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Amino acids as modulators of lipoxygenase oxidation mechanism. The identification and structural characterization of spin adducts intermediates by electron spin resonance and tandem mass spectrometry

Anna Russo [a,b], Serafina Caputo [a], Manuela Pantusa [c], Enzo Perri [b], Giovanni Sindona [a,*], Luigi Sportelli [c]

[a] Dipartimento di Chimica, Università della Calabria, Via P. Bucci, cubo 12/c, I-87036 Arcavacata di Rende, CS, Italy
[b] CRA-Istituto Sperimentale per l'Olivicoltura e l'Industria Olearia, I-87036 Rende, Italy
[c] Dipartmento di Fisica, Università della Calabria, Via P. Bucci, cubo 30/c, I-87036 Arcavacata di Rende, Italy

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ABSTRACT

Antioxidant activity is displayed by amino acids, such as tryptophan (Trp), tyrosine (Tyr) and histidine (His) in the spontaneous oxidation of linoleic acid (LA). In addition, when Trp was incubated with soybean lipoxygenase (LOX 1) and LA, a modulating effect was observed. The elucidation of the reaction pathways was achieved through the identification, by electron spin resonance (ESR) spectroscopy, of α-[4-pyridyl 1-oxide]-N-t-butyl nitrone (POBN) adducts with the selected amino acids. The latter, when electrosprayed (ESI) were detected in the gas-phase as radical cations. They were structurally characterized by tandem mass spectrometry (MS/MS) through collision-induced dissociation (CID) of the adducts. The kinetic data obtained from selected model systems suggested a reversible radical scavenging activity of tryptophan on the intermediate dienyl radical formed in the lipids lipoxygenase cascade.

1. Introduction

The direct detection of short-living species such as radicals involved in lipid oxidation can be performed by electron spin resonance (ESR) spectroscopy, a unique methodology successfully applied in the elucidation of single electron processes taking place in biological systems (Davis, 1987). Detection of free radicals in food matrices by ESR (Morello, Shahidi, & Ho, 2002) is widely used in the fields of food irradiation (Dadayli, Sunnetcioglu, Koksel, & Celik, 1997), lipid oxidation (Qian, Kenneth, Yue, Guo, & Mason, 2002; Qian, Yue, Kenneth, & Mason, 2003), antioxidants and food processing (Mazziotti, Mazzotti, Pantusa, Sportelli, & Sindona, 2006), and the characteristic *g* value of specific resonances provides enough information to distinguish the carbon-, nitrogen-, and sulphur-centred single electron species (Schaich & Karel, 1976).

Saeed and Howell (1999) and Saeed, Fawtrop, and Howell (1999) have applied the methodology to show the transfer of free radicals from fish oil to proteins. The combined methods of on-line high-performance liquid chromatography coupled with electron spin resonance and mass spectrometry (HPLC/ESR and HPLC/MS), have allowed Qian et al. to identify spin-trapped lipid-derived carbon-centered radicals formed in linoleic acid peroxidation as well as to characterize the interaction of both linoleic and arachidonic acids with soybean lipoxygenase in the presence of α-[4-pyridyl 1-oxide]-N-t-butyl nitrone (POBN). Polat and Korkmaz (2003) have shown that ESR can be used to study the kinetics of radiation induced free radicals in γ-irradiated ground soybeans. Lee, Volkov, and Lee (2001), Lee, Vitaly, Byun, and Lee (2002) have observed, by ESR, free radicals in γ -irradiated soybean paste and compared it with those of soybean protein isolate and soybean oil to examine the influences of irradiation dose, moisture content, and heating after irradiation of the free radical concentration and species. The oxidized lipids in lipid–protein systems are considered to induce polymerization and aggregation of proteins, causing undesirable changes in their nutritional and functional properties (Nakhost & Karel, 1983). It has also been demonstrated that linoleic acid oxidation products induced by lipoxygenases, are very reactive species that may

Abbreviations: AA, amino acid; Trp, tryptophan; Tyr, tyrosine; His, histidine; LA, linoleic acid; LOX, soybean lipoxygenase; ESR, electron spin resonance spectroscopy; POBN, α-[4-pyridyl 1-oxide]-N-t-butyl nitrone; ESI, electrospray ionization; MS/MS, two-stage, in line, mass spectrometric analysis; CID, Dissociation of gaseous ions after internal energy enhancement Induced by Collision with inert gases.

