INTRODUCTION

Lipoxygenases (LOX) recognize the 1,4-pentadiene structure of polyunsaturated fatty acids and incorporate single molecules of oxygen at specific carbon atoms of substrate fatty acids. The resulting hydroperoxy group is subsequently reduced by glutathione peroxidase, forming corresponding monohydroxylated fatty acids as major LOX products.\(^1\) 12-LOX, which reacts with carbon-12 (C-12) of arachidonic acid (AA), has been purified from mammalian tissues and cells, and its oxidation products have been well investigated.\(^2\) In addition, 12-hydroxyeicosapentaenoic acid (12-HEPE) and 13-hydroxyoctadecadienoic acid (13-HODD) were also found in the reaction products as minor components. Similar results were obtained by the analysis of the reaction products of AA and carp gill microsomes. These results confirm the presence of 12-lipoxygenase in carp gill microsomes.

KEY WORDS: carp, Cyprinus carpio, gills, 12-lipoxygenase, microsomes, 12-hydroxyeicosapentaenoic acid, 12(S)-hydroxyeicosatetraenoic acid, 13-hydroxyoctadecadienoic acid.

SUMMARY: A lipoxygenase was found in the crude enzyme solution from the gills of carp, Cyprinus carpio. It oxidized arachidonic acid (AA) more efficiently than linoleic acid, eicosapentaenoic acid and docosahexaenoic acid. Lipoxygenase activity was constantly detected in the gill microsomes and was found to be optimum at pH 7.2. It was not stimulated by reduced nicotinamide adenine dinucleotide phosphate and was not inhibited by SKF525A, a cytochrome P450 inhibitor. The oxygenated products extracted from the reaction mixtures of crude enzyme solution and AA were purified by reverse-phase and straight-phase high-performance liquid chromatography (HPLC). The major metabolite was identified as 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) by ultraviolet spectrophotometry, gas chromatography-mass spectrometry and chiral phase HPLC. In addition, 12-hydroxyeicosapentaenoic acid (12-HEPE) and 13-hydroxyoctadecadienoic acid (13-HODD) were also found in the reaction products as minor components. Similar results were obtained by the analysis of the reaction products of AA and carp gill microsomes. These results confirm the presence of 12-lipoxygenase in carp gill microsomes.

Original Article

Identification of 12-lipoxygenase products in the gills of carp, Cyprinus carpio

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carp gills and determine the stereoselectivity of mono-hydroxylated fatty acid metabolite by LOX activity, by gas chromatography-mass spectrometry (GC-MS) and chiral phase HPLC.

MATERIALS AND METHODS

Arachidonic acid, EPA and DHA were purchased from Nacalai Tesque (Kyoto, Japan), linoleic acid from Toyo Jyozo Co. Ltd (Tokyo, Japan), 12(R,S)-HETE and 12(S)-HETE from Cayman Chemical Co. (MI, USA). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was obtained from Dojindo Laboratories (Kumamoto, Japan). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Kohjin Co. Ltd (Tokyo, Japan), and SKF-525A HCl from Research Biochemicals Inc. (MA, USA). Silicic acid (100 mesh) for column chromatography was purchased from Mallinckrodt Inc. (KY, USA).

Enzyme preparation

Gills of freshly killed carp, Cyprinus carpio (0.6–0.8 kg), were rinsed three times stepwisely in each 1 L ice-cold 0.85% NaCl to remove blood. The following procedure was performed at 4°C. Gill filaments were homogenized in 3 volumes of 50 mM HEPES-NaOH (pH 7.4), and the homogenate was centrifuged at 15 000 g for 15 min. The resulting supernatant was used as a crude enzyme solution and was further centrifuged at 100 000 g for 60 min. The cytosol fraction (supernatant) was collected, and the microsome fraction (pellet) was suspended in 50 mM HEPES-NaOH (pH 7.4) with an equal volume of cytosol fraction. The enzyme preparation was either used immediately, or stored at −80°C after freezing in a bath of dry ice-acetone.

