Purification and Partial Characterization of Rat Liver Lipoxygenase

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Lipoxygenase was purified from rat liver cytosolic fraction by a method involving two successive chromatographic steps on Sephacryl S-200 and Phenyl Sepharose CL-4B. The enzyme has a molecular weight of 96 Kdal and it seems to be composed of two identical subunits. Chromatofocusing of the enzyme revealed a single band of activity at pH 6.3. The enzyme activity of the purified fraction showed maximum activity at pH 7.0 with a Km for linoleic acid of 1.4 μM and is competitively inhibited by the specific lipoxygenase inhibitor nordihydroguaiaretic acid. The purified enzyme shows absorption and fluorescence spectra similar to those of lipoxygenase from other sources. However, the molecular weight of lipoxygenase purified from liver is found to be different from that of the enzyme from polymorphonuclear leukocytes. It is suggested that there are different isoenzymes of lipoxygenases in mammals.

Introduction

Lipoxygenases are a group of iron containing dioxygenases widely distributed in animal and plant tissues that catalyze the hydroperoxidation of polyunsaturated fatty acids containing a cis-penta-1,4-diene system, such as linoleic acid or arachidonic acid [1]. The formation of a conjugated 1,3-diene product from a 1,4-diene substrate is the basis of spectrophotometric assays of lipoxygenase activity [2].

Although plant lipoxygenases have been known for some time, the first report for the characterization of the lipoxygenase from mammalian tissues is more recent. In 1975, Nugteren demonstrated that a suspension of blood platelets contained a soluble enzyme that converted a series of polyunsaturated fatty acids to the corresponding hydroperoxy acids [3]. Since then lipoxygenase activity has been found in several mammalian cells and tissues and their products have been implicated in several processes of both biological and clinical interest [4, 5]. The biosynthetic pathways of these compounds in mammalian cells from different tissues are not well understood, partly due to the instability of the metabolic intermediates, but also due to the lack of purified enzyme preparations. Although much is known about lipoxygenase products [6–8], little information is available regarding the physical chemical and kinetic properties of mammalian lipoxygenases.

Several tissue lipoxygenases have been purified so far, for example those from polymorphonuclear leukocytes [9], reticulocytes [7, 10] or rat testis [11], using various technical approaches like ionic exchange or affinity chromatography. However, to the best of our knowledge there is not any report about purification and kinetic characterization of liver lipoxygenase.

In the present study, we describe a purification process using a rapid and effective method involving hydrophobic chromatography and leading to electrophoretic homogeneity of the rat liver lipoxygenase. The enzyme has also been partly characterized and its molecular weight and kinetic properties established. Finally, the effects on the enzymatic activity of some reagents typical of lipoxygenases have been studied.

Experimental

Reagents

The linoleic acid and the nordihydroguaiaretic acid (4,4’-2,3-dimethyl-1,4-butanediyl)-bis(1,2-benzene diol) were supplied by SIGMA, London. Sephacryl S-200, Sephadex G-150, Phenyl Sepharose CL-4B, PBE 94 gel and Polibuffer 74 were purchased from Pharmacia Fine Chemicals, Upsala. All the other chemicals were of the highest purity available and used without further purification.

Lipoxygenase assay

The enzymatic activity was assayed at 20 °C by following the increase in absorbance at 234 nm pro-
duced by the transformation of the cis,cis-1,4 pentadiene system of linoleic acid into the conjugated hydroperoxydiene derivative cis-trans-(2). The reaction mixture typically contained 75 µl of 10 mM sodium linoleate, 0.05 to 0.1 ml of enzymatic solution and 50 mM sodium phosphate buffer (pH 7.0) to a final volume of 1 ml. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 µmol of linoleate per min under the described conditions. Specific activity is expressed as units of enzyme per mg of protein.

Protein determination
The protein determination was measured by the Lowry method [12], using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis
Analytical polyacrylamide disc gel electrophoresis has been carried out following the method of Davis [13] and the protein has been stained with coomassie blue G-250 in perchloric acid. Electrophoresis under SDS denaturing conditions and gel staining were performed according to the method of Weber et al. [14].

Molecular weight and hydrodynamic radius of the native enzyme
The chromatographic behaviour of rat liver lipoxygenase was studied using a Sephadex G-150 column (1.6×76 cm) equilibrated with 1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 10 ml/h. Cytochrome C, ovalbumin, a-chymotripsinogen-A and bovine serum albumin were used as molecular weight markers. The molecular weight of the enzyme was estimated according to the procedure of Andrews [15] and its hydrodynamic radius was calculated by the procedure of Siegel and Monthy [16].