* Corresponding author. Tel.: +39 0984 492083, Mobile: +39 335 5738465; fax: +39 0984 493307.
E-mail address: giovanni.sindona@unical.it (G. Sindona).

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interact with soybean proteins affecting in a pronounced manner their structural feature (Huang, Hua, & Aiyong, 2006; Obata, Matsuura, & Kitamura, 1996). Commercially available soybean proteins, prepared from low denatured defatted soybean flours display substantially high lipoxygenase activity (Huang et al., 2006). Likewise, the residual lipids present in soybean flours (1% typically) are easily oxidized by soybean lipoxygenases during the preparation of soybean proteins. Boatright and Hettiarachchy have found that the lipids associated with soybean protein isolates contributed to protein insolubility and protein oxidation; whereas, antioxidants added to soybean protein isolates during processing, promote a marked increase in protein solubility (Boatright & Hettiarachchy, 1995a, 1995b). Obata et al. have found that degradation of sulphydryl groups in soymilk was related to the lipid oxidative reaction of lipoxygenases during soybean grinding. Youru, Da, and Yufei (2006) have suggested that the structure of soybean proteins could be affected by the single electron transfer process taking place in the LOX oxidation of linoleic acid.

Statistical analyses of amino acid distribution patterns in integral membrane proteins reveal a pronounced enrichment of tyrosine and tryptophan in the trans-membrane domains of all major classes of membrane proteins (Moosmann & Behl, 2000). Tyrosine and tryptophan are especially expressed in the region contacting the membrane zone of the highest lipid density, which comprises the inner portion of the lipid head groups and the beginning of the hydrocarbon tails (Moosmann & Behl, 2000), whereas, phenylalanine is found mainly in the inner hydrocarbon core zones of the membrane. These distributional patterns seem to constitute a general principle of membrane protein architecture (Reithmeier, 1995) and have been incorporated into protein topology prediction algorithms (Schirmer & Cowan, 1993). The enrichment of tyrosine and tryptophan in the trans-membrane regions of integral membrane proteins has been explained by their quadrupolar moment, e.g. aiding in the translocation of cations across the membrane in cation channels, and other $\pi \pm$ cation interactions (2 Deber & Foldi, 1996; Forrest, Tieleman, & Sansom, 1992). Nevertheless, none of these models can completely explain the observed general characteristics of tyrosine and tryptophan enrichment. They are, in fact, accumulated in many diverse classes of membrane proteins independent of their primary function as, e.g., multi-span cation channel or single-span receptor; they can be found strongly enriched also in β-barrel proteins; and model studies indicate no direct influence of trans-membrane tryptophan on membrane protein insertion and topology (Killian & Heijne, 1999; Ridder et al., 2000). Thus, trans-membrane tyrosine and tryptophan do not seem to constitute a general biophysical prerequisite for membrane protein stability or correct insertion. The literature referenced above provides evidence on the formation of unpaired-spin-density species, such as phenoxy and indolyl radicals from tyrosine and tryptophan. Their relative high abundance, within membrane proteins is also well documented. Finally, many problems remain uncovered in the understanding of the mechanism of lipoxygenases oxidation of fatty acids.

We have investigated, therefore, model systems made of linoleic acid, amino acids, LOX Type I from soybean, and the POBN spin trapper, aiming at determining whether specific amino acids could act as antioxidants or redox-regulators in lipid oxidation. The selection of ESI ionization method is due to the expected specificity of the methodology in providing the observation of the spin-adduct intermediates directly sprayed to the gas-phase. The specificity of the MS/MS methodology should favor their structural determination (Benincasa et al., 2003; Cooks, 1995). In previous experiments (Mazziotti et al., 2006), the adducts formed by POBN with methyl and methoxy radicals have provided important clues into the radical scavenging role of natural antioxidants in vitro.

