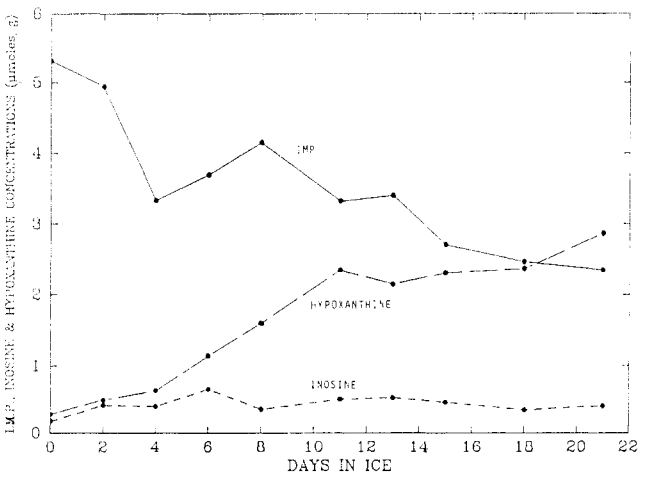

Figure 4. Changes in concentrations of IMP, inosine, and hypoxanthine in hoki during storage in ice.

Table II. Percentage Recovery of ATP and Its Breakdown Products from Standard Added to a Perchloric Acid/Fish Homogenate of Known Initial Concentration of the Relevant Compounds

	recovery, %				
	amount added, μmol/g[a]				
compd	0.60	0.30	0.15	0.08	mean
IMP	89.7	86.5	91.7	110.1	94.5
ATP	100.9	96.9	104.0	89.7	97.9
ADP	92.6	94.6	95.9	117.3	100.1
AMP	96.9	92.3	101.5	92.3	95.8
Hx	96.4	92.1	96.5	105.1	97.5
HxR	91.2	88.9	93.6	102.9	94.2

[a] μmol/g of fish/perchloric acid homogenate.

plotted due to their consistently low levels throughout the storage period. Hypoxanthine increased while IMP decreased and HxR remained constant. The *K* value also increased during this period. These chromatograms demonstrate that ATP and its breakdown products in fish muscle can be monitored by using this technique.

The mean recoveries for each compound, over the range of amounts of added standard, ranged from 86.5 to 117.3% with a mean recovery of each compound varying from 94.2 to 100.1% (Table II). Peak areas increased linearly with increasing concentrations of reference compounds up to 2 μg injected, and the minimum detectable amount was 5 ng/injection. The IMP and hypoxanthine peaks cochromatographed with guanosine 5′-monophosphate and guanine, respectively, but the latter compounds have seldom been reported in fish muscle extracts.

A radially compressed column (Waters Associates Z Module) with μBondapak C18 packing was evaluated but gave no advantages over the stainless steel column for routine analyses. In spite of the lower pressures and high flow rates associated with radially compressed columns enhancing the ease and speed of solvent changeover, the resolution between the adenosine nucleotide peaks was inferior to that achieved on the stainless steel column.

ACKNOWLEDGMENT

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Registry No. AMP, 61-19-8; ADP, 58-64-0; ATP, 56-65-5; IMP, 131-99-7; inosine, 58-63-9; hypoxanthine, 68-94-0.

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Lipid Oxidation in Fish Tissue. Enzymatic Initiation via Lipoxygenase

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Mechanisms underlying the initiation of lipid peroxidation in fish were investigated with trout skin tissue. Conversion of docosahexaenoic and arachidonic acid into polar products by skin tissue was consistent with enzymatic activity sensitive to lipoxygenase inhibitors stannous chloride and esculetin. GC/MS analyses of the monohydroxy derivatives of arachidonic acid identified 12-hydroxyeicosatetraenoic acid as the major monohydroxy product suggesting a 12 lipoxygenase activity in the skin. Thus, enodgenous skin lipoxygenase released post-mortem may constitute a significant source of initiating radicals leading to subsequent lipid peroxidation in fish tissue.