Enzyme assay and protein concentration determination

Enzyme activity was determined by monitoring the conjugated diene absorption at 234 nm. The standard assay mixture contained 50 mM HEPES-NaOH (pH 7.4) previously bubbled with O2 gas, 17 μM substrate fatty acid and 0.1 mL of enzyme solution with a final volume of 3 mL. The enzyme activity was monitored after the addition of 3 μL of 17 mM substrate fatty acid in EtOH at 25°C.

Enzyme assay was also monitored via oxygen consumption using a YSI Model 5300 biological oxygen monitor (Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA) according to the method of Hsieh et al.15 The standard assay mixture contained 50 mM HEPES-NaOH (pH 7.4), 2.5 mM substrate fatty acid and 1 mL of enzyme solution in a final volume of 2 mL. The assay mixture was equilibrated in an oxygen electrode chamber at 25°C, and the enzyme activity was initiated by the addition of 5 μL of 1 M substrate fatty acid in EtOH.

The protein concentration of the enzyme preparation was determined by the method of Lowry et al.24 using bovine serum albumin as a standard.

Product analysis

Purification of monohydroxy derivatives was performed as described elsewhere.21,25 Twenty microliters of 0.26 M AA in EtOH was added to 60 mL of the crude enzyme solution previously equilibrated at 25°C for 1 min. The reaction was initiated at 25°C for 5 min and terminated by adding 5 mL of 0.2 M citric acid. To this solution 3 volumes of diethyl ether was added, and the ethereal extract was obtained. The monohydroxy fraction was purified from the ethereal extract by silicic acid chromatography21 and was subjected to a reverse-phase HPLC on the columns of Cosmosil ODS (4.6 × 150 mm, Nacalai Tesque) and Cosmosil ODS (4.6 × 50 mm). The column was eluted with acetonitrile/water/methanol/AcOH (150 : 110 : 50 : 0.02, by volume) at a flow rate of 1 mL/min. Column effluent was monitored at 235 nm. Peaks 1, 2 and 3 (see below), separated by reverse-phase HPLC, were purified with straight-phase HPLC using a Zorbax SIL column (4.6 × 250 mm; Dupont New England Nuclear Research, Boston, MA, USA). The column was eluted with n-hexane/2-propanol/AcOH (99 : 10 : 0.1, by volume) at a flow rate of 2 mL/min. Column effluent was monitored at 235 nm. The ultraviolet absorption spectra of peaks 1, 2 and 3 purified with straight-phase HPLC were recorded on a Shimadzu dual wavelength spectrophotometer (Model UV 3000; Kyoto, Japan).

Samples (purified peaks 1, 2 and 3) were transmethylated, hydrogenated and converted to their trimethylsilyl (TMS) ether and tert-butyldimethylsilyl (t-BDMS) ether derivatives as described previously.21,25 Electron impact mass analysis of methyl ester-TMS and -t-BDMS ether derivatives of the samples was performed with a Shimadzu GC-MS QP-1000A, consisting of a 1.1 m × 3 mm column of 2% OV-17 on Chromosorb W (80–100 mesh) under isothermal conditions at 220°C. The electron energy was 70 eV in all cases. The injector, separator and ion source temperatures were kept at 230°C, and the He carrier gas flow was set at 30 mL/min. Selected ion monitoring traces of the samples were also performed with Shimadzu GC-MS QP-1000A by chemical ionization, using ammonia as a reagent gas.

Chiral separation

Purified peak 3, authentic 12(R,S)-HETE and 12 (S)-HETE were transmethylated with ethereal dia-
zomethane, and were resolved by chiral phase HPLC on a Chiralcel OJ column (4.6×250 mm; Daicel Chemical Ind., Tokyo, Japan) using n-hexane/2-propanol (99:1, v/v) at a flow rate of 0.5 mL/min. Column effluent was monitored at 235 nm.

