Lipoxygenase Activity in Pig Muscle: Purification and Partial Characterization

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Lipoxygenase from Iberian pig *Biceps femoris* muscle was purified in a process that involves two successive chromatographic steps on DEAE-Sephadex and phenyl-Sepharose CL4B. The purified enzyme had a final specific activity of 52 mU/mg, a purification factor of 3250, a molecular weight of 90 kDA, and a maximum activity at pH 5.5. The K_M values obtained for linoleic acid (K_M = 0.28 mM), arachidonic acid (K_M = 3.8 mM), and linolenic acid (K_M = 0.43 mM) reveal a preferential use of linoleic acid as substrate. When purified enzyme was incubated in the presence of linoleic acid, two main products were identified by direct-phase HPLC: 9-hydroperoxy octadecadienoic acid and 13-hydroperoxy octadecadienoic acid in the ratio of 45:55. The presence of lipoxygense activity suggest a possible participation of this enzyme in the biogenesis of flavor and aroma in hams from Iberian pigs.

Keywords: *Lipoxygenase; purification; hams; Iberian pig; lipid oxidation*

INTRODUCTION

Dry cured ham is a product manufactured using the two basic principles of stabilization through a drop in water activity and elaboration of the flavor through maturation (Berdagué et al., 1991). Because of the high level of intramuscular fat (8−10%), variations in cured Iberian ham flavor are attributed to the lipid composition of fat tissue and also to the degree of lipid breakdown during processing. Hydrocarbons, probably derived from the oxidative decomposition of lipids, and aldehydes, which may be formed by breakdown of the hydroperoxides derived from unsaturated fatty acids, are the most significant compounds generated during the ripening process of Iberian ham. Hexanal was the most abundant of the volatile compounds identified (García et al., 1991).

The sensitivity of the tissue to oxidative deterioration is a function of the polyunsaturated fatty acid (PUFA) content. This oxidation has been assumed to occur mainly through catalysis by heme iron. However, evidence for non-heme-soluble protein catalysis has been reported, and the presence of lipoxygenase-type enzymes was demonstrated in chicken muscles (Grossman et al., 1988).

Lipoxygenase catalyzes the hydroperoxidation of fatty acids interrupted by *cis*-methylene (Tappel, 1963). The unstable products generated by the enzyme are mainly metabolized to the stable hydroxy species originally discovered in blood platelets and subsequently found in lymphocytes, neutrophils, mast cells, reticulocytes, as well as in macrophages (Malle et al., 1987). Furthermore, lipoxygenases are able to cause the cleavage of the C−C bond next to hydroperoxy groups forming aldehydes and alkanes, compounds that are involved in the aroma generation process (Yamamoto, 1992).

Lipoxygenase activity has been implicated in the biogenesis of flavor and aroma compounds in vegetables. It has been reported that lipoxygenase products are desirable in many foods but may also give rise to off flavors (O'Connor and O'Brien, 1991).

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In contrast, little is known about the participation of lipoxygenase in the biogenesis of aroma in foods from animal sources. By using specific lipoxygenase inhibitors it has been demonstrated that this enzyme is present in the gill and skin of different fresh and saltwater species and that this enzyme is involved in the generation of short-chain carbonyl compounds following harvesting (Triqui and Reineccious, 1995).

In this work we show the presence of lipoxygenase in the *biceps femoris* muscle from Iberian pig, and its purification and characterization are described.

MATERIALS AND METHODS

Chemicals. DEAE-Sephadex and phenyl-Sepharose CL4B were purchased from Pharmacia Fine Chemicals. Linoleic acid, linolenic acid, arachidonic acid, phenidone, caffeic acid, nordihydroguaiaretic acid, indomethacin, and β-mercaptoethanol were obtained from Sigma Chemical Company. All the reagents used were of analytical grade.

Materials. Samples of *biceps femoris* muscle were removed from a 15−16-month-old Iberian pig, with a live weight of 160 kg, between 18 and 24 h post slaughter. The samples were wrapped in an aluminum sheet, stored in a polyethylene bag under reduced pressure, and frozen at −30 °C until use.

