LIPOXYGENASE ACTIVITY IN PIG MUSCLE. ISOLATION AND PARTIAL CHARACTERIZATION.

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SUMMARY

Lipoxygenase from Iberian pig *biceps femoris* has been purified by a process which involve two successive chromatographic steps on DEAE Sephadex and Phenyl Sepharose CL4B, obtaining a final specific activity of 30.3U/mg and a purification factor of 241.3. The enzyme has a molecular weigh of 80 kDa with maximun activity at at pH 5.4, and Km values for arachidonic, linoleic and linolenic acid of 6.43, 21.27 and 23.60 μM respectively. The enzyme show more catalytic efficiency for arachidonic acid. The presence of lipoxygenase activity in Iberian ham suggest a participation of this enzyme in the mechanism of generation of volatile compounds derived from oxidative degradation of fatty acids.

INTRODUCTION:

One of the more importants pathways of enzymatic eicosapolyenoic acid oxygenation is by lipoxygenases to yield products containing a noncyclic diene system. The initial step of this reaction is abstraction of hydrogen followed by an insertion of one oxygen molecule at the 5-, 8-, 9-, 11-, 12-, or 15-position of a basic *cis, cis*- nonconjugated diene system. This structure is a required feature for all substrate fatty acids. Addition of oxygen to arachidonic acid (AA) in the presence of lipoxygenase activity results in the formation of hydroperoxy eicosane tetraenoics derivatives. These unstables intermediates are mainly metabolized to the stable hydroxy species, originally discovered in blood platelets, were subsequently found in lymphocytes, neutrophils, mast cells, retyculocytes as well as in alveolar and peritoneal macrophages (Malle, 1987). Furthermore, 5-, 12- and 15- lipoxygenases are able to cause the cleavage of C-C bond next to hydroperoxy groups forming aldehydes and alkanes (Yamamoto, 1992). On the other hand, vegetal lipoxygenase plays an important role in the genesis of the flavour and aroma compounds in plant products. These compounds are desirable in many foods but may also give rise to off flavours (O'Connor 1991). It has been reported previously the presence in hams of volatile compounds probably derived from oxidative decomposition of lipids, being hexanal the most abundant (García, 1990). Likewise it has been identified a serie of alkanes and aldehydes similar to those derived from lipoxygenase activity. In this work we show the presence of lipoxygenase a in the *Biceps Femoris* muscle from Iberian pig and its purification and characterization are described.

MATERIALS AND METHODS.

Materials: DEAE-Sephadex and Phenyl Sepharose CL4B were purchased from Pharmacia Fine Chemicals. Linoleic acid, linolenic acid, arachidonic acid, phenidone, caffeic acid, nordihydroguaiaretic acid and indomethacin were obtained from Sigma chemical Co. All the reagents were of analytical grade.

Lipoxygenase assay: The enzymatic activity was assayed at 20°C by following the increase in absorbance at 234 nm produced by the transformation of the *cis, cis*-1,4 pentadiene sytem of the polyunsaturated fatty acid into the conjugated hydroperoxydiene derivative *cis, trans*. The reaction mixture typically contained 100 μM linoleic acid, 10 to 100 μL of enzymatic solution and 25 mM acetate buffer pH 5.5 to a final volume of 1 mL.

Polyacrylamide gel electroforesis: Analytical polyacrylamide disc gel electrophoresis has been carried out following the method of Laemmli (1970), and the protein has been stained with Coomassie Brilliant Blue R-250. Molecular weigh was estimated by comparing mobilities of the bands with of the those of the following marker proteins: β-galactosidase (116kDa),phosphorilase-b (94kDa), bovine serum albumin (67kDa), ovalbumin (43kDa) and carbonic anhydrase (30kDa).

Protein determination: During chromatography, protein was measured by the absorbance at 280 nm. For more accurate measurement the method of Lowry (1951) was used, using bovine serum albumin as standard.

RESULTS AND DISCUSSION:

The aim of the present study was to stablish the presence of lipoxygenase activity in hams from Iberian pigs. A portion of *biceps femoris* muscle was excised from Iberian pig immediately after slaughtering and stored at -20°C until use.

Purification procedure: A portion of muscle was weighed and homogenized in 3 volumes of 50 mM phosphate buffer pH 7.0 containing 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMFS), 2mM ethylenediamine tetraacetic acid. After filtration through 4 layer of gauze the resulting homogenate was successively centrifuged at 10,000xg for 15 minutes and at 100,000xg for 1 hour and gave a supernatant free of sucellular organelles. The supernatant was precipitated in the intervale 20%- 40% saturation ammonium sulphate, the pellet was redissolved in 50 mM phosphate buffer pH 7.0 and dialized against the same buffer overnight. An aliquot of the resuspended and dialyzed pellet was loaded onto a DEAE-Sephadex column (1x20 cm) equilibrated with 15 mM phosphate buffer pH 7.0. Lipoxygenase activity proteins were eluted with 0-0.5M NaCl in 50 mM phosphate pH 7.0 (Figure 1). The active fractions were collected and conditioned with $(NH_4)_2SO_4$ 5% (w/v). Pooled fractions were applied to a phenyl-Sepharose CL-4B column (1x10) equilibrated with extraction buffer containing $(NH_4)_2SO_4$ 5% (w/v) pH 7.0. Lipoxygenase activity was efficiently retained by the gel, while most of the contaminating proteins were eluted. After washing the column with this buffer an increasing gradient (0 to 50% and then 50% to 100%) of ethylene glycol and decreasing in ionic strenght (5% to 0% $(NH_4)_2SO_4$) was applied and two peaks of lipoxygenase activity were obtained (Figure 2). The purified enzyme showed a single protein band when assayed by analytical gel electrophoresis.The purification steps are summarizes in Table I. All the estudies were performed with the second peak eluted.