RESULTS

Properties of carp gill lipoxygenases

The substrate specificity of carp gill LOX was examined in the crude enzyme solution (Fig. 1). The addition of AA to the crude enzyme solution resulted in rapid oxidation. However, the reaction velocities of linoleic acid, EPA and DHA were lower than that of AA. In addition, the velocity of oxygen consumption was well correlated with the increase in OD234, using AA as a substrate (data not shown). This indicates the formation of hydroperoxide and conjugated diene of AA by the LOX activity in the crude enzyme solution. Subsequently, the crude enzyme solution was resolved into microsome and cytosol fractions, and the enzyme activities were determined in each fraction, using AA as a substrate. Lipoxygenases activity was constantly observed in the microsome fraction, but not in the cytosol fraction (data not shown). Therefore, we used the microsome fraction as an enzyme source in the present experiment. The optimum pH for LOX activity in the microsome fraction was found to be 7.2, and an increase in pH from 7 to 8 resulted in the loss of almost all the activity (Fig. 2). Lipoxygenases activity in the microsome fraction was not stimulated by the addition of NADPH and was not inhibited by SKF525A, a cytochrome P450 inhibitor, measured with an oxygen electrode (data not shown).

Fig. 1 Substrate specificity of the lipoxygenase in the crude enzyme solution of carp gills. Enzyme reactions were started by the addition of (O) linoleic acid, (●) arachidonic acid, (△) eicosapentaenoic acid or (□) docosahexaenoic acid, and the absorbance at 234 nm was monitored.

Fig. 2 Effect of pH on the lipoxygenase activity in the microsome fraction of carp gills. Reactions were started by the addition of arachidonic acid to assay mixtures at various pH, and the enzyme activity was measured by monitoring the absorbance at 234 nm. The buffer used was citric acid-Na₂HPO₄ buffer (pH 4.0–8.0).

Fig. 3 Reverse-phase high-performance liquid chromatography (HPLC) of the reaction products from the crude enzyme solution of carp gills incubated with arachidonic acid (AA). The ethereal extract obtained from the incubation mixture of AA and crude enzyme solution was purified by silicic acid chromatography and subjected to reverse-phase HPLC. Peaks denoted by the bars were pooled.
Identification of reaction product

Lipoxygenases activities in the crude enzyme solution and microsome fraction decreased almost half by the addition of glutathione (GSH), using AA as a substrate (data not shown). Therefore, we used the reaction products of AA and the crude enzyme extract of carp gills in the absence of GSH for the identification of lipoxygenase products. The monohydroxy fraction obtained from the ethereal solution of the reaction mixture of AA and the crude enzyme solution was analyzed by reverse-phase HPLC (Fig. 3). One major and two minor peaks were observed, and the retention time of peak 3 coincided with that of authentic 12-HETE standard. Three peaks denoted as peaks 1, 2 and 3 (shown by the bars in Fig. 3) were collected and purified by straight-phase HPLC (Fig. 4). Peaks 1, 2 and 3, separated by reverse-phase HPLC, were detected as one major peak, and peak 3 also coeluted with authentic 12-HETE by straight-phase HPLC. Purified peaks 1, 2 and 3 denoted by the bars in Fig. 4 were also collected, respectively. The ultraviolet spectra of purified peaks 1, 2 and 3 exhibited maximum absorption around 235 nm (data not shown), which suggests the presence of a conjugated diene in the compound. Methyl ester-TMS ether derivatives of purified peaks 1, 2 and 3 were analyzed by GC-MS. Characteristic ions of a methyl ester-TMS ether derivative of purified peak 3 were observed at m/z 295 (Fig. 5a), and those of its hydrogenated derivative at m/z 215 and m/z 301 (data not shown). These spectra were identical to those of authentic 12-HETE. The mass spectra of methyl ester-t-BDMS ether derivatives before and after hydrogenation were essentially identical to those of 12-HETE.21,25 The methyl ester-TMS ether of purified peak 1 showed the characteristic ion m/z 295 (Fig. 5b), and the characteristic ions of its hydrogenated derivative were found at m/z 215 and 301 (data not shown). The elution time (Fig. 3) and the mass spectra (Fig. 5b) of purified peak 1 corresponded to those of 12-HEPE.21,26 In addition, selected ion monitoring traces of the methyl ester-TMS ether derivatives of purified peaks 1 and 3 were performed. As a result, the molecular ion of 12-HEPE at m/z 422 [M‘+NH₄⁺] was found in purified peak 1 and that of 12-HETE at m/z 424 [M‘+NH₄⁺] in purified peak 3 (data not shown). Subsequently, purified peaks 1 and 3 were identified as 12-HEPE and 12-HETE, respectively. Purified peak 3 was further analyzed by chiral phase HPLC and was identified as 12(S)-HETE (enantiomer excess >91%) (Fig. 6). A methyl ester-TMS ether derivative of purified peak 2 exhibited mass spectrometric peaks of the following intense ions: m/z 382 [M‘], 311 and 225 (Fig. 5c). This pattern was essentially identical to that of 13-hydroxyoctadecadienoic acid (13-HODD).27
DISCUSSION

