Effect of Heat Inactivation of Lipoxygenase on Lipid Oxidation in Lake Herring (*Coregonus artedii*)

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The role of lipoxygenase in causing lipid oxidation in lake herring was studied. Lipid oxidation was measured by assaying for 2-thiobarbituric acid-reactive substances (TBARS), and lipoxygenase activity was measured by a spectrophotometric (470 nm) method. Lipoxygenase activity was highest in light muscle, lowest in skin and intermediate in dark muscle. Light muscle contained the highest percentage of phospholipids (PL) and the least total lipid. The percentage of PL was lowest and total lipids were highest in the skin. Eicosapentaenoic acid and docosahexaenoic acid were found in larger amounts in PL than in triglycerides. Heat treatment rapidly inactivated lipoxygenase. After a 5-min heating period, lipoxygenase was totally inactivated in most cases. TBARS data indicated that samples heated at 80°C for 1.5–2.0 min were more stable than samples heated at 80°C for 2.5–5.0 min, suggesting that heat accelerated nonenzymatic oxidation.

KEY WORDS: **Gas chromatography, lake herring, lipid oxidation, lipoxygenase, 2-thiobarbituric acid-reactive substances.**

Lipid oxidation in fatty fish is one of the most important factors responsible for off-flavor development and quality deterioration of both refrigerated and frozen fish. More than 20% of the total lipids in fatty fish such as herring and mackerel are polyunsaturated C20 and C22 fatty acids (1,2), and these are more susceptible to autoxidation than other fatty acids. Lipid oxidation can be initiated enzymatically or nonenzymatically (3). Once it is initiated, the quality of the fish declines rapidly.

Lipids in fish tissue are distributed unevenly. Ke and Ackman (4) found that mackerel skin contained 50% lipid, whereas muscle contained 12% lipid. The fatty acid compositions of skin and muscle in mackerel were also different. Viswanathan Nair *et al.* (5) reported that skin lipids from oil sardine *(Sardinella longiceps)* had a higher proportion of monoenoic acids and a lower proportion of polyunsaturated acids than muscle lipids. A similar pattern in the skin and muscle lipids of the Atlantic mackerel *(Scomber scombrus)* was noted by Ke *et al.* (6). Although both groups found more rapid oxidation in skin lipids, they concluded that the difference in fat content and the slight variation in fatty acids between skin and meat samples were not adequate to explain the rapid oxidation of skin lipids. Ke and Ackman (4) proposed that one or more lipid-soluble prooxidants, alone or associated with trace metals, were responsible for the high susceptibility to oxidation exhibited by skin lipids. Bosund and Ganrot (7) observed that lipid oxidation in both herring *(Clupea harengus)* and cod *(Gadus morhua)* fillets from the southern Baltic could be reduced by pre-cooking. They suggested that the decrease in lipid oxidation could be due to either enzyme denaturation or to a decrease in the permeability of the muscle tissue to oxygen. Recently, German and Kinsella (8,9), employing capillary gas-chromatography (GC), reported a lipoxygenase in rainbow trout *(Salmo gairdneri)* gill tissue that used unsaturated fatty acids as its substrate. In addition, they proposed that endogenous skin lipoxygenase, released post-mortem, may constitute a significant source of initiating radicals, leading to subsequent lipid oxidation in fish tissue. Josephson *et al.* found a lipoxygenase-like enzyme system in fish and oyster (10–13). Kanner and Kinsella (14) also indicated a myeloperoxidase-hydrogen peroxide-halide system in rainbow trout leukocytes. Other researchers (15–18) found a NADH-dependent oxidase system in fish muscle microsomes or fish tissue.

To minimize adverse changes in stored vegetables due to the activity of enzymes, vegetables are usually blanched before being frozen or stored. The effect of blanching on lipoxygenase activity in garden peas was investigated by Rhee and Watts (19). They found that lipoxygenase was rapidly inactivated by a short blanching time, and no regeneration of the enzyme occurred during frozen storage. Hsieh *et al.* (20) showed that mild heat treatment may control lipoxygenase-initiated lipid oxidation and retard quality deterioration of fish. However, Ke *et al.* (21) found microwave heating had a prooxidant effect on lipids in Atlantic mackerel. The objectives of this research were to compare lipid oxidation due to lipoxygenase with that caused by nonenzymatic lipid oxidation in lake herring fillets, and to explore the possible efficacy of heat inactivation of lipoxygenase as a means of reducing rancidity.