Enzyme Extraction and Purification. A portion of muscle was weighed and homogenized in three volumes of 50 mM phosphate buffer (pH 7.0) containing 1 mM β-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride (PMFS), and 2 mM ethylenediaminetetraacetic acid (EDTA). After filtration through four layers of gauze, the resulting homogenate was sucessively centrifuged at 10000*g* for 15 min and then at 100000*g* for 1 h. The resultant supernatant was free of subcellular organelles. The supernatant was precipitated in the range of 20−40% ammonium sulfate saturation. The pellet was redissolved in 50 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight. An aliquot of this solution was loaded onto a DEAE-Sephadex column (1 × 20 cm) equilibrated with 15 mM phosphate buffer (pH 7.0). Protein-containing lipoxygenase activity was eluted with a gradient of 0−0.5 M NaCL in 50 mM phosphate buffer (pH 7.0). The active fractions were collected and conditioned with 5% $(NH_4)_2SO_4$ (w/v). Pooled fractions were applied to a phenyl-Sepharose CL4B column (1 × 10 cm) that was equilibrated with the extraction buffer, which contained 5% (w/v) $(NH_4)_2SO_4$ (pH 7.0). After washing the column with this buffer, an increasing gradient (0−50% and then 50%−100%) of ethylene

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glycol was applied and the ionic strength (5% to 0% $(NH_4)_2SO_4$) was decreased and a peak of lipoxygenase activity was obtained. Purified lipoxygenase fractions were stored at −20 °C until use.

Lipoxygenase Assay. The substrate solution for the measurement of lipoxygenase activity was prepared by adding 140 mg of linoleic acid to 5 mL of deoxygenated double-distilled water containing 180 μL of Tween 20. The solution was kept at pH 9.0 by adding 2 N NaOH until all the linoleic acid was dissolved and the pH remained stable. Finally, distilled water was added to a final volume of 50 mL. The substrate solution was flushed with and kept under a nitrogen atmosphere.

Lipoxygenase 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 system of linoleic acid into the conjugated *cis,trans*-hydroperoxydiene derivative. The reaction mixture typically contained 75 μL of 10 mM sodium linoleate, 0.05−0.10 mL of enzymatic solution, and 50 mM citrate buffer (pH 5.5) to a final volume of 1 mL.

K_M Determinatión. The K_M values were obtained by the Lineweaver−Burk plot method of fitting the data by linear regression.

Polyacrylamide Gel Electrophoresis. Analytical disc polyacrylamide gel electrophoresis (PAGE) was carried out following the method of Laemmli (1970), and the protein was stained with coomassie brilliant blue R-250. The molecular weight of the lipoxygenase was estimated by comparing mobilities with those of the following marker proteins: β-galactosidase (116 kDa), phosphorylase-*b* (97 kDa), bovine serum albumin (66kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

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

Preparation of Lipoxygenase Products. The products of the reaction between pig muscle lipoxygenase and linoleic acid were formed by incubation of 0.26 mg of the enzyme preparation with 2 mM linoleic acid in 2 mL of 25 mM acetate buffer (pH 5.5) in the presence of 0.6 μL of Tween 20 at 20 °C for 30 min (the reaction was followed spectrophotometrically at 234 nm). The reaction mixture was acidified at pH 2 with HCl and reduced by addition of 100 mg of sodium borohydride. After 30 min, the sample was extracted with diethyl ether, evaporated to dryness under a stream of nitrogen, and dissolved in 100 μL of the SP-HPLC solvent.

To identify chromatographic peaks, a sample containing predominantly 13-HPODE, a linoleic acid hydroperoxide isomer, was prepared by incubation of 20 μg of soybean lipoxygenase with 2 mM linoleic acid in 100 mM borate buffer (pH 9.0) until no increase of absorbance at 234 nm was observed. A sample of another linoleic acid hydroperoxide isomer, 9-HPODE, was prepared by incubation of 16 μg of potato lipoxygenase, purified by the method previously reported by Reddana et al. (1990), with 2 mL of oxygen-saturated 150 mM potassium phosphate buffer (pH 6.3). Other conditions of incubation, reduction, and extraction of products were the same as those described for the soybean enzyme.

