Eggplant Lipoxygenase (*Solanum melongena*): Product Characterization and Effect of Physicochemical Properties of Linoleic Acid on the Enzymatic Activity

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Lipoxygenase (LOX) from eggplant (*Solanum melongena* L. cv. Belleza negra) was partially purified, and the products and kinetics of the enzyme were studied. Linoleic acid (LA) was the best substrate for this enzyme. Product analysis by HPLC and GC/MS revealed that, at its pH optimum (pH 7.0), the enzyme converted LA almost totally into the 9-hydroperoxy isomer, whereas the 13-hydroperoxy isomer was only a minor product. At this pH, the enzyme had K_m and V_{max} values for LA of 1.4 μM and 2.2 μmol min^{-1} (mg of protein)$^{-1}$, respectively, when the monomeric form of LA was used as substrate. The dependence of eggplant LOX activity on the physicochemical properties of LA was also studied. Experiments revealed that LA aggregates were used more efficiently than monomeric LA as substrate. The apparent substrate cooperativity observed may be due to the different activities exhibited toward monomers and aggregates. This result can be interpreted as a substrate-aggregation dependent activity.

Keywords: *Aggregation state; cyclodextrin; eggplant; HPOD; linoleic acid; lipoxygenase; Solanum melongena*

INTRODUCTION

Lipoxygenases (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) are nonheme iron-containing enzymes that use molecular oxygen in the dioxygenation of a fatty acid containing one or more 1,4-*Z*,*Z*-pentadiene systems (*1*−*3*). The main reaction products are chiral (*E*, *Z*) conjugated hydroperoxy fatty acids. These enzymes are ubiquitously distributed in animals and plants and have a key function in the formation of biologically active substances (*4*, *5*).

The oxygenation of polyunsaturated fatty acids (PUFAs) by most lipoxygenases is characterized by a high positional specificity. The linoleic acid (LA)-derived products of plant lipoxygenases usually contain hydroperoxide groups at C-9 or C-13. The relative proportion of the 9- and 13-hydroperoxide isomers depends on the enzyme source (*6*) and, to some degree, on the reaction conditions (*1*). In most plant species studied, LOXs are encoded by gene families (*7*). Isoforms differ in a number of characteristics such as substrate preference, kinetic parameters, and the positional specificity of substrate oxygenation. Although the physiological role of plant lipoxygenases is poorly understood, their products are substrates for the subsequent action of enzymes, such as hydroperoxide lyase and allene oxide synthase. Thus, the identification of the hydroperoxide isomers produced during the LOX reaction is an important step in the characterization of this enzyme and helps to clarify the role of these products in lipid metabolism. Sredni and Grossman (*8*) reported the production of 13-hydroperoxide as the major product when eggplant lipoxygenase was incubated with LA acid. This enzyme was able to oxidize LA and linolenic acid (LnA) at the same rate (*9*). In this paper, the isolation of a novel eggplant lipoxygenase producing 9-hydroperoxy linoleic acid and with a different substrate specificity is reported.

Characterization of LOX activity in plant extracts is hampered by the complex nature of the reaction system, which involves a lipid substrate and an aqueous enzyme preparation. Although the polarographic assay is useful for routine analysis of both crude and partially purified LOX extracts, it is not sufficient to fully characterize the reaction mechanism. In spectrophotometric studies of LOX reaction at neutral pH, the low solubility of fatty acids is a common problem. One way of overcoming the visual turbidity is to use detergents, such as Tween 20, that form micelles which solubilize and optically clear these fatty acid suspensions. However, the use of these surfactants may lead to misinterpretations of the results obtained (*10*−*12*). Another consequence of the low solubility of PUFA is the heterogeneity of the reaction medium: depending on the pH and on the fatty acid concentration, the substrate may be present as monomers, micelles, or bilayers, or as a separate oil phase (*13*). It is of critical importance to know which of these forms of substrate is being used by LOX and what percentage of the overall fatty acid is available for the enzyme. Recently our group applied the ability of cyclodextrins (CDs) to solubilize PUFA to the study of the LOX reaction (*14*, *15*). This method provides valuable information as regards the preferences of LOX for the physical form of the substrate and makes it possible to know the exact concentration of the fatty acid in its different forms. In the present paper CDs were used to study the influence of the aggregation state of LA on the activity of eggplant LOX.