MATERIALS AND METHODS

Preparation and analysis of samples. Lake herring or cisco *(Coregonus artedii)*, each weighing approximately 500 g, were obtained from either Kemp's Fisheries (Duluth, MN) or Bodine's Fisheries (Bayfield, WI). Fish were iced immediately upon capture and transported to the laboratory within 24 hr. They were filleted, rinsed in cold water, and dissected as indicated for each treatment. The heads, tails and belly flaps were discarded. A model 2250 food grinder (Rival Manufacturing Co., Kansas City, MO) was used to comminute tissue for analysis in all experiments.

Chemical analyses of skin, dark muscle and light muscle. Skin, dark muscle and light muscle were separated and each sample was comminuted in a food grinder. Moisture and protein were determined by AOAC methods (22). Lipids were extracted by the method of Bligh and Dyer (23). Lipid oxidation products were measured by quantitating the 2-thiobarbituric acid-reactive substances (TBARS) as described by Rethwill *et al.* (24).

Separation of phospholipids and triglycerides. Whole lipid extracts were separated into triglycerides and phospholipids (PL) by solvent partition (25). Fatty acid methyl esters (FAME) were prepared by the method of Einig and Ackman (26). The FAME were separated with

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a capillary GC (Hewlett-Packard 5890 A; Hewlett-Packard, Avondale, PA) equipped with a Supelcowax 10 column (30 m × 0.32 mm i.d.; Supelco, Inc., Bellefonte, PA) and were quantitated by using a flame ionization detector. Chromatographic conditions were as follows: injection port temperature, 200°C; detector temperature, 300°C; initial oven temperature, 125°C for 4 min, rising to 260°C at 6°C/min with a final hold time of 4.5 min. The carrier gas was hydrogen at a flow rate of 1 mL/min. Retention times and peak areas were calculated on a computing integrator (Hewlett-Packard 3393A). Compounds were tentatively identified by comparison with the retention times of known standards. Data from the foregoing experiments are summarized in Tables 1-3.

Lipoxygenase assay. Lipoxygenase was extracted by the method of German *et al.* (27) with the following modifications: samples were homogenized at speed 7 for 30 sec in a Polytron homogenizer (pcu-2 Kinematica 6 GmbH, Lucerne, Switzerland) in 8 volumes of cold 0.05M phosphate buffer, pH 7.8, containing 1 mM reduced glutathione. The crude supernatant was assayed for lipoxygenase activity by the method of Williams *et al.* (28) by monitoring the change in absorbance at 470 nm and 22°C.

Thiobarbituric acid-reactive substances. TBARS were quantitated by a modification of Rethwill *et al.* (24).

Heat inactivation of lipoxygenase and TBARS values. In Experiments 1A and 1B, lake herring from two different batches of fish (A and B) were filleted, and each fillet was divided transversely into three sections. The mean weight of the sections was 100 g. Each group of six random sections was stored in a nylon/polyethylene bag composed of 0.75 mil nylon, 2.25 mil polyethylene and 0.2 mil adhesive (Kutter Co., Avon, MA). The control group (unheated) was stored at 4°C until analysis. The remaining groups were heated in an 80°C water bath (Model 730, Fisher Scientific, Pittsburgh, PA) for time intervals of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 4.0 and 5.0 min. Lipoxygenase activity was determined immediately after heat treatment.

In Experiment 2, the same procedure was used except that heat-treatment samples were stored on trays with polyvinyl chloride (PVC) film at 4°C until analysis. Samples were prepared and analyzed for lipoxygenase activity after 0, 1, 2 and 3 days of storage. All 0-day analyses were done immediately after the heat treatment.

Effect of heat on lipid oxidation. In Experiments 3A and 3B, the fish samples were prepared and stored as in the preceding experiment. TBARS were determined after 0, 1, 2 and 3 days of storage. The fish from Experiment 2 were also used in 3A; 3B represented a separate batch of herring.

In Experiment 4, the control samples (unheated) were stored on trays covered with PVC film at 4°C until analysis. The remaining samples were wrapped in nylon/polyethylene bags and were heated in a water bath at either 50°C, 60°C, 70°C, 80°C or 90°C for 10 min, and TBARS were measured after 0, 1, 2 and 3 days of storage. All 0-day analyses were done immediately after the heat treatment (as can be seen in Table 4).