INTRODUCTION

Lipid oxidation is a very important event leading to the deterioration of foods containing highly unsaturated fats. Fish, in particular contain high levels of cellular unsaturated lipids (Kinsella et al., 1977) which readily deteriorate via peroxidation resulting in the development of objectionable odors and flavors. Although the process of lipid oxidation is highly favored thermodynamically, the direct reaction between oxygen and even highly unsaturated lipid is kinetically hindered. Hence, an activating reaction is

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necessary to initiate the free-radical chain which subsequently rapidly proliferates and accelerates lipid oxidation. For the minimization or control of lipid oxidation it is therefore essential that the mechanisms by which lipid oxidation is initiated be understood and perhaps then eliminated at the source. We recently proposed that a potential source of initiating species in fish, i.e., lipid hydroperoxides, could be generated by endogenous enzymes liberated from the tissue itself (German and Kinsella, 1983). Kanner and Kinsella (1983) demonstrated that tissue peroxidase could initiate lipid peroxidation. Lipoxygenases found in various animal tissues catalyze the insertion of oxygen into an unsaturated fatty acid forming a highly reactive hydroperoxide as the product. We have reported on a lipoxygenase in trout gill tissue with high activity toward unsaturated fatty acids (German and Kinsella, 1983; German et al., 1985). The possibility that other tissues in close proximity to muscle tissue might also contain lipoxygenases capable of reacting with unsaturated fatty acids present led us to investigate skin tissue which in mammals contains lipoxygenase activity. In this communication we report the presence of a similar lipoxygenase activity in skin tissue of fresh water trout.

MATERIALS AND METHODS

Skin tissue was removed from freshly killed, mature (3 kg) rainbow trout and homogenized in 4 volumes of cold 0.1 M phosphate buffer, pH 7.8, containing 1 mM reduced glutathione. This homogenate was centrifuged at 15 000g for 10 min to remove cellular debris and organelles. The crude supernatant was used in all subsequent analyses as the source of enzyme.

Fatty Acid Analyses. Fatty acids and their oxygenated derivatives were extracted from the supernatant with ethyl acetate, dried under nitrogen, and esterified with ethereal diazomethane as previously described (Mai et al., 1982). The fatty acid methyl esters were chromatographed on a Silar 100 capillary column (25 m, 0.25 mm ID) with a Hewlett Packard 5830 gas chromatograph. Compounds were identified by their retention times relative to known standards. Methyl 15:0 was used as an internal standard for quantification.

Separation of Radiolabeled Products. Radioactive 20:4n − 6 and 22:6n − 3 were added as tracers to identify the accumulation of oxygenated products from the enzymatic activity in the tissue. Aliqots (1 mg of protein/mL) of enzyme preparation in phosphate buffer (total volume 2 mL) were incubated with either 20:4 or 22:6, 1 μM (0.05 μCi), for 15 min at 25 °C. The reaction mixture was then acidified with HCl and extracted with equal volumes of ethyl acetate. This was evaporated to a volume of 20 L and spotted on analytical TLC plates (EM Science, Gibbstown, NJ). The plates were developed with a solvent system of chloroform–methanol–water–acetic acid (90:8:1:.8) as previously described (German et al., 1985). The thin-layer plate was then placed in an X-ray cassette, overlayed with Kodak type x-AR X-ray film, and exposed at −70 °C for 4–7 days. This X-ray film was then developed using standard chemicals (Kodak, Rochester, NY).

Gas Chromatography/Mass Spectrometry. For identification of the monohydroxy derivatives of the unsaturated fatty acids generated by the enzyme system(s) in the skin tissue, the enzyme preparation (20 mL) diluted with 100 mL of phosphate buffer containing 100 μM free arachidonic acid was incubated for 15 min. The products were extracted and separated by thin-layer chromatography as described above. The region corresponding to monohydroxy derivatives of arachidonic acid was scraped, eluted, derivatized to form the methyl ester and either the trimethylsilyl (Me_3Si) or tertiary butyldimethylsilyl (t-$BuMe_2Si$) ether, and analyzed by gas chromatography/mass spectrometry (GC/MS) with a Hewlett Packard 5995 instrument and conditions as previously described (German et al., 1985; Mai et al., 1982).