2. Materials and methods

2.1. Reagents

Linoleic acid (LA, assay = 99%GC); soybean LOX type I-B (70 800 units/mg); and the amino acids (AA), L-tryptophan (Trp); L-histidine (His); L-tyrosine (Tyr); L-phenylalanine (Phe); L-serine (Ser) and α-[4-pyridyl-1-oxide]-N-t-butyl nitrone (POBN) were purchased from Sigma Aldrich (Milan, Italy). All other reagents and chemicals were of analytical grade.

2.2. Sample preparation

Freshly prepared, mother suspension of LA (1 g) in 10 mL of Tris–HCl buffer (50 mM, pH 9) was shaken and diluted to 0.107 M with Tris–HCl buffer [50 mM, pH 9], before use. LOX suspension was prepared in phosphate buffer solution [0.1 M, pH 9]. Each Solution of (LA + POBN + AA) were prepared from 333 μl of 0,107 M LA suspension, 1:25 mol/mol POBN (138 mg) and 1:10 mol/mol of the following amino acids Tyr (64 mg), His (55 mg), Trp (72 mg) Phe (58 mg) Ser (37), diluted with two volumes of methanol and concentrated to 1 mL. Sample aliquots of 200 μl and 20 μl were used for ESR and ESI-MS measurements, respectively. The same procedure was used for the preparation of LA + POBN, LA + LOX + POBN and LA + Trp + LOX + POBN solutions, the latter two containing 392 μL of LOX Type IB suspension (1.8 mg of protein/mL).

2.3. Instrumentation

The ESR spectra were recorded with a 9-GHz Bruker (Karlsruhe, Germany) spectrometer, model ESP 300 and digitized with the spectrometer built-in computer using OS-9 compatible ESP1600 Data System spectral acquisition software. The fluid sample, containing POBN, were sealed in 1 mm (i.d.) 100-μL glass capillaries and then inserted in a 4-mm (i.d.) quartz ESR tube and were centered in a TE102 standard rectangular cavity (ER 4201 Bruker). The solid samples were transferred in the quartz tube and then inserted in the cavity. The POBN adduct ESR spectra were recorded under the following experimental conditions: 10 mW of microwave power and 1 G_{p-p} of the magnetic field modulation amplitude. The ESR spectra of solid samples were acquired with microwave power of 1 mW and modulation of 5 G_{p-p}. For all the spectra the magnetic field scan was 150 G, the receiver gain was 2×104 and the field modulation frequency was 100 kHz for phase sensitive detection. An MDS Sciex API 2000 triple quadrupole Mass Spectrometer equipped with an ESI source [Applied Biosystem, Foster City, CA] interfaced with an 1100 HPLC system (Agilent Technologies, Waldbron, Germany) was used to carry out solution state samples analyses. Aqueous part per millions solutions of the analytes were delivered to the turbospray source by direct injection at a flow rate of 10 μL/min. The experiments were performed in positive mode at curtain gas (CUR) and source gas (GS1, GS2) pressures of 20, 20, 0 psi, respectively, while the ionspray voltage, the declustering potential, the focusing potential and the entrance potential were set to 4500, 20, 200 and 10 V, respectively. The tandem mass experiments were performed with collision energy] of 20 V while the CID parameter was set to 2.

3. Results and discussion

The radical trapping technique, available within the ESR methodologies thoroughly exploited in this work, have provided for the first time clues into the role played by selected amino acids as radical scavenger in the complex lipid oxidation mechanism (Fig. 1).