The SP-HPLC analysis of the products was carried out on a LDC analytical apparatus, equipped with a 12.5 × 4 mm Lichrospher Si-60 column, with 5-μm particle size (Merck, Darmstadt). The samples were eluted isocratically at 1.5 mL/min with a solvent system of *n*-hexane/2-propanol/acetonitrile/acetic acid (983:15:1:1). The effluent was monitored at 234 nm with a spectromonitor 3200 (LDC analytical) and registered, and the chromatograms were registered and integrated in a Milton Roy CI 4100 computing integrator.

RESULTS AND DISCUSSION

The presence of lipoxygenase enzyme in hams from Iberian pigs was established in this study. The enzyme was purified by a method involving two chromatographic steps in DEAE-Sephadex (ion-exchange chromatography) and phenyl-Sepharose CL4B (hydrophobic chromatography). Lipoxygenase activity is efficiently retained by the ion-exchange support and eluted as a broad peak with enzymatic activity after the application of an ionic strength gradient (see Figure 1). The fractions showing the best ratio of enzymatic activity to protein concentration were pooled. The resulting mixed eluate typically contained protein at 0.89 mg/mL and had an enzymatic activity of 1.53 mU/mg. This eluate was loaded onto a phenyl-Sepharose CL4B column. Lipoxygenase activity was efficiently retained (Figure 2), whereas most of the contaminating protein was eluted. After applying an increasing gradient (0−50%) of ethylene glycol and decreasing the ionic strength [25 to 0% $(NH_4)_2SO_4$], a sharp peak of lipoxygenase activity was recovered, with a protein concentration of 0.01 mg/mL and a specific activity of 52 mU/mg. The purification process is summarized in Table 1. The data illustrate the high efficiency of the hydrophobic chromatography step, with a purification factor of 3250. These results are corroborated by those of Macias et al. (1987), who previously demonstrated the use of this method for purification of lipoxygenase from animal sources.

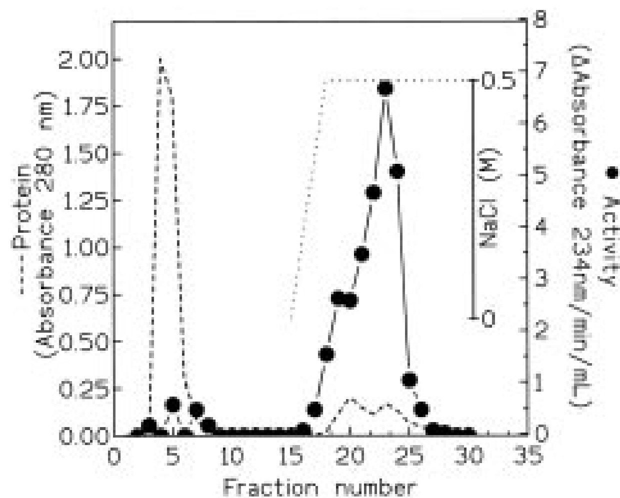

Figure 1. Elution profile of lipoxygenase from Iberian pig *biceps femoris* muscle on a DEAE-Sephadex column. Fractions of 3 mL were collected at a flow rate of 6 mL/h.

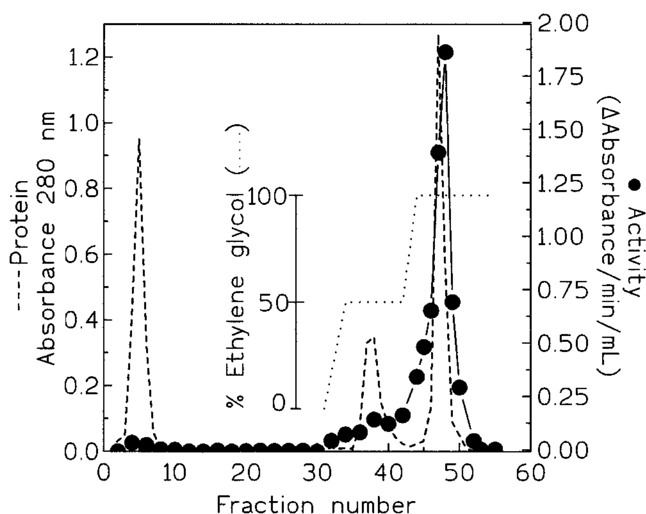

Figure 2. Hydrophobic chromatography, on a phenyl-Sepharose CL4B column, of lipoxygenase from Iberian pigs *biceps femoris* muscle. The flow rate was 6 mL/h, and 3-mL fractions were collected.