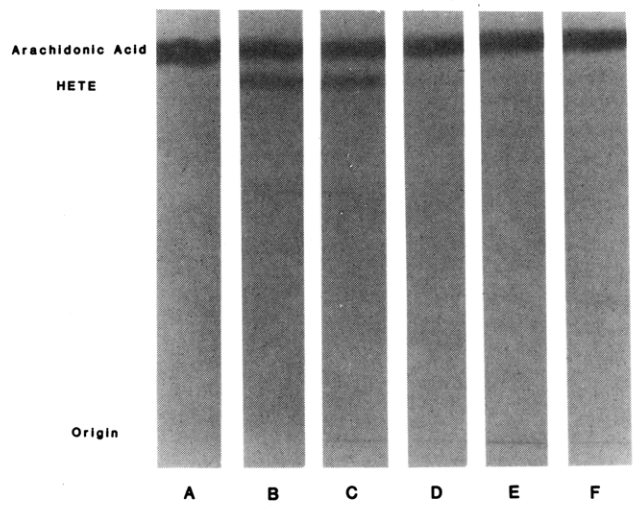

Figure 1. Autoradiogram of the thin-layer chromatogram showing separation of products produced from radiolabeled arachidonic acid by skin tissue homogenates in the presence and absence of lipoxygenase inhibitors. Aliquots (2 mL) of tissue homogenate (2 mg of protein)/(enzyme) were incubated as described in methods with radiolabeled arachidonic acid. (A) Boiled tissue preparation (no products). (B and C) Crude enzyme plus arachidonic acid, 12-hydroxy fatty acid band (HETE). Enzyme plus arachidonic acid plus: (D) EGTA (1 mM). (E) Stannous chloride (5 mM). (F) Esculetin (100 μM).

RESULTS

The oxygenation of polyunsaturated fatty acids by the skin tissue was initially investigated with radiolabeled arachidonic or docosahexaenoic acid incubated with skin tissue homogenates. The rate and extent of conversion of radiolabelled arachidonic acid and docoashexaenoic acid were similar in these studies. Hence, conversion of arachidonic acid is shown in the figures because the lack of significant quantities of arachidonic acid in the tissue verifies the enzymatic conversion of exogenously added substrate, and reference standards and mass spectra of arachidonate metabolites are readily available. Separation of the products generated by the enzymatic activity in the trout skin tissue by thin-layer chromatography (Figure 1) demonstrated a significant conversion of exogenous fatty acid to prodcts of greater polarity than the original fatty acid. Note lane B and C (Figure 1). These compounds cochromatographed with monohydroxy fatty acids such as prostaglandins (Lokesh et al., 1984).

The existence of an enzymatic activity was demonstrated by the complete inhibition of oxygenation following boiling of the enzyme preparation for 30 s (Figure 1 lane A). Furthermore, the effect of several specific inhibitors on the production of radiolabeled products is consistant with an enzyme mediated conversion. The compounds stannous chloride and esculetin are known inhibitors of mammalian and trout gill lipoxygenases (German et al., 1985; Wallack and Brown, 1981). The presence of these compounds in the reaction mixture dramatically reduced the conversion of exogenous 1-^{14}C-labeled fatty acids into more polar products. The evidence argued in favor of a lipoxygenase and against involvement by nonspecific enzymatic initiators such as cytochrome P450 (McDonald et al., 1979). Similarly, ethylene glycol bis(aminoethyl ether) $N,N,$-

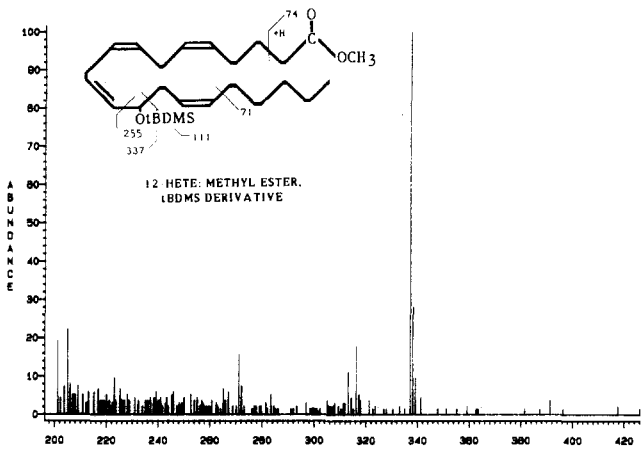

Figure 2. Mass Spectrum of the methyl ester t-BuMe$_2$Si derivative of the major product produced from arachidonic acid by skin tissue homogenates. 12-Hydroxyeicosatetraenoic (HETE) and its major mass spectra fragments are shown in the insert.

