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ORIGINAL PAPER

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Enzymatic oxidative activity in sardine (*Sardina pilchardus*) and herring (*Clupea harengus*) during chilling and correlation with quality

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Abstract Enzymatic oxidative activity of two fatty fish species, sardine (*Sardina pilchardus*) and herring (*Clupea harengus*), was studied during chilled storage. Lipoxygenase enzyme activity was isolated and tested by measuring the hydroperoxides produced after induced oxidation of arachidonic and docosahexaenoic fatty acids. The most abundant degradation products of the hydroperoxides formed were 12- and 16-hydroxy acids which were detected by HPLC. Lipoxygenases were concentrated in the skin tissue of fish, and were active for up to 48 h of chilled storage. The pro-oxidative activity due to haem proteins continued for longer than that due to lipoxygenase. Trends of fluorescent formation resulting from interaction between oxidation products and biological amino constituents were compared with the pro-oxidative activities to establish correlations with quality loss during chilling.

Key words Herring · Sardine · Chilling · Lipoxygenase · Lipid oxidation

Introduction

During processing and storage, fish quality may decline as a result of several factors. In fatty fish species, one of the most important is the oxidation of highly unsaturated lipids which produce off-flavours and odours in foods [1, 2]. Endogenous enzymes liberated from the fish tissue itself can be a potential source of species responsible for the initiation of the peroxidation [3, 4]. In particular, peroxidases and lipoxygenases catalyse the formation of highly reactive hydroperoxides which can propagate the lipid oxidative chain reaction, especially on storage at high temperature.

Lipoxygenase activity has been reported in some fish species and evidence supports the presence of different types of these enzymes in fish with varying distribution, activity and stability [5–7]. The ability of lipoxygenases to product hydroperoxides and the instability of the enzymes in general has led to the proposal that they can affect flavour generation, especially fresh flavours. The unique pattern of flavours which result from enzymatic activity in raw, healthy tissues will rapidly diminish as the enzymes deteriorate and autooxidative reactions predominate. However, the mechanism of lipid degradation during fish storage is very complex involving not only enzymatic oxidation but also oxidation catalysed by other factors such as the presence of metals and haem pigments [8]. Thus, a knowledge of the mechanisms involved in fish oxidation is critical to the design of technological treatments for preserving freshness and quality.

The aim of this work was to study lipoxygenase activity in two fatty fish species, herring and sardine, which have high consumption rates in Europe. Lipoxygenase enzymatic activity was monitored during chilling by testing its effect on the production of hydroxides, breakdown products from hydroperoxides [9]. The effect of lipoxygenase on lipid damage was also tested by fluorescent compound formation [10, 11]. Measurements of interaction compounds formed by the reaction of oxidation products and biological amino constituents (proteins, peptides, free amino acids and phospholipids) have been commonly employed for quality determination [12, 13]. The oxidative activities due to lipoxygenases and haem proteins were correlated with the formation of fluorescent compounds during chilling to evaluate their contribution to quality deterioration of fatty fish.

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Materials and methods

Materials. Atlantic herring (*Clupea harengus)* and sardine (*Sardina pilchardus*) were supplied by a local supplier in Guildford, UK. Whole fish were stored in ice at 4 °C, and samples taken at 0, 1, 2, 4 and 6 days. For each day, two fish were analysed individually. The fatty acids arachidonic acid (ARA) (20:4*n*-6) and docosahexaenoic acid (DHA) (22:6*n*-3) and 5-, 9-, 12- and 13-hydroxyoctadecaenoic acid standards were purchased from Sigma (Poole, Dorset, UK). All reagents used were analytical and HPLC grades.

Lipoxygenase extraction. Lipoxygenase was extracted by a modification of the method of German and Kinsella [5] Skin or muscle tissue (20 g) was removed from fish and homogenised in 5 volumes of 0.05 M potassium buffer (pH 7.4) containing 5 mM glutathione. The homogenate was centrifuged at 12 000 *g* for 10 min. The mixture was stirred on ice for a further 30 min and then adjusted to 45% saturation with solid ammonium sulphate. The insoluble protein was sedimented by centrifugation at 105 000 g for 60 min. The pellet was redissolved in 2 ml of potassium buffer (pH 7.4) and used as the source of enzyme (first extract). To ascertain the effect of the haem proteins**,** a second extraction was carried out by adjusting the supernatant to 90% saturation with solid ammonium sulphate and the extraction was repeated as described above giving the second extract.