The results of the SDS-PAGE (Figure 3) showed that only one major band is present following Coomassie blue

Table 1. Summary of Purification of Lipoxygenase from *Biceps femoris* Muscle of Iberian Pig[a]

step	volume (mL)	protein (mg/mL)	protein (mg)	activity (units)[b]	recovery (%)	activity (mU/mg)	purification (fold)
crude extract	220	19	4180	0.70	100	0.016	1.0
20–40% $(NH_4)SO_4$	12	29	348	0.23	32	0.66	41.2
DEAE-Sephadex	11	0.89	9.8	0.015	2.13	1.53	95.6
phenyl-Sepharose	2.5	0.01	0.025	0.0013	0.18	52	3250

[a] The data given in this table correspond to those of a typical preparation; similar results have been obtained in three different preparations. [b] One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of linoleate per minute under the described conditions; concentration of the enzymatic product has been calculated with ϵ = 25 000 M^{-1} cm^{-1} for hydroperoxy linoleic acid.

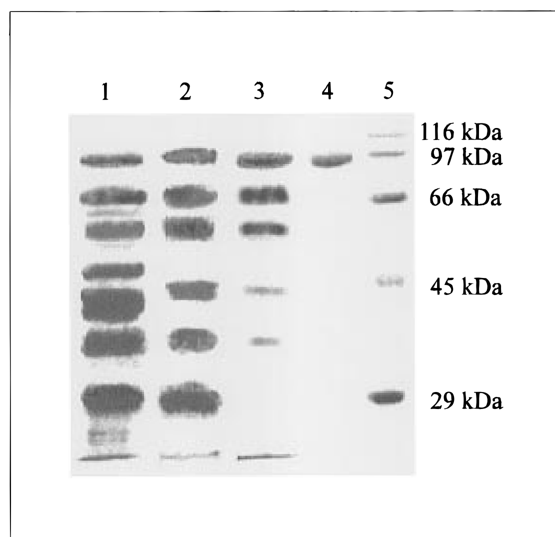

Figure 3. Analysis of samples from the purification process by 10% SDS-PAGE. Lane 1 is the supernatant from centrifugation at 100000*g*. Lane 2 is the sample from 20–40% saturation ammonium sulfate. Lane 3 is the sample eluted from DEAE-Sephadex. Lane 4 is the sample eluted from phenyl-Sepharose CL4B. Lane 5 are molecular weight markers.

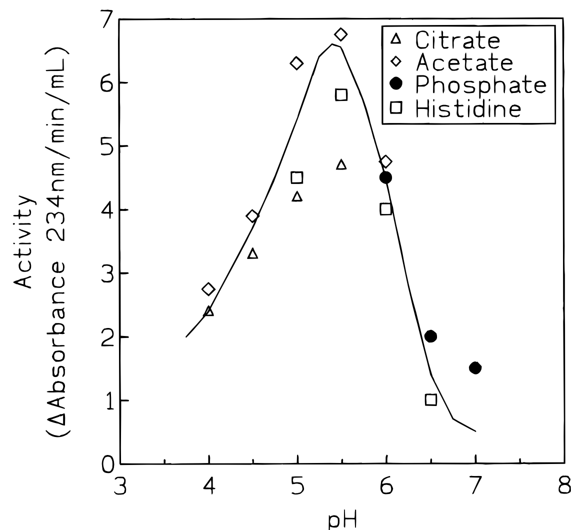

Figure 4. pH–activity profile of the purified fraction using linoleic acid as substrate. Measurements of lipoxygenase activity were performed as is indicated in Materials and Methods.

staining, indicating the high purity level of our preparation. From the migration pattern of the purified fraction we can infer a molecular weight ~90 kDa. This value is similar to those reported for other lipoxygenases, such as arachidonic acid 15-lipoxygenase (Boyington et al., 1993)

Optimal enzymatic activity for the purified protein, using linoleic acid as a substrate, was centered at pH 5.5 (Figure 4), and pH values for half-maximal activity were 4.3 and 6.2. This behavior is similar to that reported for 15-lipoxygenase from rabbit leukocytes, showing a pH optimum near 6, with arachidonic acid as substrate (Malle et al., 1987).