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MATERIALS AND METHODS

Plant Material. Purple eggplant fruits (*Solanum melongena* L., cv. Belleza negra) at harvest maturity were obtained from the local market.

Reagents. Linoleic acid (LA), linolenic acid (LnA), and arachidonic acid (AA) were purchased from Cayman Chem. Co (Paris, France). Diphenylhexatriene (DPHT), a fluorescent probe, was a product of Fluka (Madrid, Spain). Palladium on calcium carbonate was from Janssen Chimica (Geel, Belgium). β-Cyclodextrin (β-CD), pyridine, 1,1,1,3,3,3-hexamethyldisilazane, and trimethylchlorosilane were obtained from Aldrich (Madrid, Spain). Octadecyl solid-phase extraction columns (1 mL, 100 mg) were purchased from J. T. Baker Inc. (Deventer, The Netherlands). Sodium borohydride and soybean lipoxygenase-1 type IV were from Sigma (Madrid, Spain). Potato lipoxygenase was purified according to the method of Mulliez et al. (*16*) and had a specific activity of 27 μmol O_2 min^{-1} (mg of protein)$^{-1}$. Methanol (Lab-Scan, Dublin, Ireland) and tetrahydrofuran (THF) (Merck, Darmstadt, Germany) were of HPLC grade. All other chemicals were of analytical grade.

Enzyme Preparation. All processes were performed at 4 °C. Peeled eggplant fruits (150 g) were diced and homogenized in a blender with 200 mL of 50 mM phosphate buffer pH 7.0. The resulting homogenate was filtered through four layers of nylon cloth and then centrifuged at 5000*g* for 5 min at 4 °C. The supernatant was discarded and the pellet was solubilized by resuspension in 150 mL of 100 mM sodium phosphate buffer pH 7.0. This was then sonicated for 2 h in an ultrasonic bath (Branson 1200). The solution was then centrifuged at 10 000*g* for 15 min and the pellet was discarded. The supernatant was brought to 40% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60 000*g* for 1 h at 4 °C. The supernatant was discarded and the pellet was resuspended in phosphate buffer 100 mM pH 7.0 to give a final volume of 20 mL. This solution (3.8 U/mL) was employed for the enzymatic activity assays. One enzymatic unit (U) is defined as the ammount of enzyme that gives rise to the formation of one μmol of conjugated diene per min, using linoleic acid as substrate in 0.1 M phosphate buffer pH 7.0.

Analysis of LOX Products. *HPLC Analysis.* For product analysis, 30 mL of a 100 μM solution of LA in 100 mM phosphate buffer (pH 7.0) containing 1% ethanol was incubated with 100 μL enzyme extract at 4 °C and under constant aeration. After 30 min the reaction was stopped by acidification to pH 4.0 with HCl, and the products were extracted with an octadecyl solid-phase extraction column eluted with methanol. These compounds were analyzed with a Kontron-420 HPLC on an Inertsil-2-ODS column (GL Science Inc, 5 μm particles, 4.6 × 150 mm) and detected at 234 nm using a Kontron 430 UV detector. Samples were injected using a Reodyne 7125 loop injector (20 μL). Products were eluted isocratically with THF/methanol/water/acetic acid (25:30:45:0.1, v/v/v) (*12*) at a flow rate of 1 mL/min. The ratio between 9- and 13- HPOD was calculated from the peak areas, assuming a molar absorbance of 25 000 M^{-1} cm^{-1} at 234 nm for both compounds. The products of eggplant LOX on LA were identified by HPLC coinjecting standards of 13- and 9-HPOD. 13-HPOD was prepared by incubation of 0.1mM LA with 15 μL of soybean LOX-1 (1 mg/mL) in 30 mL of 0.1M sodium borate buffer pH 9.0; 9-HPOD was obtained by incubation of 0.1 mM LA with 500 μL of 5-LOX potato (4.6 mg/mL) in 30 mL of 0.1 M sodium phosphate buffer pH 6.3. Both compounds were extracted using the procedure described above and stored at −20 °C flushed with N_2.