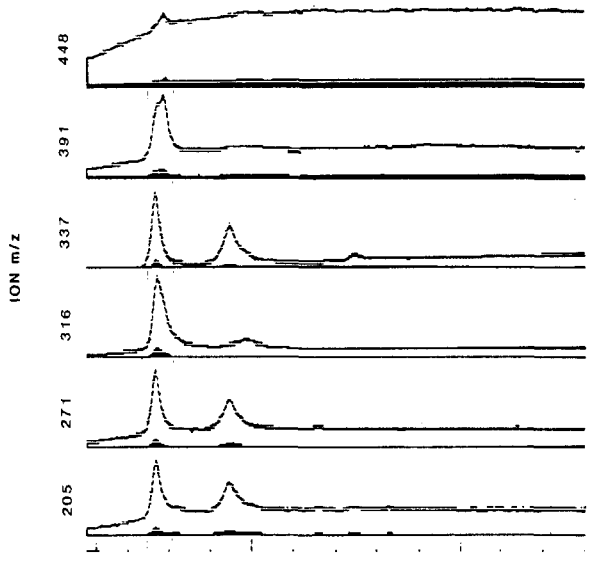

Figure 3. Single ion chromatograms of six ion fragments diagnostic for 12 HETE. (GC/MS analyses performed as described in methods.)

N',N'-tetraacetic acid (EGTA) (5 mM) a potent metal chelating agent also reduced the activity suggesting a metal cofactor or requirement by the enzyme. These observations suggested that the skin tissue contained a lipoxygenase-like enzyme.

From further analyses we obtained evidence that the monohydroxy compounds produced are consistant with the presence of a 12-lipoxygenase previously described in trout gill tissue and various mammalian cell types, platelets (Hamberg, 1974), lungs (Yokoyama et al., 1983), and skin (Ruzicka et al., 1983). The positional specificity of the enzyme was determined by GC/MS analyses of the products cochromatographing on the TLC system with standard monohydroxy derivatives of 20:4; i.e., the 5, 12, and 15 isomers of hydroxy eicoasatetraenoic acid. The mass spectrum of the major product isolated from this region on the TLC plate is shown in Figure 2. Both the mass spectrum and the retention time on the capillary column used are consistant with 12 hydroxyeicosatetraenoic acid (HETE). Further support for the production of the 12 HETE was provided by the single ion chromatogram (Figure 3). Ions diagnostic for the 12 HETE methyl ester, t-BuMe$_2$Si derivative are 205, 271, and 337 and these fragments all clearly cochromatographed to identical retention times. The ions 391 (M − 57), 316 (M − 189), and 448 (M+) are characteristic of monohydroxy t-BuMe$_2$Si derivatives of 20:4 but are not position specific. These also peaked at the retention time of the HETE standard, though there was some evidence of an additional monohydroxy peak distinct from the 12 isomer. We are currently investigating the possibility of additional lipoxygenases present in the tissue responsible for the production of other positional monohydroxy fatty acids.

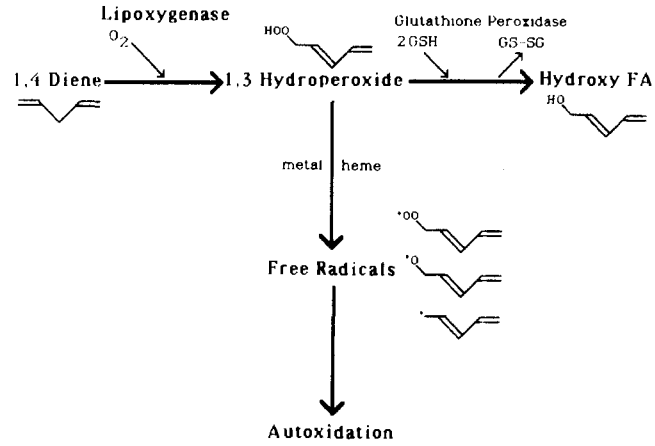

Figure 4. Proposed mechanism for initiation of tissue lipid peroxidation via lipoxygenase activity. Normal physiologically controlled pathway is summarized in the horizontal direction, while the proposed consequences of tissue destruction and lipoxygenase product proliferation are suggested in the vertical direction.