Chromatofocusing
Chromatofocusing of the enzyme was performed at 4 °C according to Sluyterman [17]. A total volume of 50 ml of purified lipoxygenase, previously dialzed using 25 mM Imidazol-HCl buffer (pH 7.4) was loaded onto a column of polybuffer exchange PBE-94 and eluted with polybuffer 74.

Absorbance and fluorescence spectroscopy
The absorbance spectra have been recorded using an Uvikon 8109 spectrophotometer. Fluorescence spectra were measured on a Hitachi Perkin Elmer spectrofluorimeter, Mod. 650-40, at room temperature (20—22 °C), operating in ratio mode. The emission spectra were recorded using quartz cells of 1 cm path-length in 50 mm potassium phosphate buffer at pH = 7.0. The scan rate was 60 nm/min and the spectra shown are uncorrected.

Results
Purification procedure of rat liver lipoxygenase and criteria of purity
Liver has been isolated from wistar rats anestheized with ethyl ether. The rats typically weighed between 250 and 350 g. The liver was thoroughly perfused with 0.9% cold NaCl to wash out the remaining blood cells, weighed and homogenized in 50 mM sodium phosphate buffer (pH 7.5) with an ultraturrax (3,000 r.p.m.) and then sonicated for 2 min at 50 W. The homogenate was successively centrifuged at 10,000×g for 15 min and at 100,000×g for 1 h and gave a supernatant free of subcellular organelles. The supernatant was fixed to 20 mg/ml of protein and precipitated with ammonium sulphate between 60% and 80% saturation. The final pellet was redissolved in 50 mM phosphate buffer (pH 7.0) at a final protein concentration of 28 µg/ml yielding 366 mU/ml.

The method of purification presented here is based on a hydrophobic chromatography using Phenyl Sepharose CL-4B [18]. The procedure can be summarized as follows: an aliquot of 5 ml of the resuspended pellet is loaded onto a Sephacryl S-200 column (1×87 cm), preequilibrated with 50 mM potassium phosphate buffer and eluted with the same buffer. The fractions showing lipoxygenase activity were pooled, and the resulting mixed eluate was typically found to contain 0.92 µg/ml of proteins and an enzymatic activity of 82.5 mU/ml. This eluate of lipoxygenase (typically 1.65—1.90 units) was diluted with 20 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 25% saturated ammonium sulphate, and then loaded onto a Phenyl Sepharose CL-4B column equilibrated with 50 mM potassium phosphate buffer. The lipoxygenase was efficiently retained by the gel, while most of the contaminating proteins were eluted (see Fig. 1). After
washing the column with this buffer, an increasing gradient (0 to 50%) of ethyleneglycol decreasing in ionic strength (25% to 0% (NH₄)₂SO₄) was applied. As shown in Fig. 1, a very sharp peak of lipoxygenase activity was recovered. The purified enzyme showed a single protein band when assayed by analytical gel electrophoresis (results not shown).

Characterization of rat liver lipoxygenase

The absorbance spectrum of the purified enzyme shows a characteristic profile in the UV range and does not show any strong absorbance band in the visible. This is in agreement with the spectral properties described for other lipoxygenases obtained from mammals [9] and plants [19].

On the other hand, soybean lipoxygenase shows a characteristic fluorescent spectrum identical to that of soybean enzyme, with a maximum emission wavelength at 335 nm (λₑₓ = 280 nm). When the purified rat liver lipoxygenase is incubated in presence of linoleate, a quenching of the fluorescence band centered at 335 nm is observed. We have also found that the purified soybean lipoxygenase (type V from SIGMA, London) in these experimental conditions behaves similarly to the rat liver lipoxygenase (data not shown). In addition, the purified protein does not exhibit any intense absorbance band in the 400–450 nm wavelength range, as it has been shown previously to occur with other lipoxygenases.

The molecular weight of the purified rat liver enzyme, subunit molecular weight and estimated subunit composition of the native enzyme are given in Table II. These values are similar to those described for lipoxygenases from other sources [3, 9, 20].

The optimum pH of the rat liver lipoxygenase activity was determined to be pH = 7.0. Lipoxygenase

Figure 1. Chromatographic elution pattern of the extract loaded on a Sephacryl S-200. Prior loading the extract was partially purified by ammonium sulphate precipitation (60–80% saturation).

Figure 2. Phenyl Sepharose CL-4B chromatography elution pattern of the lipoxygenase partially purified by chromatography on Sephacryl S-200.