Characterization of the enzyme: The absorbance spectrum shows the characteristic profile in the UV range and does not show any strong absorbance band in the visible (data not shown). This is in agreement with the spectral properties described for lipoxygenases (Macías, 1987). This point allow us to discard the possibility that the enzyme purified should be an hemeprotein such as citochrome P450. From the analytical electrophoresis we can inferred a molecular weigh near to 80 kDa that is coincident with described for mammalian lipoxygenases (Yamamoto, 1992).

The pH dependence of the enzymatic reaction of purified protein using linoleic acid as substrate exhibit a broad range of pH activity with a maximun at 5.4 and pH values for half maximal activity of 4.3 and 6.2 (Figure 3), this behaviour is coincident with that reported for some other lipoxygenases, for instance, 15-lipoxygenase from rabbit leukocytes show a pH optimum near to 6 using arachidonic acid as substrate (Malle, 1987). We have checked substrate specificities of purified fraction by determination of kinetic parameters k_M, V_{MAX} and Kcat/Km using linoleic, linolenic or arachidonic acid as substrates. From Table II we can deduce that the enzyme reacts predominantly with C-20 polyunsaturated fatty acids, this is coincident with substrate specificities reported for 5-, and 15 lipoxygenases from mammalians. It is to be noted that 12-lipoxygenase from porcine leukocytes has a broader substrate specificity reacting with C18- and C22-unsaturated fatty acids as fast as arachidonic acid (Yamamoto, 1992).

A wide number of natural occuring and synthetic compounds have been reported to inhibit lipoxygenases from several sources. Figure 4 shows the effect of NDGA, caffeic acid and phenidone on lipoxygenase activity of purified fraction. It is remarkable that indomethacin, an inhibitor of cyclooxygenase activity do not inhibit the lipoxygenase activity. Therefore our results show the absence of cytochrome P-450 and cyclooxygenase, being lipoxygenase activity the only present in our preparation. From both ionic exchange and hydrophobic chromatography elution profiles we can to expect that more than one lipoxygenase activity is present in pig muscle. We have purified the fraction which possess the better ratio activity-protein concentration. This activity may correspond to a 5- 12- or 15-lipoxygenase. Additionally it is known that arachidonic acid can be oxygenated not only at a major site (C-12 for 12-lipoxygenase and C-15 for 15-lipoxygenase) but also at alternative sites (C-15 for 12-lipoxygenase and C-12 for 15-lipoxygenase) and that the primary hydroperoxy product of some lipoxygenases can be further oxygenated at additional carbon atoms by the same enzyme (Yamamoto, 1992). In consequence there are a lot of possibilities to obtain compounds with aromatic implications as volatile aldehydes. Has been reported that mammalian lipoxygenase cleave C-C bonds next to hydroperoxy groups forming aldehydes and alkanes. Plant lipoxygenase produces 6-carbon aldehydes hexanal or cis-3-hexenal, these compound are derived from 9- or 13-hydroperoxyderivatives when linolenic or linoleic acid are used as substrate (O'Connor, 1991). On the other hand has been reported that hexanal was the most abundant of the volatile compounds identified in the hams from Iberian pigs, being this compound derived from the oxidative decomposition of lipids. (García, 1991). We have incubated the purified fraction of lipoxygenase from Iberian pig with linoleic acid and the products were analyzed by reversed - phase HPLC (data not shown). The cromatogram reveal that lipoxygenase from Iberian ham produces 9- and 13-

hydroperoxylinoleic acid. The fact that linoleic and linolenic acids are good substrates for Iberian ham lipoxygenase (see table II) strongly suggest that an important amount of volatile compounds present in Iberian ham may be derived from the oxidative decomposition of lipids by the lipoxygenase pathway.

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LEGENDS FOR FIGURES

Figure 1. Elution profile of lipoxygenase from Iberian pig *biceps femoris* muscle on a DEAE Sephadex column. Fractions of 3 mL were collected at a flow of 6 mL / hour.

Figure 2. Hydrophobic chromatography on a Phenyl Sepharose CL4b of lipoxygenase from Iberian pig *biceps femoris* muscle. Flow rate was 6 mL/hour and 3 mL fraction were collected.

Figure 3. 10%SDS-PAGE of samples of purification process of lipoxygenase from biceps femoris from Iberian pigs. Lane 1, supernatant 100000xG. Lane 2, 20-40% saturation ammonium sulphate. Lane 3, eluted from DEAE-Sephadex. Lane 4, eluted from Phenyl Sepharose CL4b. Lane 5, molecular weigh markers.

Figure 4. pH - activity profile of *biceps femoris* lipoxygenase using linoleic acid as substrate. All buffers were 25 mM.

Figure 5. Inhibition of lipoxygenase activity by specific inhibitors. The compounds tested were added to reaction mixture inmediately prior to the enzyme addition. 100% activity correpond to 1.5 units of increase of absorbance at 234 nm per min. and mL of enzymatic solution.