Inhibition of lipoxygenase activity. Lipoxygenase activity was inhibited by boiling the enzyme solution at 90 °C or by adding 5 mM of stannous chloride [14].

Measurement of pro-oxidative activity of tissue extracts. ARA or DHA (3 mg) was dispersed in 3 μl of Tween 20 and 1 ml of 0.05 M phosphate buffer (pH 7.4) by sonicating for 9 min. One millilitre of tissue extract (4 mg/ml of protein) was incubated with the fatty acid preparation for 5 min in a water bath shaker at 37 °C and oxygen was bubbled through. The reaction was stopped by adding 1 ml of formic acid (3%) and the hydroperoxides were extracted three times in freezing ethyl acetate. The extracts were pooled together, concentrated in a flash evaporator and dissolved in 200 μl of the HPLC mobile phase.

Analysis of hydroperoxides by HPLC. Hydroxy compounds were separated according to Saeed and Howell [9] on a reversed phase KR100 column (5 μm, 4.6 mm i.d. × 25 cm, Hichrom), coupled with a Waters HPLC 600A liquid chromatograph, and a Waters AS 3000 variable wavelength UV detector set at 235 nm. The chromatographic separation was achieved by an isocratic elution with methanol: water: acetic acid (65:35:0.01, v/v/v). The flow rate was 1 ml/min. The identities of the peaks were verified by comparing their relative retention times with those of standards (Sigma). Analyses were performed in duplicate.

Derivatisation of the fatty acid hydroperoxides to trimethylsilyl ethers. The hydroperoxide fraction was dried under a stream of nitrogen. Then, 100 μl of dry pyridine and 100 μl of *N*,*O*-bis(trimethysilyl) acetamide and 1% trimethylchlorosilane were added, while flushing with nitrogen, and the vial was sealed and vortexed. The sample was injected in the gas chromatography-mass spectrometry (GC-MS) system after a reaction time of 3–4 h at room temperature.

GC-MS analysis. The identities of the hydroxides were confirmed with a Fisons MD800 GC system equipped with an electron impact (EI) ion source operated at 70 eV. A Fisons GC 8000 GC system with a DB-1 fused silica gel capillary column (30 m × 0.32 mm i.d.) was used for sample preparation. The injector temperature was kept at 280 °C and the temperature programmed to increase from 50 °C to 220 °C at a rate of 20 °C/min and then to increase at 5 °C/min up to 285 °C.

Fluorescence measurements. Fluorescence formation (Perkin-Elmer LS 3B) at 393/463 nm and 327/415 nm was studied according to previous experience [10, 11]. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the sample fluorescence at each excitation/emission maximum, and F_{st} is the corresponding fluorescence intensity of a quinine sulphate solution (1 μg/ml in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence shift (dF) was calculated as the ratio of both RF values: $dF = RF_{393/463\ nm}/RF_{327/415\ nm}$, and was analysed in the aqueous (dF_{aq}) and organic (dF_{or}) phases resulting from the lipid extraction of fish tissues [15].

Protein concentration. The protein content was determined according to Bradford [16].

Determination of haem content of extracts. The haem protein concentration was estimated according to Hudzik [17].

PAGE separation. The tissue extracts were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with separating gels (140 × 120 × 1.5 mm) of 10% total acrylamide concentration [10% T (20 acrylamide + bisacrylamide), 2.6%C (20 bisacrylamide)] with 3% stacking gels. Samples were diluted in the sample buffer (glycerol, 10%; SDS, 2%; MCE (mercaptoethanol), 5%, Tris buffer (pH 6.8), 0.5%; bromophenol blue, 0.001%, H_2O, 50%) to be loaded onto the gel. Electrophoresis was performed in a LKB [apparatus (pharmacia)] at 40 mA per slab at 10 °C. The gel was stained with coomassie blue (0.1%) and trichloroacetic acid (10%) in 40% methanol for 30 min. Excess stain was removed with several washes of destaining solvent (40% methanol, 10% acetic acid). A molecular mass standard mixture ranging from 14 kDa to 120 kDa was employed (Sigma).