Table I. Summary of the purification steps of rat liver lipoxygenase.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume [ml]</th>
<th>Protein [mg/ml]</th>
<th>Enzymatic activity [mU/ml]</th>
<th>Specific activity [mU/mg]</th>
<th>Yield [%]</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>76</td>
<td>20.000</td>
<td>47.6</td>
<td>2.3</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>6</td>
<td>28.000</td>
<td>366.6</td>
<td>20.0</td>
<td>61</td>
<td>8.7</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>20</td>
<td>0.910</td>
<td>82.5</td>
<td>90.0</td>
<td>45</td>
<td>39.0</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B</td>
<td>45</td>
<td>0.055</td>
<td>16.6</td>
<td>300.0</td>
<td>20</td>
<td>130.4</td>
</tr>
</tbody>
</table>

a The data given in this Table correspond to those of a typical preparation. Similar results have been obtained in three different preparations; b the purification factor is taken as the increase in specific activity of the enzyme relative to that of the crude preparation.
activity drops more than 50% below pH 5.5 as well as above pH 8.5.

The isoelectric point of the purified enzyme was determined by chromatofocusing. We have obtained a pI value of 6.3 which is in good agreement with that found for the soybean lipoygenase by Galliard et al. [25]. Fig. 3 shows the dependence of the activity of the purified rat liver lipoygenase upon addition of nordihydroguaiaretic acid (NDGA), a selective and potent inhibitor of mammalian lipoygenases from several sources, such as kidney [21], polymorphonuclear leukocytes [22] and platelets [23].

The inhibition produced by NDGA on the rat liver lipoygenase is time and inhibitor concentration dependent as shown in Fig. 3. This behaviour is qualitatively identical with the effect of NDGA on other lipoygenases [21, 22, 24]. The quantitative differences are likely to arise from the differences in experimental procedures. We have measured rat liver lipoygenase activity using a purified enzyme preparation and the above referred reports have been carried out with cellular suspensions. The kinetic analysis of this inhibition revealed that it is competitive with substrate.

Table III summarizes the effects of several relevant compounds on the purified enzyme. The fact that KCN does not significantly inhibit the enzyme can be taken as an evidence for the absence of heme proteins as contaminants in the final preparation [20]. Indomethacin and aspirin, two potent inhibitors of the cyclooxygenase activity [25], do not significantly inhibit the lipoygenase activity. Therefore, our results show the absence of cyclooxygenase as a possible contaminant in our lipoygenases preparations.

Sulphydryl groups have been shown to be important in determining lipoygenase activities from various sources. Table III. Effects of various compounds on purified rat liver lipoygenase activity.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Relative activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCN 1 mM</td>
<td>100</td>
</tr>
<tr>
<td>KCN 10 mM</td>
<td>100</td>
</tr>
<tr>
<td>Aspirin 0.5 mM</td>
<td>130</td>
</tr>
<tr>
<td>Aspirin 5 mM</td>
<td>136</td>
</tr>
<tr>
<td>Indomethacin 1 μM</td>
<td>100</td>
</tr>
<tr>
<td>Indomethacin 10 μM</td>
<td>110</td>
</tr>
<tr>
<td>Iodoacetamide 0.1 mM</td>
<td>95</td>
</tr>
<tr>
<td>Iodoacetamide 10 mM</td>
<td>50</td>
</tr>
<tr>
<td>Parahydroxymercuribenzoate 0.1 mM</td>
<td>60</td>
</tr>
<tr>
<td>Parahydroxymercuribenzoate 10 mM</td>
<td>40</td>
</tr>
</tbody>
</table>

The compounds tested were preincubated for 15 min at 4 °C with 10 μg of purified rat liver lipoygenase in 50 mM potassium phosphate buffer (pH 7.0). The results are expressed as the activity relative to the control.
ous sources [26]. Thus, we have checked the effects of iodoacetamide and parahydroxymercurybenzoate being well known sulphhydryl blocking agents on the reaction catalyzed for the rat liver lipoxygenase. Our results suggest the involvement of $-\text{SH}$ groups in rat liver lipoxygenase activity. These groups obviously maintain a functionally active state of this enzymatic system.

**Discussion**

The aim of the present study was to establish the presence of lipoxygenase activity in rat liver. The results reported herein largely confirm this point. We have purified this enzyme from rat liver by a rapid method involving ammonium sulphate precipitation and two chromatographic steps, firstly gel filtration, followed by hydrophobic chromatography on Phenyl Sepharose CL-4B and thus obtained an apparently homogeneous product.