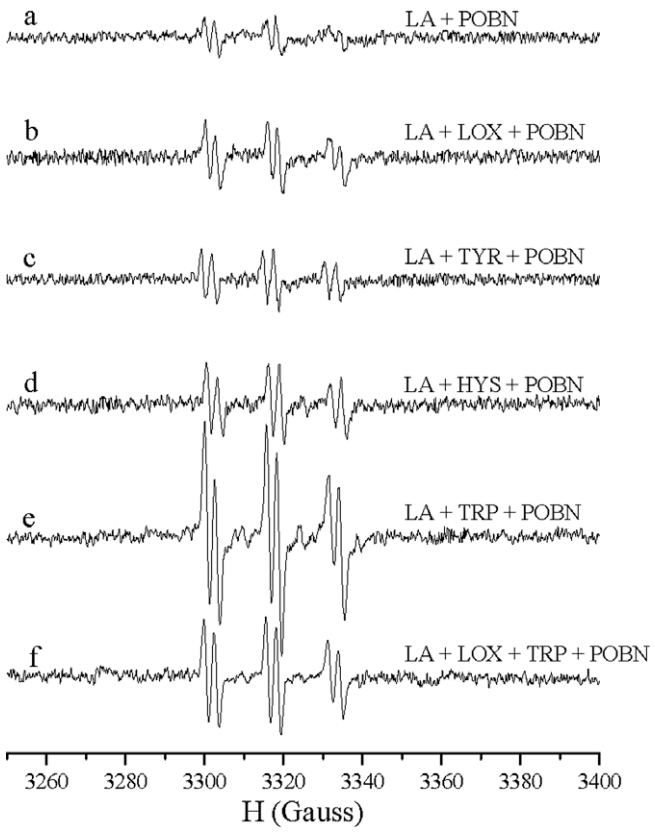

Fig. 1. ESR Spectra taken from methanol/water 2:1 solutions of (a) LA + POBN; (b) LA + LOX + POBN; (c) LA + Tyr + POBN; (d) LA + His + POBN; (e) LA + Trp + POBN; (f) LA + LOX + Trp + POBN.

The incubation of LA fatty acid with POBN, in the absence, therefore, of any catalyst, produces the radical species displayed by the ESR spectrum (Fig. 1a) as a six lines pattern with hyperfine coupling of aN = 15.7 G and aH = 2.5 G. It can be confidently assumed that the autoxidation of the unsaturated fatty acid promotes the formation of a transient unpaired-spin-density species that is transformed, after interaction with POBN, into the stable radicals displayed in the spectrum (Qian et al., 2002, 2003). The latter was used as control for the experiments performed on ternary and quaternary systems (Fig. 1).

The same shaped lines were present in the ESR spectrum of the ternary mixture which includes LOX in the ratio 1/14 w/w to LA (Fig. 1b). The intensity of the lines, however, was 1.8 higher than those present in the spectrum of Fig. 1a. The relative ratio of the resonance lines became 1.4, 2.2 and 5.6, when LOX was replaced by the amino acids Tyr, His and Trp, respectively, sampled at 1/10 mol/mol ratio with respect to LA (Fig. 1c–e).

Since the same POBN adducts were obtained when the three different amino acids were incubated as described, it should be considered that they interact similarly with the azomethine oxide scavenger. Therefore, with reference to the hyperfine constant values, the formation of the observed adducts requires a radical intermediate whose unpaired-spin-density is centered on the α carbon of each amino acid. An intermediate situation was observed in the case of the quaternary system containing Trp and LOX as well as LA and POBN (Fig. 1f), where the relative intensity ratio of the central lines was 3.2. Although the ESR resonances of the LA and AA suggest the establishment of identical connectivity within the adducts, they can not provide clues into their structure.