In Experiment 5, fish samples were prepared and stored as in the preceding experiment. The control samples were stored at 4°C until analysis. The remaining samples, wrapped in nylon/polyethylene bags, were heated in a water bath at 60°C or 80°C for either 10 min or 1 hr. TBARS were determined after 0, 1, 2, 3 and 4 days of storage on trays covered with PVC film. All 0-day analyses were done immediately after the heat treatment.

Statistical analysis. The data were analyzed by analysis of variance (ANOVA), and least significant differences were calculated by using the statistical analysis system (29).

RESULTS AND DISCUSSION

Comparison of skin, dark muscle and light muscle. The proximate compositions, TBARS and lipoxygenase activity of lake herring skin, dark muscle and light muscle are summarized in Table 1. The subcutaneous fat layer was included in the skin lipid. Other researchers (4-6) have found that fatty fish, such as herring, store reserve lipid in the muscle tissues. Mackerel skin contains 200% more lipid than the dark muscle (4); however, in the lake herring the amount of lipid in the skin and the dark muscle was approximately the same. The skin had the lowest water content of the three components and approximately the same protein content as the light muscle. The percentage of triglycerides (TG) decreased in the following order: skin > dark muscle > light muscle. However, TG were always the major components of the lipid fraction. Even though the light muscle contained the largest percentage

TABLE 1

Proximate Composition, TBARS and Lipoxygenase (LOX) Activity of Skin, Dark Muscle and Light Muscle of Lake Herring

Composition	Skin	Dark muscle	Light muscle
Distribution (%)[a]	11.23 ± 2.08[b]	9.17 ± 1.89	79.60 ± 3.82
Protein (%)	23.81 ± 2.13	15.18 ± 0.29	21.00 ± 0.71
Water (%)	40.52 ± 2.42	56.10 ± 0.93	74.90 ± 0.08
Lipid (%)	17.97 ± 2.75	16.02 ± 1.89	1.33 ± 0.34
Triglycerides (%)	16.33[c]	13.03	0.92
Phospholipids (%)	0.49	1.26	0.33
TBARS (ppm)[d]	1.77 ± 0.16	1.26 ± 0.13	0.84 ± 0.06
LOX activity (ΔA_{470})[d,e]	0.10 ± 0.04	0.47 ± 0.06	0.66 ± 0.07

[a]Weight as percentage of soft tissue.
[b]Mean ± S.D. of three composite samples.
[c]One determination.
[d]Measured from fresh samples.
[e]Change in absorbance at 470 nm.

of PL, the total amount of lipid in the light muscle was so low that it contained the lowest amount of PL.

The fatty acid compositions of total lipids in skin, dark muscle and light muscle are presented in Table 2. All three components contained approximately the same percentage of total saturated and unsaturated fatty acids. The skin contained slightly more monoenes than did the light or dark muscle. Ke *et al.* (30) showed that the monoenes also could be oxidized fairly rapidly. The TG fraction of the skin contained more monoenes than did the PL (Table 3). Generally, the TG fraction of all three components contained slightly more polyenoic fatty acids

TABLE 2

Fatty Acid Compositions^{a-c} of Skin, Dark Muscle and Light Muscle Lipids of Lake Herring^{d}