Results and discussion

Lipoxygenase activity in skin and muscle tissues of herring and sardine

The lipoxygenase activity in skin and muscle tissues was investigated by incubating ARA and DHA with either the skin or the muscle homogenates extracted from herring (first extracts). Figures 1 and 2 show the HPLC

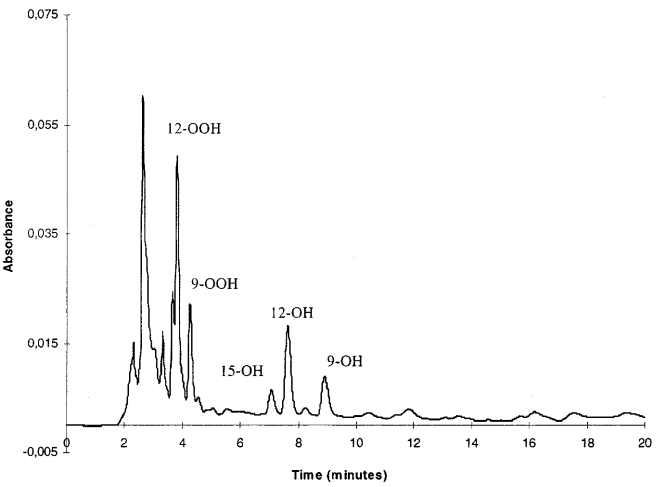

Fig. 1 HPLC separation of products formed after incubation of the enzyme solution extracted from herring skin with arachidonic acid

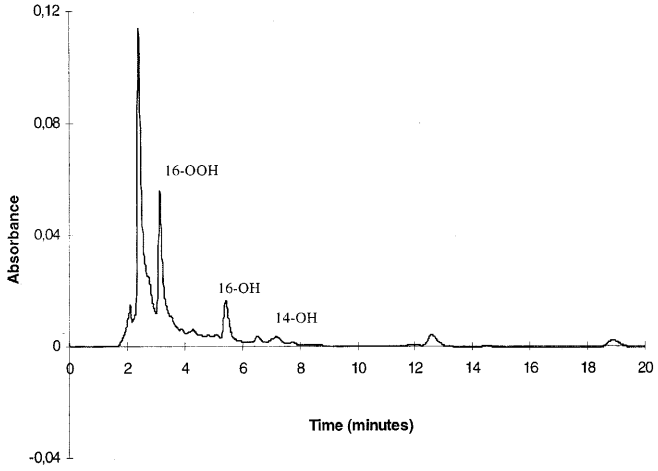

Fig. 2 HPLC separation of products formed after incubation of the enzyme solution extracted from herring skin with docosahexaenoic acid

separations of the oxidation products resulting from incubation with the acids for 5 min with the enzyme solution extracted from skin. As a result of oxidation of ARA, the formation of 9- and 12-hydroperoxyeicosatetraenoic acids (9-OOH and 12-OOH respectively) and 9-, 12- and 15- hydroxyeicosatetraenoic acids (9-OH, 12-OH and 15-OH respectively) was evident (Fig. 1). With DHA oxidation, 16-hydroperoxydocosahexaenoic acid (16-OOH) and the 16- and 14- hydroxydocosahexaenoic acids (16-OH and 14-OH respectively) were detected. These results indicate the presence of different kinds of lipoxygenases active in the skin tissues. The presence of enzymatic activity was demonstrated by the virtually complete inhibition of oxygenation following boiling of the enzyme preparation (Table 1). Furthermore, the presence of a known inhibitor of animal and trout lipoxygenases such as stannous chloride [5, 18] reduced the conversion of DHA into its corresponding degradation products (Table 2).

