocarbons represented by squalene and β-carotene, tocopherols by α-tocopherol, triterpenes by β-amyrin, and sterols by β-sitosterol. However, unsaponifiable matter from the URAO and avocado seed oil had an additional fraction. This fraction, called component C, was located between the tocopherols and the triterpenes (R_f = 0.38).

The content of unsaponifiable matter and its components in the various avocado oils is presented in Table I. Unsaponifiable content in the RAO and URAO is 1.5 and 2%, respectively, while that of the seed oil reached 50%. It can be seen that the content of unsaponifiable components in the seed differs from that of the RAO and URAO. The hydrocarbon content in the RAO and the URAO was found to be double and the sterol content was as much as 5-fold higher than that of the avocado seed oil. However, tri-

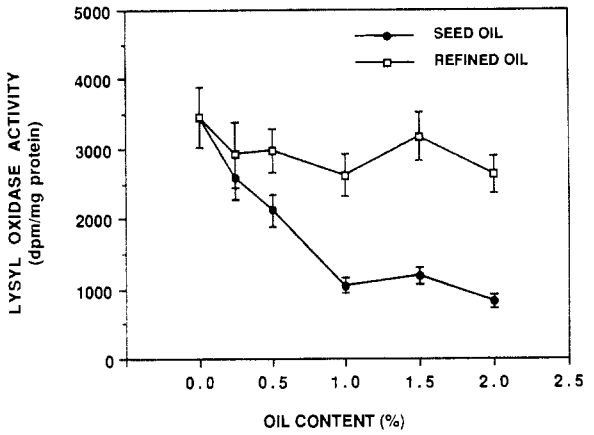

Figure 2. Influence (in vitro) of avocado seed oil on lysyl oxidase activity from rat skin. Values are the means ± SD of six determinations for each concentration.

Table II. Influence (in Vitro) of Unsaponifiable Components from Avocado Seed Oil on Lysyl Oxidase Activity from Rat Skin and Tibia of Chick Embryo[a]

unsaponifiable components	content per assay, μg/mL	lysyl oxidase activity, dpm/mg of protein	
		chick tibia	rat skin
hydrocarbons	2800	3150 ± 280	3130 ± 400
tocopherols	1000	2780 ± 195	3345 ± 290
component C	600	1090 ± 375	1200 ± 230
triterpenes	2100	3400 ± 350	3120 ± 225
sterols	450	2900 ± 350	2770 ± 400
origin	2500	2910 ± 410	
control (Tween 80)	20000	3100 ± 380	3565 ± 395

[a] Values are the means ± SD for six determinations of each component.

terpene content in the seed oil was double that of the other oils, and the content of the fraction that remained at the origin was double that of the URAO and 6 times that of the RAO. The content of component C in the seed was double that of the URAO, while in the RAO this fraction was not found. Our previous in vivo studies with growing rats fed various avocado oils (Werman et al., 1990) showed that avocado oil contains an inhibitory factor of lysyl oxidase and that this factor probably originates in the seed.

Figure 2 shows that the inhibitory effect of avocado seed oil can be achieved also in vitro. Avocado seed oil applied to the lysyl oxidase assay system, in a final concentration of 0.5%, significantly decreased enzyme activity by 30% as compared to the RAO. Seed oil content of 1% decreased lysyl oxidase activity by 70%; this effect was also observed at 2% seed oil content. Enzyme activity was not affected by the presence of the RAO, at any concentration tested. To identify the active factor, each of the seed oil unsaponifiable components was applied (in vitro) to the reaction at a final concentration corresponding to 2% seed oil. Table II summarizes the effect of the seed oil unsaponifiable components on both rat and chick lysyl oxidase activity. Component C, which is typical only to the seed oil, was the only unsaponifiable component to affect lysyl oxidase activity. Enzyme activity was decreased by 65% in both rat skin and chick tibia. These results indicate, undoubtedly, that the factor influencing lysyl oxidase activity is associated only with component C of the seed oil unsaponifiables. The molecular weight of the rechromatographed component C was obtained by mass spectra (Figure 3) and was found to be 248 at 280 °C. The IR spectrum of this component dissolved in chloroform (15 mg/mL) and the NMR chart are given in Figures 4 and