Fatty acid	Skin	Dark muscle	Light muscle
14:0	5.6 ± 1.0^{a}	5.0 ± 0.6^{a}	4.4 ± 0.2^{a}
16:0	17.7 ± 2.3^{a}	16.8 ± 1.3^{a}	16.6 ± 0.7^{a}
18:0	2.6 ± 0.2^{a,b}	2.4 ± 0.1^{b}	2.7 ± 0.1^{a}
Total saturated	25.9 ± 3.4^{a}	24.2 ± 1.9^{a}	23.7 ± 1.0^{a}
16:1	5.5 ± 0.7^{a}	5.0 ± 0.4^{a}	4.6 ± 0.2^{a}
18:1	20.9 ± 1.6^{a}	18.6 ± 0.6^{b}	18.6 ± 0.8^{b}
20:1	1.4 ± 0.1^{a}	1.1 ± 0.0^{a}	1.1 ± 0.2^{a}
Total monoenoic	27.8 ± 2.5^{a}	24.8 ± 1.1^{a}	24.3 ± 1.2^{a}
18:2	11.6 ± 0.8^{a}	10.9 ± 0.3^{a}	10.5 ± 0.4^{a}
18:3	12.7 ± 0.9^{a}	12.2 ± 0.3^{a}	11.9 ± 0.6^{a}
18:4	3.5 ± 0.2^{a}	3.4 ± 0.1^{b}	3.4 ± 0.2^{a,b}
20:2	1.4 ± 0.0^{a}	1.4 ± 0.0^{b}	1.3 ± 0.2^{b}
20:3	2.1 ± 0.2^{a}	1.9 ± 0.0^{c}	2.0 ± 0.2^{b}
20:4	5.9 ± 0.3^{a}	5.8 ± 0.2^{a}	6.1 ± 0.7^{a}
20:5	4.7 ± 0.2^{b}	4.9 ± 0.1^{a,b}	5.5 ± 0.7^{a}
22:5	2.4 ± 0.1^{b}	2.7 ± 0.2^{a}	2.5 ± 0.3^{a,b}
22:6	4.2 ± 0.1^{c}	5.2 ± 0.1^{b}	5.8 ± 0.6^{a}
Total polyenoic	48.4 ± 2.9^{a}	48.3 ± 1.3^{a}	49.0 ± 3.9^{a}

$^{a-c}$Values in the same line with different superscript letters are significantly different ($p<0.05$).

^{d}Mean ± S.D. of three determinations; values were calculated as wt% of FAME from the total lipids.

TABLE 3

Fatty Acid Compositions^{a-c} of Triglycerides (TG) and Phospholipids (PL) of Skin, Dark Muscle and Light Muscle of Lake Herring^{d}

	TG			PL		
Fatty acid	Skin	Dark muscle	Light muscle	Skin	Dark muscle	Light muscle
14:0	5.1^{a}	4.0^{b}	5.1^{a}	2.8^{a}	2.8^{a}	2.0^{a}
16:0	16.1^{a}	14.4^{a}	16.0^{a}	19.7^{a}	29.5^{a}	30.8^{a}
18:0	2.0^{c}	2.5^{b}	2.6^{a}	3.0^{a}	2.4^{b}	3.3^{a}
Total saturated	23.2^{a}	20.9^{a}	23.7^{a}	25.5^{a}	34.8^{a}	36.1^{a}
16:1	4.6^{b}	4.8^{a,b}	5.1^{a}	2.4^{a}	1.7^{b}	5.1^{c}
18:1	16.3^{c}	22.1^{a}	20.2^{b}	13.2^{a}	9.9^{b}	6.9^{c}
20:1	1.1^{c}	1.4^{a}	1.1^{b}	0.7	—^{e}	—^{e}
Total monoenoic	22.0^{b}	28.3^{a}	26.4^{a}	16.3^{a}	11.6^{b}	7.5^{c}
18:2	11.5^{a}	11.2^{a}	11.4^{a}	7.0^{a}	4.1^{b}	4.0^{b}
18:3	12.5^{a}	12.8^{a}	13.1^{a}	7.1^{a}	5.4^{b}	5.3^{b}
18:4	3.3^{b}	3.2^{c}	4.0^{a}	1.5^{a}	0.6^{b}	—^{e}
20:2	2.0^{a}	1.5^{b}	1.1^{c}	1.2^{a}	0.5^{b}	—^{e}
20:3	3.1^{a}	2.0^{c}	2.1^{b}	2.0^{a}	0.7^{b}	—^{e}
20:4	6.2^{b}	6.1^{b}	6.6^{a}	8.8^{a}	6.1^{c}	6.8^{b}
20:5	4.3^{c}	5.0^{b}	5.7^{a}	7.3^{b}	6.9^{b}	10.2^{a}
22:5	3.0^{a}	2.8^{b}	2.9^{b}	3.0^{a}	2.4^{b}	2.1^{c}
22:6	4.9^{b}	4.3^{c}	5.2^{a}	15.5^{b}	19.4^{a}	15.4^{b}
Total polyenoic	50.7^{a}	48.9^{a}	52.0^{a}	53.3^{a}	46.0^{a}	43.8^{a}

$^{a-c}$Values in the same line of TG and PL columns with different superscript letters are significantly different ($p<0.05$).

^{d}Mean value of three determinations; values were calculated as wt% of FAME from the total lipids.