The hydroxy compounds produced after incubation with ARA showed the presence of a more active 12-lipoxygenase rather than 15- and 9-lipoxygenases in skin tissue. The existence of 15- and 12-lipoxygenases has been described in trout skin tissue and various mammalian cell types [5, 19]. The enzyme was concentrated predominantly in the skin tissue of herring (Table 1). Results obtained after enzymatic oxidation of DHA showed the presence of 16- and 14- lipoxygenases concentrated in the skin tissue as well.

ARA and DHA were also incubated with the first skin homogenate of sardine, resulting in the same pattern of hydroperoxides as indicated for herring, but present in minor quantities. The production of hydroperoxides was also inhibited by boiling the homogenate and after the addition of stannous chloride, confirming enzymatic activity.

Table 1 Oxidation products formed from arachidonic acid incubated with the first muscle and skin tissue homogenates from herring and percentage inhibition of the oxidation by boiling of the enzyme preparation

Compound	Muscle[a]	Inhibition (%)	Skin[a]	Inhibition (%)
12-OOH	1223898	68.2±3.2	4035914	62.5±4.1
9-OOH	276482	41.4±1.5	2341965	45.5±1.6
15-OH	355543	82.2±1.7	1385782	70.5±2.5
12-OH	900922	74.8±2.0	3298430	66.8±2.8
9-OH	243403	54.5±0.8	1879184	48.0±1.7

[a] Data are expressed as peak area (means of two independent samples). The coefficient of variation was always less than 3%
[b] % Inhibition (%) = $[(C-S)]/C] \times 100$ where C = peak area corresponding to no boiling sample and S = peak area of boiling sample. Results are expressed as mean ± standard deviation

Table 2 Oxidation products formed from docosahexaenoic incubated with the first muscle and skin tissue homogenates from herring and percentage inhibition of the oxidation by stannous chloride

Compound	Skin[a]	Inhibition (%)
16-OOH	148634	62.1±1.5
16-OH	43486	50.3±2.8
14-OH	48432	81.1±3.2

[a] Data are expressed as peak area (means of two independent samples). The coefficient of variation was always less than 3%
[b] Inhibition (%) = $[(C-S)]/C] \times 100$ where C = peak area corresponding to no boiling sample and S = peak area of boiling sample. Results are expressed as mean ± standard deviation

Oxidative stability during chilling

The oxidative stability of fish during chilled storage was studied by means of the enzymatic activity of skin homogenates and the fluorescent compounds formed between oxidation products and amino constituents.

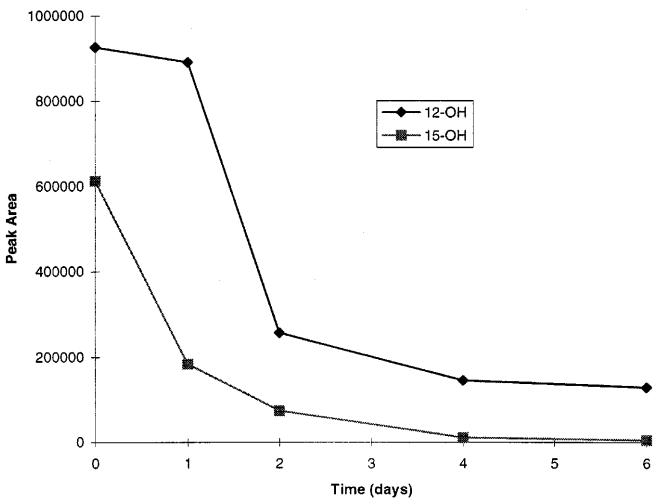

Fig. 3 Enzymatic oxidative activity as a function of the amounts of 15- and 12-hydroxyeicosatetraenoic acid during chilled storage of sardines (6 days at 4 °C)

Figure 3 shows the enzymatic oxidative activity on the basis of 15-OH and 12-OH produced after 6 days of chilled storage of sardines. 12-OH was the predominant product showing a high enzymatic activity due to 12-lipoxygenase during the first 24 h at 0 °C after which it decreased significantly. However, the half-life of the 15-lipoxygenase was less than 24 h and its activity decreased notably after the 1st day of storage. There were no significant differences in the protein concentrations of the skin homegenates during storage. Thus, results obtained in this work indicate differing stabilities of these enzymes which is in agreement with data previously published [7]. Differences between the activities of lipoxygenases have also been described in other fish species [7]. Skin tissues extracted from trout exhibited very high 12- lipoxygenase activity and virtually undetectable 15-lipoxygenase activity, while in those extracted from carp, both 12-lipoxygenase and 15-lipoxygenase were abundant. Other studies carried out with sturgeon showed 15-lipoxygenase as the predominant enzyme.