Derivatization and GC/MS. For GC/MS analysis the fatty acid hydroperoxides were dissolved in methanol and reduced to the corresponding hydroxy fatty acids with $NaBH_4$ at 0 °C under N_2. After 20 min, water was added and the reaction mixture was acidified with HCl to pH 4.0. The products were extracted with an octadecyl solid-phase extraction column and eluted with methanol. The solvent was evaporated and the residue was dissolved in 2 mL of ethereal diazomethane at room temperature. After 30 min the ether was evaporated to dryness under a N_2 stream. GC/MS analysis was performed on hydrogenated and non-hydrogenated samples. For catalytic hydrogenation of the double bond, half of each sample was dissolved in methanol and, after the addition of a small amount of palladium on calcium carbonate, H_2 was bubbled through for 30 min. The catalyst was removed by filtration through cotton wool and the methanol was evaporated with N_2. Prior to GC/MS analysis, hydrogenated and non-hydrogenated samples were derivatized to the trimethylsilyl (TMS) ether by reaction with a mixture of pyridine/hexamethylsilazane/trimethylchlorosilazane (5:1:1, v/v/v) for 30 min. The resulting compounds were analyzed on a mass spectrometer (Fisons Instruments MD 800 mass lab spectrometer, GC 8000 series Interscience), equipped with a DB1-fused-silica capillary column (30 m × 0.32 mm, J&W Scientific). The temperature program was 2 min isothermal at 140 °C, 140 °C to 280 °C (6 °C/min), and 2 min isothermal at 280 °C; runtime, 28 min. All mass spectra were recorded under electron impact with an ionization energy of 70 eV.

Fluorimetric Determination of Critical Micellar Concentration (CMC). CMC is defined as the LA concentration value at which the fatty acid starts to aggregate. The CMC value of LA at pH 7.0 was determined by means of fluorescence spectroscopy (*17*). Briefly, 2-mL samples containing 0.1 M phosphate buffer pH 7.0, 0.88 μM DPHT (supplied in 2 μL THF), 1% (v/v) ethanol, and different concentrations of LA were flushed with N_2 and incubated for 30 min in the dark at 25 °C for equilibration and to reverse the photoisomerization of the fluorescent probe. Fluorescence intensity was measured at 430 nm (excitation wavelength, 358 nm) by means of a Kontron SFM-25 spectrofluorometer thermostated at 25 °C. The relative values of the fluorescence were plotted against the LA concentration, and the CMC was determined as the intersection between the lines defining the fluorescence tendency in the premicellar and postmicellar regions.

Enzyme Activity Assays. *Spectrophotometric Determination of LOX Activity in the Presence of CDs.* To determine the effect of pH on LOX activity, the enzymatic activity was followed at 25 °C, monitoring the increase in absorbance at 234 nm in a Hewlett-Packard 8452A diode array spectrophotometer. To maintain the monomeric concentration of linoleic acid (LA_f) constant (8 μM) at each pH, different amounts of fatty acid were dissolved with β-CD 1mM in the corresponding buffer. The total concentration of linoleic acid (LA_t) necessary to yield the desired amount of LA_f at each pH was calculated using the set of constants determined in a previous work (*17*) and the following equation.

$$[LA]_t = [LA]_f (1 + K_1 [CD] + K_1 K_2 [CD]^2 \qquad (1)$$

The buffers used were 0.1 M sodium phosphate containing 1% ethanol for pH 5.0−7.5 and 0.1 M sodium borate containing 1% ethanol for pH 7.5−9.0. The complexes LA-β-CD were prepared by dissolving β-CD in the corresponding buffer containing 1% ethanol, followed by the addition of fatty acid prepared in the same buffer. The samples were flushed with N_2 to prevent LA oxidation during the preparation. The reaction was started by adding 10 μL of enzyme to 1 mL of LA-β-CD.