^{e}Not detectable.

than did the PL. However, the skin PL contained more polyenoic fatty acids than did the TG. All three components contained more C20:5 and C22:6 in the PL than in the TG. The relatively high values of C20:5 and C22:6 in Lake Superior lake herring is in agreement with our previous research (31). Other studies showed that lipid oxidation occurred more rapidly in the skin than in the light muscle (5,6). However, results from the current study showed lipoxygenase had the highest activity in the light muscle (Table 1). Trace metals could also play an important role in the catalytic oxidation of skin (4).

Effect of heat on lipoxygenase and TBARS. Figure 1 shows the inactivation of lipoxygenase by heat in Experiments 1A and 1B. After 80°C heat treatment for 5 min, the enzyme was fully inactivated in most cases. In both experiments the lipoxygenase activity was reduced at least 50% after heating at 80°C for 1.5 min. However, after heating for 0.5 to 1.5 min, enzyme activity increased slightly. A similar increase in lipoxygenase activity in soybean was found by Pour-El *et al.* (32) and Wang and Toledo (33). Pour-El *et al.* (32) suggested that this might result from increased enzyme extractability during the assay rather than from an increase in activity.

Lipoxygenase activity was higher after storage at 4°C for 0, 1, 2 or 3 days than immediately after heating (Fig. 2). It is possible that the enzyme regenerated after the initial heat shock. However, after a 5-min heat treatment, no evidence of a "regeneration" was observed. Figures 3A and 3B show the lipid oxidation in lake herring after heat treatment and storage at 4°C for 0, 1, 2 or 3 days in two experiments. The differences in TBARS between the two experiments may be due to a difference in fillet thickness between the two lots of fish. Lipid oxidation was lowest on day 0 and increased during storage. However, heating for 1.5–2.0 min appeared to decrease lipid oxidation on all stored samples. Though heat can disrupt membranes and/or subcellular structures, thus liberating the enzymes and their substrates (12), heat treatment could also be considered a blanching treatment, similar to that given to vegetables before freezing. TBARS measures both enzymatic and nonenzymatic oxidation. While heat may reduce enzymatic oxidation, it also may accelerate nonenzymatic oxidation. Since lipoxygenase activity also decreased during this time, the initial decrease in oxidation may be due to inactivation of lipoxygenase. TBARS were higher in stored samples than in the control. A short heat treatment of 1.5 min in Experiment 3A (Fig. 3A) and 2.5 min in experiment 3B (Fig. 3B) minimized the increase during storage.

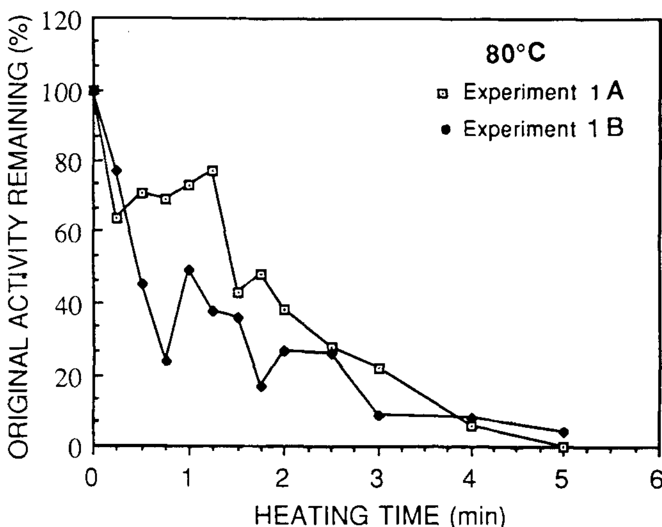


FIG. 1. Inactivation rate of lipoxygenase in lake herring fillets at 80°C in two experiments (Experiment 1A and 1B).