To obtain direct proofs on the presence of radicals or spin-adduct radicals in the linoleic acid oxidation in the absence of LOX, mass spectrometric methodologies were exploited for their known (Napoli, Aiello, Di Donna, Moschidis, & Sindona, 2008) specificity in the structural identification of gaseous ions. The direct transfer of analytes from solution to the gas-phase coupled with their ionization can be conveniently achieved by spray methods such as ESI-MS. Both even and odd-electron ionic species can be thus produced (Manicke, Wiseman, Ifa, & Cooks, 2008) whose structure can be evaluated with the classic tools of gas-phase ion chemistry, following the specific dissociations of selected precursor ions displayed by the MS/MS spectra. In the present study the positive (+) ionization mode was preferred in relation to the expected structure of the neutral POBN unpaired-spin adducts which should easily undergo nitrogen protonation, during the spray ionization procedure, to afford nitrogen-centred stable radical cations (Fig. 2).

The ESI-MS spectrum of Fig. 3a, showed an intense peak at m/z 473 with a molecular mass corresponding to the protonated (LA + POBN)$^+$ adduct. The protonated (Tyr + POBN)$^+$ adduct was the only cation displayed at m/z 376 (Fig. 3b), when the ternary system LA-POBN-Tyr was submitted to mass spectrometric analysis. This was the first direct evidence on the formation of amino acid radicals during the incubation of tyrosine with linoleic acid. The same result was obtained when tyrosine was replaced by histidine and tryptophan. Their MS spectra displayed, in fact, the ionic species at m/z 350 and 399, corresponding to the (Hys + POBN)$^+$ and (Trp + POBN)$^+$ adducts, respectively (Fig. 3c and d). The last two spectra showed, however, the presence of two additional peaks at m/z 156 and 205, respectively. The latter have the elemental composition of the corresponding amino acids which should be present in the gas-phase as odd electron species. Two possible mechanisms can be envisaged for their formation, one due to a classic gas-phase α-cleavage directed by the nitrogen radical site (Fig. 4, path a) and the other due to the cationization by ESI of the radicals formed during the incubation processes. The gas-phase process, however, should not be affected by the chemical properties of the group R attached at the α-position of the amino acid, therefore, it can be suggested that the cations at m/z 156 and 205 (Fig. 3c and d) are formed by direct ionization of the existing amino acid radicals in the reacting mixture.

The lack of similar species in the case of tyrosine can be due to the different stability of neutral radicals of tyrosine with respect to those of histidine and tryptophan, hence to the lower concentration of these species in the reaction milieu. When a high molecular weight protein such as BSA is degraded by oxygen radicals induced by cobalt radiation, two tryptophan units present within the primary structure, are about the only amino acid residues involved in the oxidation step (Davies, Delsignore, & Lin, 1987). A clear cut

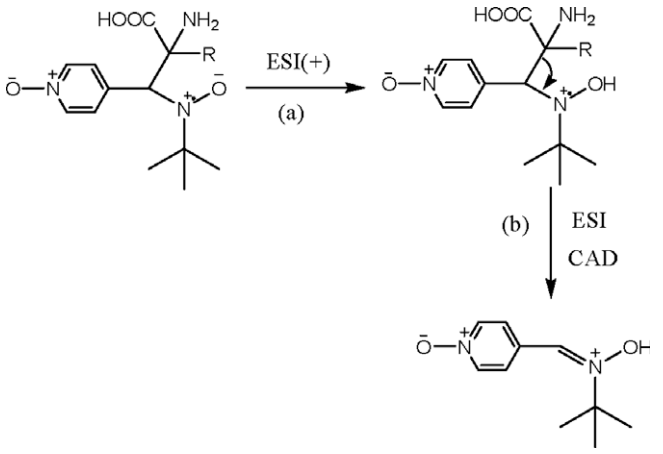

Fig. 2. ESI-MS/MS. Fragmentation pattern of the [M + H]$^+$ POBN adducts with incubated amino acids after collisions with nitrogen at 20 eV kinetic energy.