Polarographic Determination of LOX. Substrate specificity and the effect of LA concentration on LOX activity were determined by measuring oxygen consumption with a Clark-type electrode. The 1-mL samples consisted of 0.1 M phosphate buffer pH 7.0, 1% (v/v) ethanol and different concentrations of fatty acid. They were vigorously shaken before use so that they would become air-saturated, and were transferred to the stirred, thermostated oxygraph chamber (Hansatech Ltd. Norfolk, UK) where the reaction was started by adding 10 μL of enzyme. Quantitative measurements were made by using an oxygen calibration method (*18*).

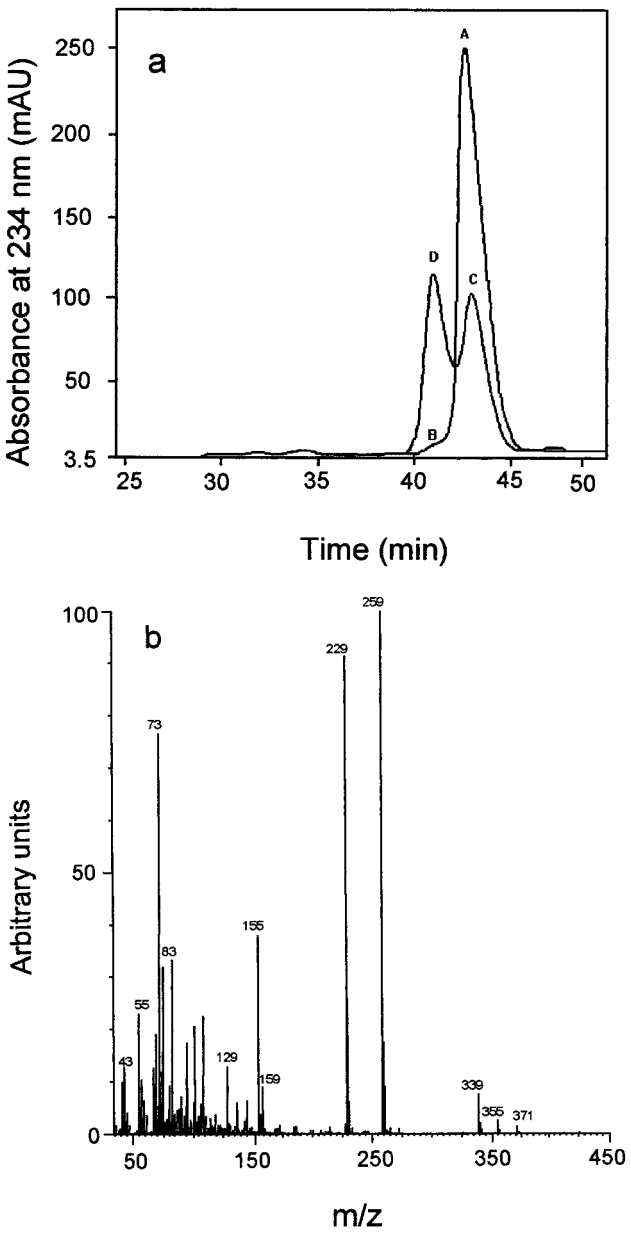

Figure 1. Analysis of products formed by eggplant LOX from linoleic acid. (a) Reversed-phase HPLC analysis of 9-HPOD from eggplant (peak A), 13-HPOD from eggplant (peak B), 9-HPOD from potato (peak C), and 13-HPOD from soybean (peak D). The eluent system is THF/methanol/H_2O/acetic acid (25:30:45:0.1). (b) Mass spectrum of the catalytically hydrogenated, $NaBH_4$ -reduced, TMS-derivatized methyl ester of peak A.