Fluorescent compounds

The measurement of fluorescent compounds in the aqueous phase of the Bligh and Dyer extraction has proved to be one of the most sensitive methods for evaluating fish freshness and oxidation [10, 11]. Furthermore, this index has shown higher correlation with the duration of chilled storage than other commonly employed parameters such as the total volatile basic nitrogen content for fatty and lean fish species [10, 20]. Figure 4 shows a significant increase in the formation of fluorescent compounds after an induction period of 1 day. The rate of oxidation was higher in skin tissue than in muscle tissue, corresponding to factors such as increased contact with atmospheric oxygen. The significant increase in fluorescent compounds after the first 24 h seems to indicate than the enzymatic activity was not the only source of new hydroperoxides, since the enzyme was less active after the 1st day of storage. Other factors such as the presence of haem groups are also considered to contribute to the development of rancidity during storage [21]. Figure 5 indicates the oxidative activity of the second tissue extract which contained a high concentration in haemoglobin (data not shown). Oxidative activity was high in the initial 2 days of storage, with no significant decrease until the 2nd day, and was high even after lipoxygenase activity had declined after the 1st day (Fig. 3). Results of this work are in agreement with those showing that haemoglobin is a major contributor to lipid peroxidation compared with lipoxygenases during refrigerated and frozen storage of mackerel fillets [21].

However, hydroperoxides resulting from lipoxygenase activity at the outset will activate the process of lipid oxidation. Lipid peroxidation by haem compounds exhibits an induction period which has been postulated to be dependent on preformed hydroperoxides [22]. Although the redox state of haem proteins can influence the catalysis of lipid peroxidation, Baron et al. [23] found that the presence of lipid hydroperoxides was the crucial factor rather the redox state of the protein.

Preliminary identification of lipoxygenase

The electrophoretic analysis of the first skin homogenate showed the existence of a band (approximately 65 kDa) during the initial 24 h of chilled storage (Fig. 6). Previous studies report 12-lipoxygenase in trout skin tissue with an apparent molecular mass of 70 ± 5 kDa, consistent with that found for mammalian lipoxygenases but significantly smaller than the soybean enzyme [7, 24]. After 24 h there was a dramatic drop in intensity of the band which became very diffuse by the end of the storage period. This result is in agreement with the diminution of enzymatic activity found

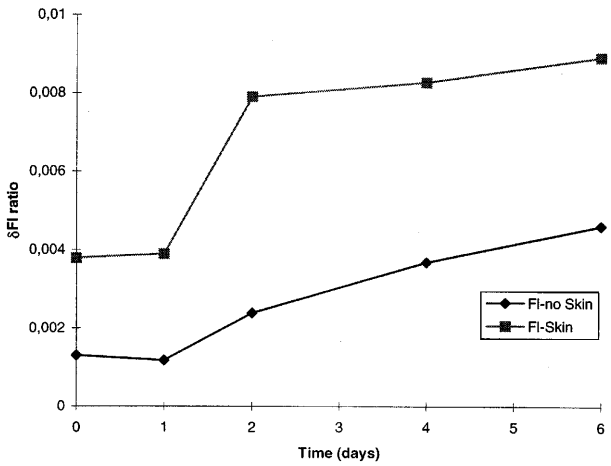

Fig. 4 Fluorescence formation during 6 days chilled storage of sardines (6 days at 4 °C)