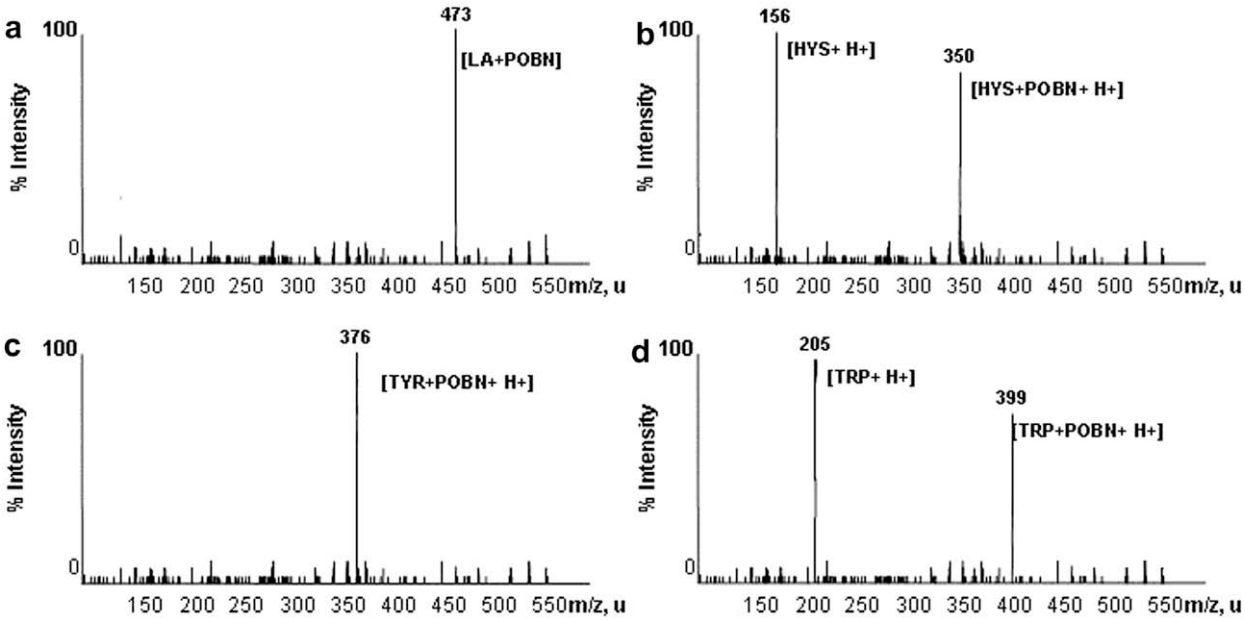

Fig. 3. ESI-MS/MS spectra of the $[M + H]^+$ (a) LA + POBN, (b) Tyr + POBN, (c) His + POBN, (d) Trp + POBN adducts, taken from methanol/water 2:1 solutions.

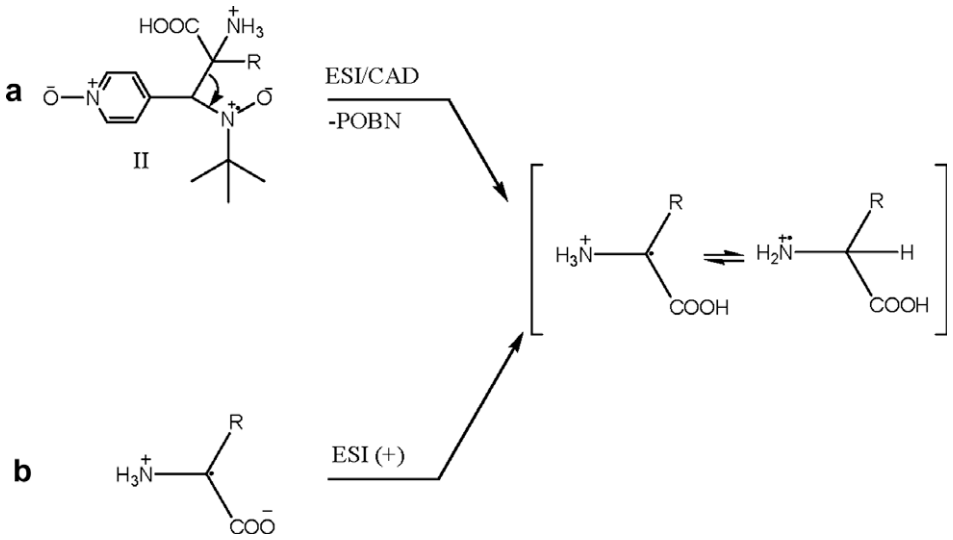

Fig. 4. Proposed mechanism of the gas-phase formation of amino acids radical cations from POBN adducts.