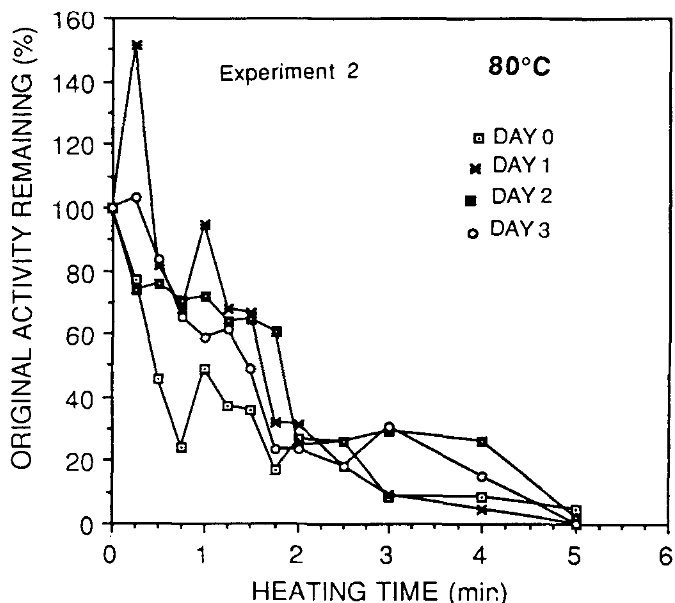
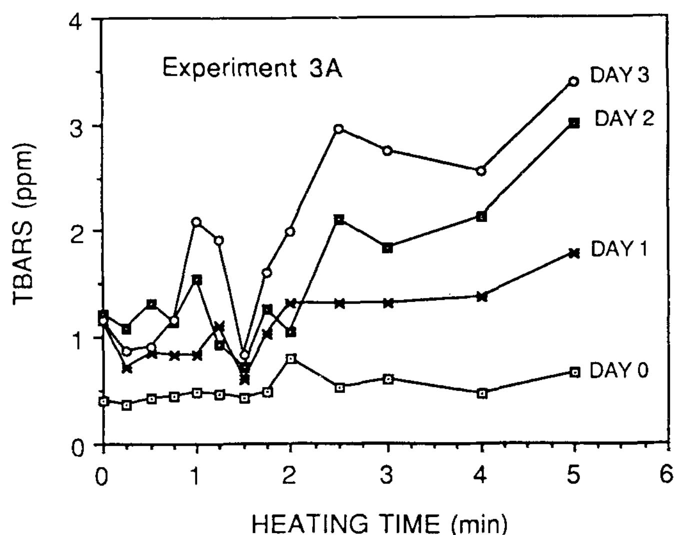


FIG. 2. Lipoxygenase activity in lake herring fillets heated for different lengths of time at 80°C and then stored at 4°C. Day 0 data were measured immediately after heat treatment: □, day 0; *, day 1; ■, day 2; and ○, day 3 (Experiment 2).

After 1.5 min in Experiment 3A (Fig. 3A) and 2.5 min in Experiment 3B (Fig. 3B), the TBARS of stored samples increased. However, the results of Experiments 1A, 1B and 2 showed that the lipoxygenase activity decreased during this time. Oxidation at this point may have been accelerated by heat (18); therefore, the increase in nonenzymatic oxidation exceeded the decrease in enzymatic oxidation and total oxidation increased.

In Experiment 4, lake herring heated for 10 min at 60°C and stored at 4°C exhibited significantly lower ($p<0.05$) TBARS values than samples heated at any other temperatures for 10 min (Table 4). TBARS for samples heated at 50°C were generally significantly lower ($p<0.05$) than those heated at 70°C or 90°C. TBARS for samples heated at 80°C and the control samples were significantly higher ($p<0.05$) than the TBARS for samples from other treatments.

Figure 4 shows TBARS in lake herring that were heated at 60°C or 80°C temperatures for either 10 min or 1 hr and stored at 4°C. Samples heated at 60°C for 10 min had TBARS significantly lower ($p<0.05$) than the TBARS for samples from other treatments. The TBARS of the samples that were heated at 60°C for 1 hr were generally significantly higher ($p<0.05$) than the TBARS for samples from other treatments. It was also interesting to note that TBARS for samples heated at 80°C for either 10 min or