Substrate specificities of the purified fraction was checked by determination of the K_M value by the Lineweaver–Burk plot (Table 2). The results indicate a marked preference toward linoleic acid as substrate. Althoug the K_M value obtained for linoleic acid (2.8×10^{-4} M) is higher than that reported for mammalian lipoxygenases, with arachidonic acid as substrate, it agrees with the K_M value reported for lipoxygenases from other sources, with linoleic acid as substrate (2.0×10^{-3} M for pea seed lipoxygenase, 3.5×10^{-4} M for rice bran, and 7.6×10^{-4} M for canola seed; Bisakowski et al., 1995). The difference among K_M values obtained for Iberian pig muscle lipoxygenase and that obtained for lipoxygenase from other sources might be due to minor differences in the assay procedure or differences in sample composition, which can infuence micelle formation.

Yamamoto (1992) reported that 12-lipoxygenase from porcine leukocytes has broader substrate specificity,

Table 2. Summary of Characteristics of Purified Lipoxygenase from *Biceps femoris* Muscle of Iberian Pig

molecular mass (kDa)	90
pH optimum	5.5
pH range	broad
K_M (apparent) for linoleic acid (mM)	0.28
K_M (apparent) for arachidonic acid (mM)	3.8
K_M (apparent) for linolenic acid (mM)	0.43
reaction products[a]	9-HPOD, 13-HPOD

[a] From linoleic acid.

reacting with C18 unsaturated fatty acids, than other mammalian lipoxygenases, which react predominantly with C20 fatty acids. Lipoxygenase from Iberian pig muscle shows a substrate specificity close to that described for porcine leukocyte type lipoxygenase.

A wide number of naturally occurring and synthetic compounds have been reported to inhibit lipoxygenases from several sources. The effect of the catecholic compounds NDGA (nordihydroguaiaretic acid) and caffeic acid (3,4-dihydroxycinnamic acid) were evaluated. These compounds inhibit active ferric enzyme by reducing it to the catalytically inactive ferrous form (Kemal et al., 1987; Sud'ina et al., 1993). Phenidone (1-phenyl-3-pyrazolidone), a pyrazoline derivative that has long been known to be a good inhibitor of lipoxygenases (Cucurou et al., 1991), was also evaluated. The results in Table 3 show that purified lipoxygenase from Iberian pig was significantly inhibited by these compounds, whereas indomethacin (a characteristic inhibitor of the cyclooxygenase pathway) did not have a significant effect.

Sulfhydryl groups have been shown to be important in determining lipoxygenase activities from various sources (Means and Feeny, 1971). Thus, the effects of iodoacetamide and β-mercaptoethanol, two well-known

Table 3. Inhibition of Lipoxygenase from *Biceps femoris* Muscle of Iberian Pig by Several Compounds

compound	concentration	residual activity (%)
NDGA	0.03 μM	50
NDGA	2.5 μM	2
phenidone	0.12 μM	50
phenidone	1.2 μM	2
indomethacine	55 μM	90
caffeic acid	0.01 μM	55
caffeic acid	0.1 μM	4.5
iodoacetamide	0.12 mM	80
iodoacetamide	1.2 mM	37
β-mercaptoethanol	0.2 mM	42
β-mercaptoethanol	1.4 mM	3.5
KCN	73 μM	44
KCN	0.36 mM	3.5