evidence of the formation of LA + POBN and AA + POBN spin adducts came from ESI-MS/MS experiments. In this condition, an ion selected from the bulk of the species formed in the ionization step, transfers by collision with inert gas part of its kinetic energy into internal energy, thus activating selected fragmentation pathways. In the experiments reported in this investigation, the instrumental parameters allowed low-energy collisions, only; therefore the fragments are formed through low activation energy processes. The ESI-MS/MS spectra obtained from each spin adduct ion at m/z 473, 376, 350, and 399 (Fig. 3), displayed the common fragment at m/z 195 corresponding to $(POBN + H)^+$, only. The latter is formed by pure gas-phase process through α-cleavage to the nitrogen of the original azomethine oxide moiety of the un-protonated radical scavenger (Fig. 2, path b). Mass spectrometry provides, therefore, clear results very useful to fulfill the goal of the present investigation. The MS spectra of the product of incubation of Tyr and His with linoleic acid (LA), in the absence of any biocatalyst, gave a

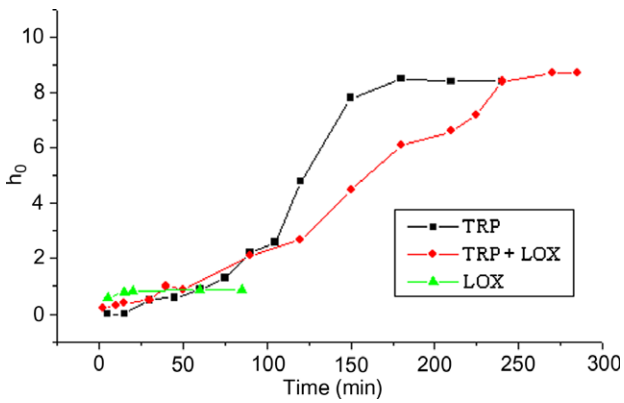

Fig. 5. Modulating effect of tryptophan in the oxidation of LA in the presence or absence of LOX.

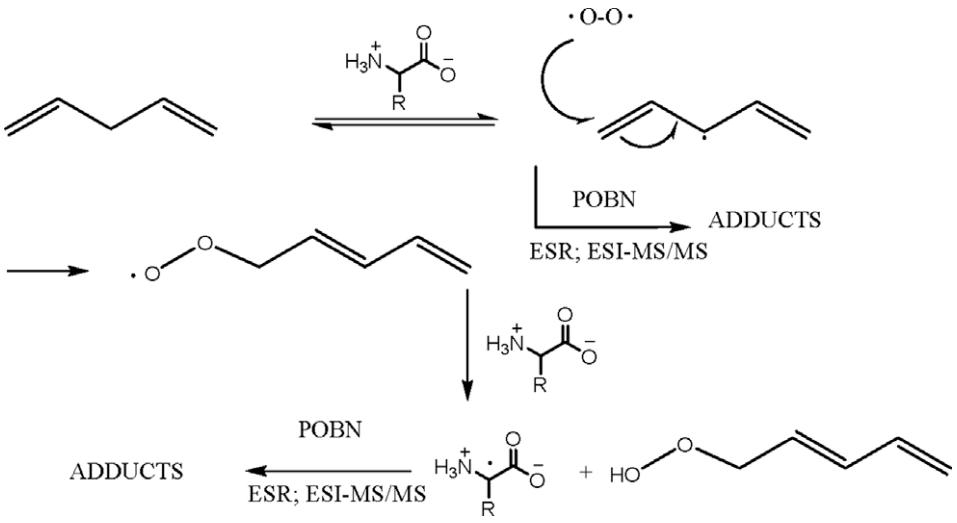

Fig. 6. Proposed pathway for LA autoxidation driven by amino acids.