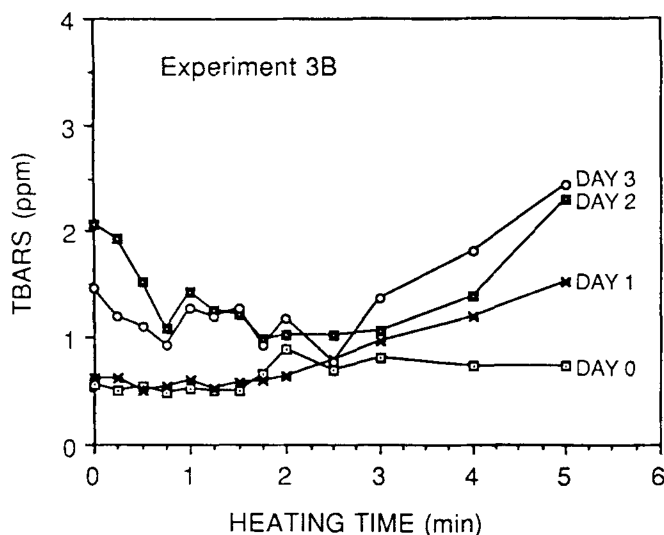


FIG. 3. TBA-reactive substances (TBARS) in lake herring fillets heated at 80°C for different lengths of time and then stored at 4°C for three days in two experiments. Day 0 data were measured immediately after heat treatment (Experiment 3).

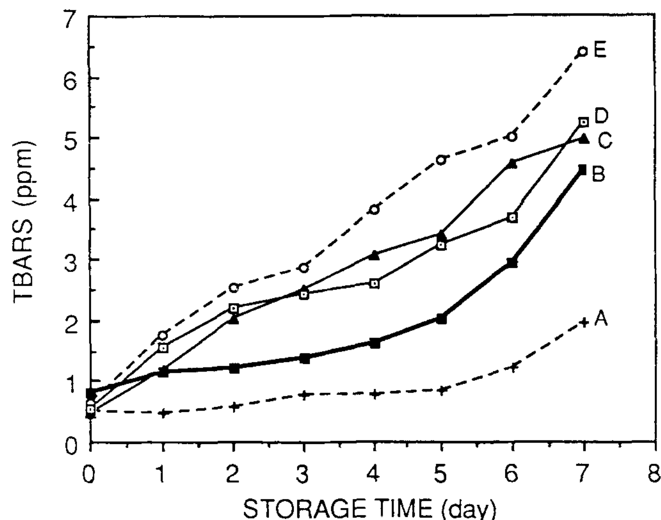


FIG. 4. TBA-reactive substances (TBARS) in lake herring fillets heated at 60°C for 10 min or 1 hr, or 80°C for 10 min or 1 hr and then stored at 4°C: (A) 60°C, 10 min; (B) raw; (C) 80°C, 10 min; (D) 80°C, 1 hr; (E) 60°C, 1 hr (Experiment 5).

TABLE 4

TBARS^{a-e} in Lake Herring Fillets^{f} Heated for 10 min and Stored at 4°C for Three Days

Treatment	Days 0	1	2	3
Control	0.67^b	1.90^a	2.85^a	3.47^a
50°C	0.50^c	0.72^c	1.26^d	$1.90^{b,c}$
60°C	0.42^d	0.57^c	0.63^e	0.84^d
70°C	0.54^c	1.56^b	$2.02^{b,c}$	1.70^c
80°C	0.57^c	1.85^a	$2.42^{a,b}$	3.77^a
90°C	0.86^a	1.54^b	1.91^c	2.34^b

$^{a-e}$Values in the same column with different superscript letters are significantly different ($p<0.05$).

^{f}Fillets were prepared as described in Materials and Methods.

1 hr were generally not significantly different ($p<0.05$) from each other.

It has been demonstrated that NADH is required in catalyzing lipid oxidation in fish systems (15–18). The NADH-requirement is evidence that lipid oxidation is enzymatic in nature. Recently, myeloperoxide-like and lipoxygenase-like enzyme systems were found in fish (8–14). Slabyj and Hultin (15) showed that lipid oxidation was reduced in light- and dark-muscle microsomes of herring *(Clupea harengus)* by using a heat treatment. Josephson *et al.* (34) also reported that oxygenase activity inhibitors and heat inhibited the lipid oxidation in both emerald shiners *(Notropis antheroides)* and rainbow trout *(Salmo gairdneri)*. In the current study a heat treatment that can reduce lipid oxidation and a lipoxygenase-like system were discovered in lake herring. These results are similar to the reports of German and Kinsella (8,9) and Slabyj and Hultin (15).

The present study indicated that a surface blanching treatment decreased the rate of oxidation in lake herring. The skin and dark muscle, which contain the largest amount of fatty acids substrate, are located closest to the surface. It is possible that inactivation of lipoxygenase in the region that contained the largest amount of substrate contributed to this decrease in lipid oxidation. Heat also may have accelerated nonenzymatic lipid oxidation in lake herring.

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