DISCUSSION

Although lipid oxidation has long been considered one cause for the deterioration of the quality of fish, the mechanisms underlying this process have previously been believed to be primarily nonenzymatic autoxidation. Certainly, the proliferation of volatile products of peroxidation are consistant with a random autoxidative process. Nevertheless, the mechanism responsible for the initiation of oxidation as an essential first step in this process which generates the initial free radicals leading to the propagation phase has not been well established. While several enzymatic and nonenzymatic mechanisms exist in biological tissues capable of generating reactive oxidizing species (Kanner and Kinsella, 1983; McDonald et al., 1979; Slabyj and Hultin, 1982; Shewfelt and Hultin, 1983; Schaich, 1981) few of these have been unequivocally described as rate limiting or even strongly participating in the deterioration of actual food tissues. The discovery of a lipoxygenase activity in gill tissue led us to propose that the postmortem release of this enzyme from endogenous constraints on its activity could generate significant quantities of reactive lipid hydroperoxides. These, in conjunction with metallic catalysts would serve as a potent source of initiating free-radical species for oxidation of the lipids present in the tissue, according to the mechanism summarized in Figure 4. Critical to this overall scheme is the release of both substrate and enzyme from normal physiological constraints and the exhaustion of endogenous hydroperoxide scavengers such as glutathione peroxidase. Recent reports from studies on the volatiles released from fish tissue and their inhibition with lipoxygenase inhibitors (Josephson et al., 1984; Josephson et al., unpublished results) strongly support the concept that endogenous oxygenase enzymes in fish tissue may be significantly responsible for the initiation of peroxidation. The demonstration of a lipoxygenase activity in skin reinforces the potential for endogenous enzymes to accelerate

lipid deterioration in underlying muscle tissue.

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Registry No. 20:4*n*–6, 506-32-1; 22:6*n*–3, 6217-54-5; lipoxygenase, 9029-60-1; esculetin, 305-01-1; stannous chloride, 7772-99-8.

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Protein Hydrolysates from Soy Grits and Dehydrated Alfalfa Flour

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The chemical and amino acid composition of hydrolyzed vegetable proteins (HVP) in filtrate, paste, and powder from soy grits and dehydrated alfalfa flour has been analyzed. The protein content in HVP from soy grits was an average of two times greater than the protein content in HVP obtained from dehydrated alfalfa flour. The results obtained are compared with those from beef extract. The glutamate content as a meat flavor enhancer in the HVP from soy grits is 2.3 times greater than that in beef extract and 3.3 times greater than that of HVP from dehydrated alfalfa flour. The HVP from dehydrated alfalfa flour is suitable for dark colored food articles and also has a specific flavor.

Hydrolyzed vegetable protein (HVP) is widely utilized in food production, particularly in soups, sauces, and flavorings, as well as a flavor enhancer in boiled, roasted, and technologically processed meat. It is added in amounts of 1–5% according to weight to ready-to-eat products, sausages, meat spreads, stewed meat, bacon, canned meat, and other products; e.g., are Bačurskaja and Guljaev (1976), Polič et al. (1982), and Trumič et al. (1982). HVP is used as a raw material for the isolation of amino acids, as a substitute for sodium glutamate (published in *Food Eng. Int.* (1983)) and as an antioxidant in the meat industry as well as in the confectionary and baking industries.

The customary raw materials and protein sources for the manufacture of HVP are wheat gluten, defatted soy grits with 44–54% protein, and other plant products among which algae and cereals are of significance. Light HVP derived from wheat gluten is used for high quality products of light color, while HVP from corn gluten differs only insignificantly in flavor and aroma. Nevertheless, HVP derived from the acid hydrolysis of defatted soy flour or grits has the broadest range of application, as proposed from the Commission du Codex Alimentarius (1978). The factors dictating the selection of raw materials for the production of HVP are price and the chemical, physical, organoleptic, and toxicological properties of the finished product as published by Olsman (1979).

The industrial manufacture of HVP is carried out in three ways: biologically (Bačurskaja and Guljaev, 1976; Konrad and Lieske, 1979), with alkalies (Lyall, 1965), and with acids (Stroszel et al., 1971). As a new source of raw material for obtaining HVP, the possibility of dehydrated alfalfa flour was investigated because alfalfa flour is the most inexpensive source of protein.

EXPERIMENTAL SECTION

Materials. This study was conducted in the industrial plant of Agrokomerc Complex Organization of Associated Labor, Velika Kladuša, which has a capacity of 4000 dm^3 per load. Forty-four percent defatted soy grits was manufactured by the Soy Factory of Zadar, according to the specifications of the Yugoslav Bureau for Standardization (1963, 1981). The alfalfa flour was produced by Agrokomerc Complex Organization of Associated Labor, Velika Kladuša, and met the specifications of the Yugoslav Bureau for Standardization (1978).

The hydrochloric acid (33%) and the sodium carbonate used were products of SODASO Tuzla and met the technical requirements stipulated for use in the food industry.

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