RESULTS AND DISCUSSION

Product Specificity of Eggplant LOX. The products formed upon incubation of LA with eggplant LOX at pH 7.0 were analyzed by reversed-phase HPLC, which showed one main product (Figure 1a, peak A) with a UV spectrum typical of conjugated dienes (λ_{max} = 234 nm). The retention time of this product compared with those of standards of 13- and 9-HPOD (Figure 1a) showed that the main product (96%) of eggplant LOX is 9-HPOD, the small amount of 13-HPOD (Figure 1a, peak B) present probably being the result of the autoxidation of LA during the incubation and extraction procedures. No differences on the positional specificity of the enzyme were observed when the analysis was carried out at a LA concentration lower than the CMC.

Table 1. Substrate Specificity of Eggplant LOX[a]

	eggplant LOX activity (nmol 0_2/min)		
substrate	linoleic acid	linolenic acid	arachidonic acid
17.8 μM	3.1 ± 0.15	0.12 ± 0.01	n.d.[b]
35.5 μM	6.2 ± 0.2	0.23 ± 0.04	n.d.

[a] Reaction medium contained the indicated PUFAs concentration in 0.1 M sodium phosphate pH 7.0 plus 1% ethanol at 25 °C. The reaction was started by adding 0.038 U of enzyme. [b] n.d., not detected.

To confirm its identity, the main peak was isolated by HPLC, reduced with $NaBH_4$, derivatized to the methyl esters, silylated, and analyzed by GC/MS. Ions in the mass spectrum included the molecular ion at *m/z* 382 and fragment ions at *m/z* 367 (M^+-15), 351 (M^+-31), 311 (M^+- 71), 292 (M^+-90), and 225 (M^+-157), indicating that the compound is a C_{18} monohydroxy fatty acid containing two double bonds (*19*). Evidence for the position of the hydroxyl moiety in the compound was obtained by hydrogenating the product prior to silylation. The mass spectrum showed major ions at *m/z* 259 and 229 (Figure 1b), indicating the presence of the TMS derivative of a hydroxyl moiety at C_9, and cleavage of the carbon bond between C_9−C_{10} and C_8−C_9, respectively (*19*). These data confirm the identity of the product as 9-HPOD. The fact that eggplant LOX produces 9-HPOD almost exclusively is in agreement with the results obtained for LOXs extracted from other Solanaceaes such as tomato fruit (*12*) and potato tuber (*16*). However, Sredni and Grossman (*8*) reported a molar ratio of 97% of 13-HPOD and 3% of 9-HPOD when eggplant LOX was incubated with LA. The purification method used in this work involves the sonication of a chloroplastic fraction in the presence of 0.1 M sodium phosphate buffer, whereas Sredni and Grossman (*8*) added Triton X-100 and used the whole homogenate as LOX extract. The discrepancy in product specificity (Figure 1) between the data reported in this paper and those of Sredni and Grossman (*8*) can be attributed to differences in the eggplant variety (*20*) or the existence of different LOXs in eggplant fruit.

Substrate Specificity. LA and LnA acids are the most abundant PUFA in plants, and are therefore the most likely substrates for lipoxygenase catalysis (*21*). Furthermore, certain plant LOXs are also able to act on arachidonic acid (*16*, *22*, *23*). The activity of eggplant LOX at pH 7.0 on these three substrates is given in Table 1.