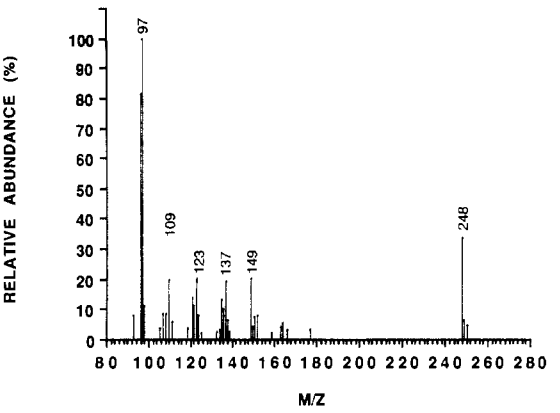

Figure 3. Mass spectrum of component C.

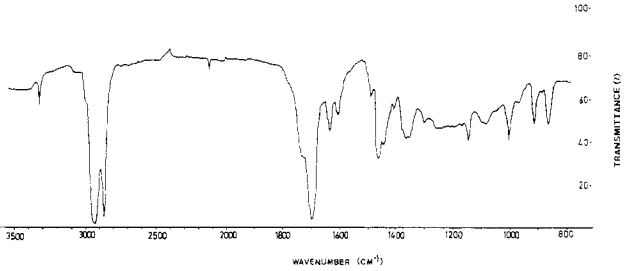

Figure 4. Infrared spectrum of component C.

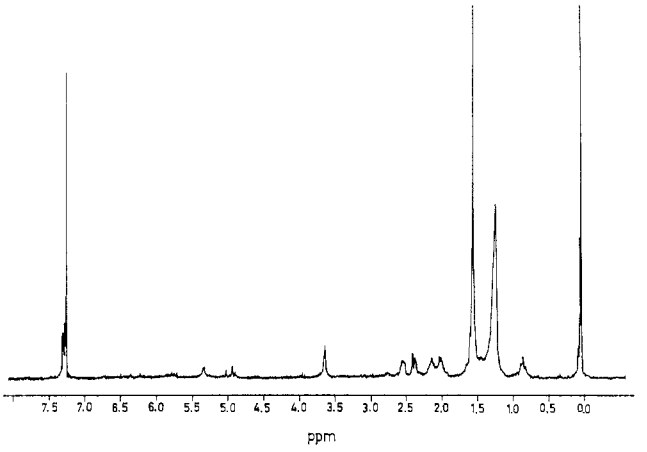

Figure 5. Nuclear magnetic resonance spectrum of component C.

5, respectively. The effect of increasing levels of component C on rat skin lysyl oxidase as compared to penicillamine is presented in Figure 6. A decrease of approximately 40% in enzyme activity was observed at a concentration level of 0.625 mM component C. Increasing levels of component C up to 2.5 mM decreased enzyme activity by 70%. However, penicillamine at the same tested levels did not affect lysyl oxidase activity. An identical inhibition was observed by comparing the effect of the seed oil (Figure 1) with that of the corresponding level of component C (Figure 6) on lysyl oxidase activity. For example, component C level of 1.25 mM and 1% avocado seed oil both inhibited lysyl oxidase activity by 65%.

DISCUSSION

Avocado oils are mainly produced commercially by two procedures, organic solvent extraction, which utilizes mature but hard intact fruit, or centrifugal separation, which utilizes mature, hard or soft, intact or cored fruit (Werman and Neeman, 1987). Besides differences in extraction procedures, effects on collagen metabolism might

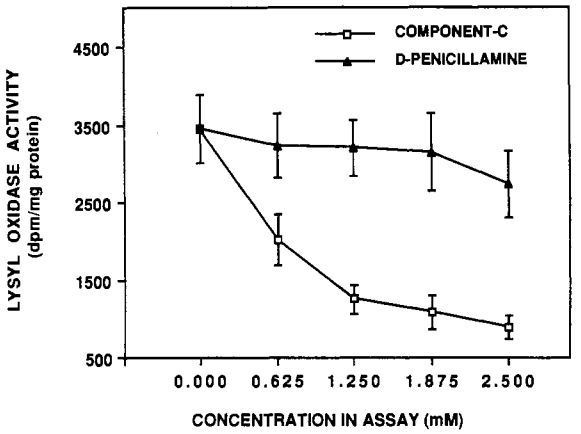

Figure 6. Influence (in vitro) of component C and penicillamine on lysyl oxidase activity from rat skin. Values are the means ± SD of six determinations for each concentration.