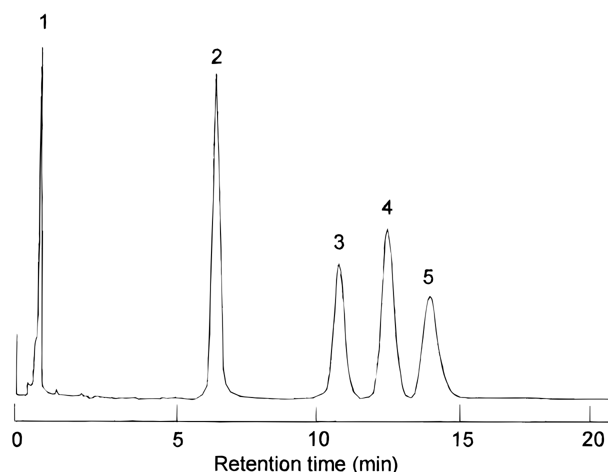

Figure 5. Direct-phase HPLC chromatograms of reduced linoleic acid hydroperoxidation products formed by purified fraction. Preparation of reduced products was as described in Materials and Methods. Peaks assignments: (1) Front of the solvent; (2) 13(*Z,E*)-hydroxy-9,11-octadecadienoic acid; (3) 13(*E,E*)-hydroxy-9,11-octadecadienoic acid; (4) 9(*E,Z*)-hydroxy-10,12-octadecadienoic acid; (5) 9(*E,E*)-hydroxy-10,12-octadecadienoic acid.

sulfhydryl blocking agents were evaluated. The results suggest the involvement of -SH groups in lipoxygenase activity, which is in agreement with the behavior described for other lipoxygenases (Macías et al., 1987).

The lipoxygenase-inhibiting activity of KCN was also evaluated. Previously, Bonnet and Crouzet (1977) demonstrated little or no effect, whereas others report significant inhibition (Hidaka et al., 1980; Khalyfa et al., 1990). Our data reveal a significative inhibition of Iberian pig muscle lipoxygenase by cyanide. These discrepancy between the results obtained by us and the aforementioned authors has been attributed to the fact that the degree of inhibition could be influenced by several factors, such as pH, temperature, nature, and concentration of buffers and enzyme source (Bisakowski et al., 1995). We can deduce from the data that the properties of the proteinic purified fraction from Iberian pig muscle are similar to properties described for lipoxygenases from several sources.

The final identification of the enzyme as a true lipoxygenase came from the characterization of the reaction products. Both linoleic acid hydroperoxide isomers, 9-HPOD and 13-HPOD, are the reaction products of Iberian pig lipoxygenase (see Figure 5). These compounds were identified from the coelution of authentic hydroxyderivatives of linoleic acid that were obtained by incubation of linoleic acid with soybean or potato lipoxygenase, as described in Materials and Methods. In addition, the time of retention obtained for these compounds in the HPLC analysis are similar to those reported for lipoxygenases from animals (Lomnitski et al., 1995), microorganisms (Iny et al., 1993), or plants (Wu and Robinson, 1995). The quantitative ratios of four isomers were 35% for 13ZE-HODE, 20% for 13EE-HODE, 29% for 9EZ-HODE, and 16% for 9EE-HODE. These ratios are consistent with those reported for soybean lipoxygenase 2 (Christopher et al., 1972), lipoxygenase from the green algae *oscillatoria sp.* (Beneytout et al., 1989), and lipoxygenase from microorganisms (Iny et al., 1993).

It can be concluded that according to the classification scheme proposed by Yamamoto (1992), a "leukocyte type" lipoxygenase is present in biceps femoris Iberian pig muscle. This enzyme uses linoleic acid as substrate but has a low activity with arachidonic acid, in contrast with 12-lipoxygenase, which oxygenates both linoleic and arachidonic acids. The concentration of linoleic acid in Iberian hams is 20−30 higher than that of arachidonic acid and the concentration of free fatty acids is high because of the lipolytic degradation of unsaturated fatty acids (García et al., 1991). Therefore, the presence of a lipoxygenase that preferentially uses linoleic as substrate is logical.

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Received for review March 4, 1996. Revised manuscript received June 4, 1996. Manuscript accepted June 7, 1996.[⊗] This work was supported by a grant (BIO92-0288) from the Spanish CICYT, and by a grant (EIA94-26) from the Junta de Extremadura.

JF960149N

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.