twofold evidence of the presence in the milieu of amino acid radicals provided by (i) the appearance of a protonated adduct with POBN, and (ii) by the presence of a peak corresponding to the amino acid radical cation. The MS/MS experiments performed on all the (AA + POBN) radical cations, produced in the ionization step, unambiguously proved the structure of the spin adducts, whose presence were already suggested by the ESR spectra reported in Fig. 1. Other amino acid, such as phenylalanine (Phe) and serine (Ser) have been incubated with LA in the presence of POBN, but no spin adduct formation was detected by ESR. The data discussed so far point towards a role of amino acids as radical scavenger in the lipid oxidation mechanism, which could take place even in the absence of LOX. Complementary information were obtained from the kinetic of formation of the spin adducts (a) LA + LOX + POBN; (b) LA + Trp + POBN and (c) LA + LOX + Trp + POBN, followed by plotting the normalized ESR signal intensity h_0, corresponding to the central line height versus the reaction times (Fig. 5).

In the ternary systems (a) and (b) the maximum intensity signal (h_0) is reached approximately after 60 and 175 min, respectively. The shape of the curve described by the system (b) is "sigmoid", whereas is "parabolic" that described by (a). These different shapes suggest that Trp could modulate the LOX catalysis as an allosteric inhibitor. The specific role of LOX enzyme is evident, even if not remarkable. In the quaternary system (c), thus in the presence of both LOX enzyme and tryptophan amino acid, the maximum signal intensity h_0 is reached at time $t = 250$ min. An active role of Trp even in LOX catalysis is, therefore, displayed, in addition to the observation that they can drive, alone, the oxidation of unsaturated fatty acid. The classic oxidation triad, fatty acid, LOX and spin trapper, displayed the kinetic burst of a pre-steady state enzyme catalyzed process, taking place in the first few seconds of the reaction (Fig. 5a). In the absence of any catalyst, tryptophan can start playing its role after the autoxidation of LA takes place.

The reaction is much slower since requires the spontaneous formation of reactive oxygen species (Fig. 5b). The kinetic of formation of Trp-POBN spin adduct in the model system (Fig. 5c) lights up the way how tryptophan carries out its catalytic action.

The interpretation of the data above discussed requires a link to some of the work previous done by many scientists and, among others, (i) to the mechanism of catalysis of LOXs, deeply investigated in the last few decades, quite recently disclosed by Brash (Coffa & Brash, 2004) through the elucidation of the stereochemistry of oxygen addition to LA radical species, generated, in the catalyzed process, by the redox action of the not-heme iron; (ii) to the mechanism of oxygen diffusion through the enzyme, brilliantly evaluated by Saam, Ivanov, Walther, Holzhutter, and Kuhn (2007); (iii) to the oxygen damage of a variety of protein (Davies et al., 1987) and, finally, (iv) to the soybean protein aggregation in the LOX catalyzed oxidation of LA (Huang et al., 2006).

The present investigation clearly shows that autoxidation of linoleic acid can be better driven by the action of some amino acid which act as radical scavenger leading likely to the formation of the reactive hydroperoxide derivative, a typical intermediate in lipoxygenase fatty acid cascade, and to a neutral-radical amino acid captured, in part, by POBN. Moreover, it has been reported that covalent modification of the accessible four tryptophan residues, in soybean LOX 1 enzyme, at pH 4.0 under non-denaturing conditions resulted in complete loss of its activity (Srinivasulu & Rao, 2000). Actually, Trp amino acid plays an allosteric negative modulating effect when incubated in the presence of LOX and LA (Fig. 5c) through reversible interaction with dienyl radicals thus preventing the accumulation of $LOO^{\cdot}$ species as suggested for β-carotene (Fig. 6) (Serpen & Gokmen, 2006).

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