The LOX of eggplant used in this paper shows a strong preference for LA, but much less activity was noted with LnA acid (less than 4% of that obtained with LA). This contrasts with the results reported by Grossman (*9*), who observed a similar activity on linoleic acid and linolenic acid, and supports the possibility that different lipoxygenases are involved. Further studies showed that the enzyme used in this work is membrane associated and has an apparent molecular mass of 97 kD after SDS−PAGE (*24*). Unlike the results reported for LOXs from another Solanaceae (*25*, *26*), no activity was detected when eggplant LOX was incubated with arachidonic acid.

Effect of pH. It is well-known that pH determines not only the protonation but also the aggregation state of fatty acids (*13*). At pH 7.0 LA CMC is approximately 20 μM (Figure 3). Thus, when LA concentrations below

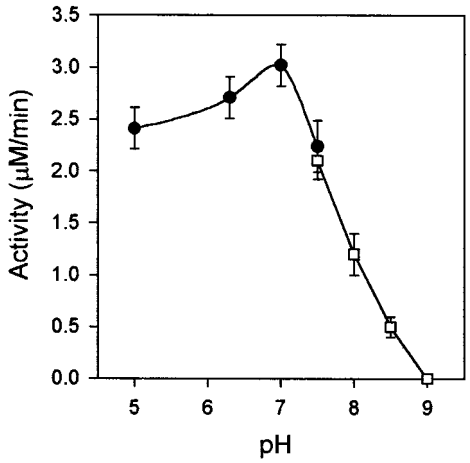

Figure 2. Effect of pH on eggplant LOX activity. The reaction medium consisted of 8 μM LA_f at each pH, 0.038 unit of LOX, and 0.1 M sodium phosphate (pH 5.0−7.5, circles) or 0.1 M borate (pH 7.5−9.0, squares). Both buffers contained 1% ethanol. The mean of three determinations ± SE is presented. See Materials and Methods for details.

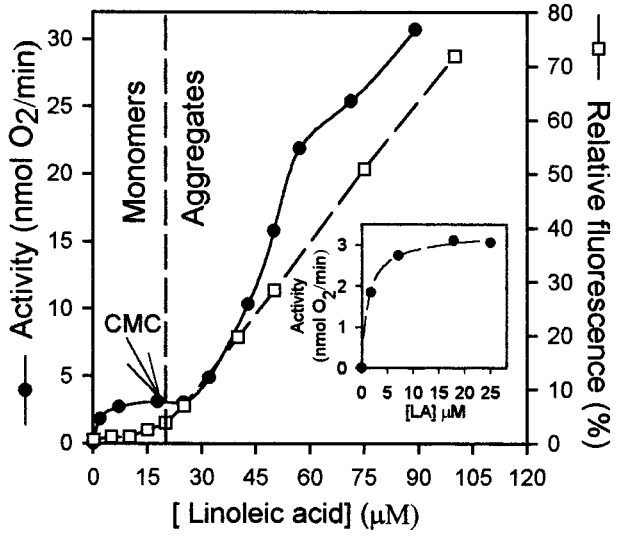

Figure 3. Dependence of oxygen consumption rate of eggplant 5-LOX on LA concentration. The reaction was started by adding 0.038 unit of LOX to a medium containing 0.1 M phosphate buffer pH 7.0 plus 1% ethanol. Dependence of relative fluorescence intensity of DPHT at 430 nm (excitation wavelength 358 nm) on LA concentration. Inset, effect of LA concentration on eggplant LOX activity in the pre-micellar region of LA.

this value are used, the substrate is quickly consumed and it is difficult to measure the LOX activity in this region. On the other hand, when the LA concentration is above CMC, fatty acids form a micellar phase and scattering makes the spectroscopic determination of LOX activity similarly difficult.