be influenced by the presence of the avocado seed during oil extraction, since the seed contains some toxic factors (Neeman et al., 1970; Werman et al., 1989). Indeed, in a previous in vivo study (Werman et al., 1990) carried out in our laboratory, we have demonstrated that collagen metabolism in growing rats was affected only by feeding avocado oils which contain, to some extent, the lipid fraction of the avocado seed. Feeding rats with avocado unsaponifiables (Robert et al., 1975) raised the possibility that the factor responsible for the effect on collagen metabolism and lysyl oxidase activity originates in this lipid fraction. It was noticed by some of the investigators that the unsaponifiable components furnish a fingerprint of the oil, useful for the detection of foreign admixtures in an investigated oil (Gutfinger and Letan, 1974; Joseph and Neeman, 1982). Separation of unsaponifiables by the TLC procedure demonstrated that besides the main unsaponifiable components (e.g., hydrocarbons, tocopherols, triterpenes, and sterols) an additional component, component C, was observed in the URAO and the seed oil (Figure 1). However, this component was not present in the cored RAO, suggesting that component C might serve as an indication for the presence of avocado seed oil. Kashman et al. (1969) have separated eight compounds shown to belong to the same group of long-chain aliphatic compounds. These compounds may be grouped into four pairs. Each pair contains two compounds that differ only in having a double or triple bond at the end of the chain. One of these compounds, 2-(tridec-12-enyl)furan, has a molecular weight of 248, the same as component C (Figure 3). However, from the NMR spectrum (Figure 5) the ratio between the allylic (δ 2.0–2.5) and the vinylic (δ 5.0–5.5) hydrogens is higher than the calculated value for 2-(tridec-12-enyl)furan isolated by Kashman et al. (1969). These results indicate that component C is not pure. Impurity is further confirmed by the IR spectrum (Figure 4). Absorbances at 3300 and 2120 cm^{-1} indicate the presence of a terminal triple bond, and that at 1700 cm^{-1} indicates a carbonyl group. These groups do not originate from the furan-containing component. However, from the absorbances at 870 and 910 cm^{-1} one cannot rule out the presence of the characteristic double bond and the furan ring, respectively.

The present study demonstrated that avocado seed oil is responsible for the unique inhibition for lysyl oxidase activity, observed in vivo (Werman et al., 1990) as well as in vitro (Table II), and that of all the seed unsaponifiables, component C is the active factor. The similar influence of avocado seed oil both in vivo and in vitro reveals that the active factor is present in the seed and is not a result of any metabolic pathway in the rat. Furthermore, the similarity observed between the effect of seed oil (Figure 1) and that of equal concentrations of isolated component C (Figure 2) on lysyl oxidase activity (in vitro) suggests that seed oil does not contain any factors which might interfere with component C activity. A comparison of the influence, in vitro, of component C to that of penicillamine (Figure 3) showed that penicillamine in the tested concentrations had no effect on lysyl oxidase activity. These results are in agreement with those of Nimni (1983) and Siegl (1977).

Penicillamine is known to inhibit lysyl oxidase activity by reacting with copper, the enzyme cofactor, but only in concentrations higher than 10^{-2} M, while at all concentrations this drug reacts with allysine or hydroxyallysine residues obtained by the oxidative deamination of lysyl oxidase, thus preventing further progress in crosslinking formation (Nimni, 1983). Another known inhibitor of lysyl oxidase is the lathyrogen BAPN (Rucker and Murray, 1978), but comparing I_{50} values of BAPN (Tang et al., 1983) and component C (Figure 6) reveals a difference of approximately 3 orders, 5 μM compared to 1 mM, respectively.

In conclusion, avocado seed oil contains a unique unsaponifiable fraction that inhibits lysyl oxidase activity. This active fraction is probably a mixture of some polyalcoholic compounds, one of them having a molecular weight of 248 and being composed of a 17-carbon aliphatic chain with a furan ring. Experiments are in progress to isolate and separate between these compounds and to determine their exact structure. These steps are needed to reveal the mechanism by which this fraction exerts its effect on lysyl oxidase.

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