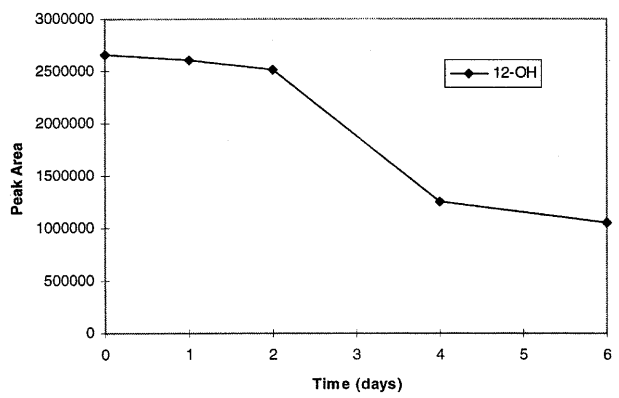

Fig. 5 Pro-oxidative activity of the second extract from sardines during 6 days chilled storage expressed as amount of 12-OH formed after incubation with arachidonic acid

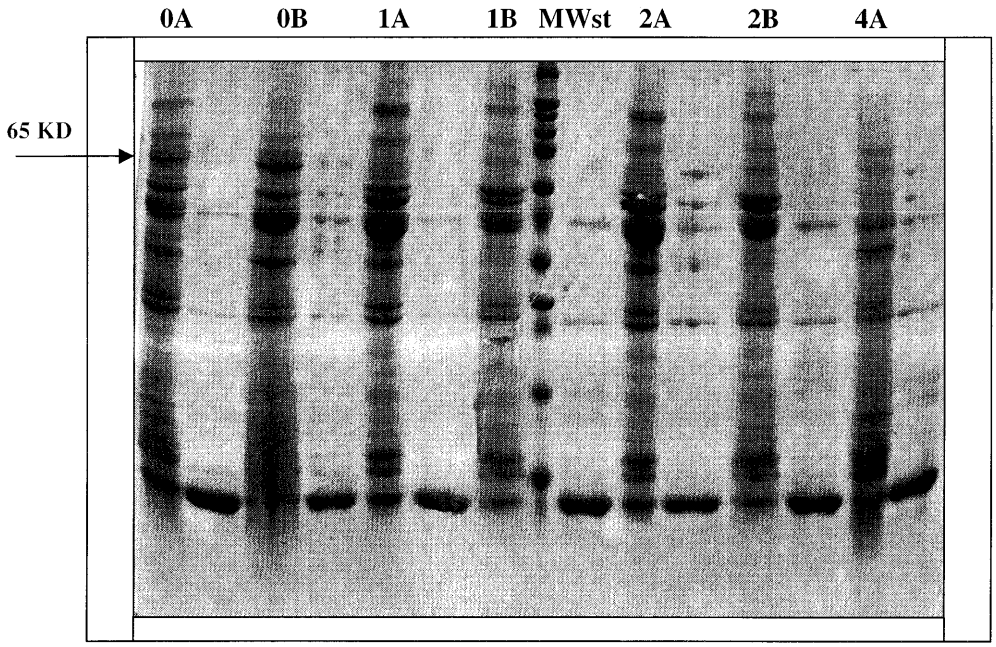

Fig. 6 SDS-PAGE separation of the enzyme solution. Samples are labelled as follows: *0*, *1*, *2*, and *4*, indicating days of storage, and *A* and *B* corresponding to the duplicate samples. *MWst*, Molecular weight standard

throughout the incubation with ARA and DHA. In the second tissue extracts, the major protein observed was an intense band around 14 kDa which was present during the whole storage period and is consistent with the apparent molecular mass of haemoglobins. There were minor amounts of other proteins and no 65 kDa band in the second tissue extract.

This preliminary study confirms the presence of different lipoxygenases having different activities which are located predominantly in the skin tissues of fatty fish species such as herring and sardine. The role of these enzymes on the development of rancidity during refrigerated storage is likely to be influenced by their half-lives, which were less than 24 h at 0 °C. These results also showed an important pro-oxidative activity by haem proteins, which made a major contribution to the production of hydroperoxides during chilled storage following enzymatic activity. A knowledge of mechanisms involved in lipid oxidation is vital to the identification of treatments used to prevent rancidity in fatty fish.

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