A LOX activity was found in the crude enzyme solution of carp gills. The enzyme of carp gills oxidized AA more efficiently than EPA and DHA (Fig. 1), slightly different from that of rainbow trout\(^8\)\(^9\) and red sea bream\(^1\) gills whose enzymes oxidized AA, EPA and DHA almost equally. The LOX activity was constantly detected in the microsomes and was found to be optimum at pH 7.2 (Fig. 2). The optimum pH for heme proteins such as catalase, peroxidase and cytochrome C was found to be around 4–5.\(^{28,29}\) and the LOX activity at pH 5 may be due to the heme protein. The enzyme activity was not stimulated by NADPH and was not inhibited by SKF525A. The result shows the presence of LOX in carp gill microsomes.

The stereoisomers of 12-HETE appear to be produced by two different enzymatic pathways. The S enantiomer at the asymmetric carbon atom, C-12, is generated by the action of 12-LOX.\(^1\)\(^2\) On the other hand, the R enantiomer is the product of allylic oxidation by a mono-oxygenase reaction of cytochrome P450. 12(R)-HETE was mainly produced in the presence of NADPH by the rat liver cytochrome P450,\(^30\) human skin from psoriatic lesions\(^31\) and bovine corneal epithelial microsomes.\(^32\)\(^34\) In addition, LOX with R stereospecificity have recently been found.\(^35\)\(^36\) In the present experiment, 12(S)-HETE was isolated as a major monohydroxylated product from the reaction mixture of AA and crude enzyme extract of carp gills in the absence of GSH (Figs 3–5). In addition, 12(S)-HETE was also detected in the reaction mixture of AA and carp gill microsomes in the absence of GSH (data not shown). Siegel et al. partially purified two forms of LOX, 100 and 160 kDa, from human platelets and found that the latter had an associated peroxidase activity which reduces 12-hydroperoxyeicosatetraenoic acid (12-HPETE) to 12-HETE in the absence of GSH.\(^37\) It is suggested from the above aspects that AA is converted to 12-HPETE and subsequently reduced to 12-HETE by 12-LOX itself in carp gills. Linoleic acid and EPA are

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Fig. 5  Mass spectra of the non-hydrogenated methyl ester trimethylsilyl ether derivatives of the compounds, (a) peak 3, (b) peak 1 and (c) peak 2.
12-LOX was detected in the microsomes, which is similar to 12-LOX-like enzyme from red sea bream gills. It remains unclear whether carp gill 12-LOX is a membranous type enzyme or if it translocates from cytoplasm to microsomes during the extraction procedure. In addition, we identified 12(S)-HETE in the monohydroxy fraction from the ethereal extract of the reaction mixture of AA and crude enzyme solution or microsome fraction of carp gills, but we have not yet identified 12-HPETE. Further study is needed to purify the enzyme from gill microsomes and characterize its properties, including conversion of AA to 12-HPETE and subsequent reduction of 12-HPETE to 12-HETE by the purified enzyme.

REFERENCES

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