The hydrophobic chromatography on Phenyl Sepharose CL-4B has been previously used for lipoxygenase purification from soybean [18]. The combination of hydrophobic chromatography and the application of gradients from 0% to 50% ethyleneglycol and 25% to 0% (NH$_4$)$_2$SO$_4$, seems to provide a good procedure for rat liver lipoxygenase purification. In addition, this procedure is faster than those previously used for purification of mammalian lipoxygenases [9]. Our way of purification allows to obtain a lipoxygenase having a specific activity of 300 mU/mg in a final yield of 20% being much higher than that previously reported by Narumiya in the purification of rabbit reticulocyte 15-lipoxygenase involving ionic exchange chromatography, gel filtration chromatography and hydroxyapatite chromatography [9].

On the other hand, SDS disc-gel electrophoresis shows only one major band on coomassie blue staining, thus, indicating a high purity level of our lipoxygenases preparations.

Characteristic UV and fluorescence spectra of lipoxygenase from different sources have been reported previously by several groups [10, 20, 24, 27]. We have checked the purity of our rat liver lipoxygenase preparations spectroscopically and we have found spectra similar to those reported for other lipoxygenases. It has also been shown that lipoxygenases contain a non-heme iron atom in the protein molecule although it does not present any strong absorbance band around 410 nm [27]. Further, it has been reported that KCN, a typical inhibitor of heme proteins, does not inhibit lipoxygenases [20]. Our results indicate that the purified rat liver lipoxygenase (see Table III) also shares these properties of lipoxygenases.

The results presented in this study suggest that the enzyme from rat liver is a dimer of ca. 96 Kdal composed of two subunits of molecular weight of ca. 50 Kdal (see Table II). These data are quite similar to those previously reported for the soybean lipoxygenase using gel filtration chromatography [19], centrifugation [24], and SDS gel electrophoresis [28]. It has to be noted, however, that Narumiya [9] has reported a molecular weight of 61,000 daltons for lipoxygenase from rabbit peritoneal polymorphonuclear leukocytes. Similarly, Cifuentes [29] has reported a molecular weight of 54,000 and 40,000 daltons for two isoenzymes of *Evernia prunastri* lipoxygenase. Thus, it appears that a polipeptidic chain of ca. 50,000 daltons is a common feature among different lipoxygenases, although the oligomeric state can vary from one to another enzyme.

The optimum pH of rat liver lipoxygenase activity has been found to be around pH 7.0 similarly to that of yeast lipoxygenase [20] and to that of polymorphonuclear leukocytes lipoxygenase [9]. This is in contrast to the optimum pH at 9.0 [30] of the typical plants lipoxygenases, *i.e.* soybean lipoxygenase. In summary, rat liver lipoxygenase appears to be a lipoxygenase of type 2, according to the classification proposed by Galliard [23].

The possibility of a contamination of our preparation by lipoxygenases from blood cells is considered unlikely on the basis of the extensive 0.9% NaCl perfusion carried out on the livers used for isolation of lipoxygenase. On the other hand, our purified lipoxygenase from liver presents some important differences, *i.e.* molecular weight, with respect to that of lipoxygenase from polymorphonuclear leukocytes (Table II).

The possibility of cyclooxygenase contamination has been explicitly considered. The effects of several characteristic reagents on cyclooxygenase and lipoxygenase have also been checked. So, while we have not found an effect of indomethacin and aspirin on our purified protein preparation, NDGA, a specific inhibitor of plant and mammalian lipoxygenases [3, 9, 20] was found to completely inhibit it, at concentrations similar to those found to inhibit other...
lipoxygenases [22, 31]. This inhibition was competitive with the substrate, linoleic acid. Therefore, we consider that cyclooxygenase contamination in our purified system preparation is negligible.

We have assayed the effects of characteristic thiol reagents such as iodoacetamide and parahydroxymercurybenzoate. Both reagents inhibit the activity of purified rat liver lipoxygenase (see Table III). These results are in agreement to those found by Grossman [32, 33] and Dreesen [34], and strongly suggest that –SH groups play an important role in maintaining the lipoxygenase activity. The different behaviour of the enzyme against both reagents is worth mentioning: the effect of iodoacetamide is considerably more slow than that of parahydroxymercurybenzoate, probably due to a decreased accessibility of a functionally relevant –SH group to the former reagent. It remains to be established whether the different rate of these agents is due to structural requirements for an active conformational state of lipoxygenase, or whether, alternatively, this reflects that –SH groups directly participate in the catalysis. Work is actually underway in this laboratory to further elucidate this important point.

In summary, in this paper we report a rapid procedure for purification lipoxygenase of rat liver. Its kinetic and some of its physical-chemical characteristics are similar to those described for lipoxygenase from other sources. In addition, it appears that our lipoxygenase is different from that isolated from polymorphonuclear leukocytes, suggesting the presence of lipoxygenase isoenzymes in different mammalian tissues.

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