To avoid these problems, the pH optimum was determined using a method based on the formation of the inclusion complex between PUFAs and CDs and previously reported by our group (*14*, *15*). The transparency of the inclusion-complex solution and the low absorption of β-CD, even at very low wavelengths, permitted the UV characterization of the reaction. In addition, the use of CDs permits an increase in total substrate concentration while the free fatty acid concentration is kept constant (*14*, *15*). This increases the length of the linear region of the product accumulation curve without affecting the constant rate and consequently improves the determination of LOX activity. We set the conditions (total LA and CDs) necessary to obtain a very low concentration of free substrate (8 μM) (*17*). At this concentration, LA was in the monomeric form at every pH assayed. The pH profile depicted in Figure 2, with an optimum at pH 7.0 and a strong decrease in the region of basic pH, agrees well with that obtained by Grossman et al. (*9*), and is typical of other LOXs.

Aggregation of LA: Effect on the Eggplant LOX Activity. Recently, Bru and García-Carmona (*27*) studied the activation of potato 5-LOX by substrate aggregation. LA aggregation can be monitored by measuring the fluorescence emission of the probe DPHT, the quantum yield of which increases dramatically when surrounded by an apolar environment, such as that created when LA monomers aggregate. As can be seen in Figure 3, the fluorescence intensity is low in the so-called premicellar region and increases suddenly when LA monomers aggregate. The same figure shows how the eggplant LOX activity is strongly affected by the aggregation state of LA. When the enzyme uses monomers of LA as substrate (premicellar region), eggplant LOX shows a Michaelian behavior (Figure 3, inset) and the kinetic parameters K_m and V_{max} can be determined (1.4 μM and 2.2 μmol min^{-1} (mg of protein)$^{-1}$, respectively). However, when LA aggregates (postmicellar region) a strong increase in the enzymatic activity can be observed. The LA concentration at which the eggplant LOX is activated coincides approximately with the LA CMC value for this pH, as is shown in Figure 3. The response of eggplant LOX in the presence of both monomeric and aggregate states of LA reflects an apparent cooperativity probably generated by the cooperativity of LA aggregation. The observed kinetics can be interpreted as a phenomenon of the *substrate-aggregation dependent activity* of eggplant LOX very similar to the behavior described for 5-potato LOX (*27*) and also described for other enzymes (*28*−*30*). The results presented in Figure 3 suggest that LOX catalysis is more efficient at the lipid−water interface than in solution; this response would be in agreement with the membrane localization of the eggplant LOX used in this paper (*24*). The K_m and V_{max} values obtained in this paper cannot be compared to those reported by Nakayama et al. (*31*) for soluble eggplant LOX as these authors employed a different method in their determination.

The physiological meaning of this behavior could be similar to that reported for potato 5-LOX (*27*).

CONCLUSIONS

The analysis by HPLC and GC/MS of the primary products of eggplant lipoxygenase acting on LA revealed that 9-HPOD is the main isomer produced. Moreover, eggplant LOX shows a pH optimum of around neutrality. These are two features of solanaceous plant LOXs, such as potato 5-LOX, but unlike in potato tuber (*16*), eggplant LOX cannot metabolize arachidonic acid, which is a good substrate for LOX from potato tubers. On the other hand, eggplant LOX shows a strong dependence on the physicochemical properties of LA. When LA is in the monomeric form (below the CMC concentration), the enzyme shows Michaelian kinetics. However, at LA concentrations above its CMC, the LA

forms aggregates that activate eggplant LOX. This double kinetic behavior, due to the distinct activities exhibited below and above the CMC concentration (monomers and aggregates, respectively) results in an apparent substrate cooperativity, which may be interpreted as a substrate-aggregation dependent activity.

ABBREVIATIONS USED

AA, arachidonic acid; CD, cyclodextrin; CMC, critical micellar concentration; DPHT, diphenylhexatriene; HPOD, hydroperoxy octadecadienoic acid; LA, linoleic acid; LA_f, free linoleic acid; LA_t total linoleic acid; LnA, linolenic acid; LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; THF, tetrahydrofuran; TMS, trimethylsilyl ether; U, enzymatic unit.

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