Antioxidant Activity of Water-Soluble Fractions of Salmon Spermary Tissue

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The antioxidant potential of chum salmon spermary tissue was evaluated using a sardine triacylglycerol emulsion system. Intact and high-molecular weight water-soluble fractions of spermary tissue were found to accelerate both autoxidation and iron/ascorbate-catalyzed oxidation. The low-molecular weight (LMW) fraction inhibited autoxidation and oxidation catalyzed by iron/ascorbate and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). Inhibition of iron/ascorbate-catalyzed oxidation by the LMW fraction decreased with decreasing pH until no activity was observed at pH ≤6.4. Activity of the LMW fraction was not strongly influenced by pH (5.0−7.0) in the presence of AAPH. Antioxidants in the LMW fraction, including spermine, putrescine, hypoxanthine, xanthine, and glutathione, both alone and in combination, exhibited less antioxidant activity than the LMW fraction, indicating that other unidentified antioxidants were present.

Keywords: *Antioxidants; lipid oxidation; polyamines; fish oil; seafood*

INTRODUCTION

Lipid oxidation reactions are detrimental to foods since they cause the production of off-flavors, alterations in color and texture, and loss of nutritional quality. Since oxidative reactions are common, numerous endogenous antioxidant defense systems are found in foods and biological tissues. These systems include lipid-soluble antioxidants (e.g. tocopherols, ubiquinone, and carotenoids), antioxidant enzymes (e.g. superoxide dismutase, glutathione peroxidase, and catalase), iron binding proteins (e.g. ferritin and transferritin), and low-molecular weight water-soluble compounds (e.g. polyamines, histidine-containing dipeptides, ascorbic acid, nucleotides, and glutathione) (Matsushita et al., 1963; Kanner et al., 1987; Lovaas, 1991; Chan and Decker, 1994). The endogenous antioxidants found in foods and biological tissues have excellent potential for use as "natural" food additives. However, use of these antioxidants is sometimes impractical due to availability, cost restrictions, physical characteristics (e.g. color and solubility), and susceptibility to inactivation.

Oxidative deterioration of foods has been postulated to be accelerated by activated heme proteins, transition metal-dependent generation of reactive oxygen species, enzymes, singlet oxygen-generating systems, and the thermal degradation of lipid peroxides (Nawar, 1985; Kanner et al., 1987). Lipid-soluble antioxidants are commonly used in many foods, but their effectiveness can be limited in low-fat products and in processed muscle foods where they are difficult to incorporate into cellular membrane lipids. In these cases, water-soluble antioxidants can be more effective than lipid-soluble antioxidants (Decker and Crum, 1991) since they are easy to incorporate into the aqueous portion of foods where they can neutralize water-soluble oxidation catalyst and reactive oxygen species. An additional advantage of water-soluble antioxidants is that they can effectively protect against the oxidation of other water-soluble compounds (e.g. myoglobin; Decker and Crum, 1991). While water-soluble antioxidants have excellent potential as food additives, the high water content of many foods results in excessive dilution, thereby making them effective only at high concentrations.

If natural water-soluble antioxidants are to be used in foods, compounds with high activity must be identified. In addition, economical sources of these compounds must be available. Salmon spermary represents an underutilized byproduct of the salmon industry. Sperm cells exhibit high levels of oxidative metabolism which in turn increases the risk of oxidative damage. Spermary tissue contains a multicomponent antioxidant defense system which consists of α-tocopherol, ubiquinones, antioxidant enzymes, and the polyamines, spermine and spermidine (Lucesoli and Fraga, 1995). Lovaas (1991) reported that spermine inhibits the oxidation of fish oils more effectively than α-tocopherol, butylated hydroxyanisole, *tert*-butylhydroquinone, and ascorbyl palmitate. The antioxidant activity of the polyamines increases with increasing amine content, and therefore, spermine > spermidine > putrescine. The antioxidant mechanism of spermine has been postulated to be due to both iron chelation and free radical scavenging (Droplet et al., 1986; Tadolini, 1988; Lovaas, 1991).

Since fish spermary tissue contains high amounts of polyamines, it represents a potential source of natural water-soluble antioxidants. The objective of this research was to evaluate the antioxidant properties of different water-soluble fractions of salmon spermary tissue using an emulsified sardine triacylglycerol system. The role of spermine and other low-molecular weight water-soluble antioxidants in the antioxidant activity of the salmon spermary fractions was also investigated.

MATERIALS AND METHODS

Materials. Chum salmon spermary tissue was obtained from a local fish-processing facility, vacuum packaged, and stored at −20 °C. Refined sardine oil was obtained from

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Nippon Chemical Feeds Co. Ltd. (Hakodate, Japan). Ascorbate, spermine, spermidine, putrescine, hypoxanthine, xanthine, adenosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine 5′-monophosphate, uric acid, *o*-phthalaldehyde, phenyl isothiocyanate, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), ethylenediaminetetraacetric acid (EDTA), silicic acid, Celite, and thiobarbituric acid (TBA) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Tween 20 was from Kanto Chemical Co. (Tokyo, Japan). All other chemicals were reagent grade or purer.

Methods. *Isolation of Spermary Fractions.* Frozen salmon spermary tissue was chopped coarsely with a knife and homogenized with 3 parts of 30 mM, pH 7.4 phosphate buffer in a Nissei Excel Auto Homogenizer (Tokyo, Japan) at 8500 rpm for 3 min. The homogenate was centrifuged in a Hitachi CR20B2 refrigerated centrifuge (Tokyo, Japan) at 10000*g* for 15 min at 4 °C. The resulting supernatant (water-soluble fraction) was filtered through four layers of cheesecloth and left intact or fractionated into high- and low-molecular weight components. The low-molecular weight (LMW) fraction was isolated by ultrafiltration in an Advantec (Tokyo, Japan) UHP-90K stirred cell using Advantec UK-10, 10 000 molecular weight cutoff (MWCO) membranes. The high-molecular weight (HMW) fraction was isolated using Viskase 12-14000 MWCO dialysis tubing. One part of the water-soluble fraction was dialyzed against 1000 parts of 50 mM, pH 7.4 phosphate buffer, a total of three times, after 4, 6, and 14 h of constant stirring at 4 °C. All salmon spermary fractions were used within 8 h, except for experiments involving dialysis where fractions were used within 32 h.

Isolation of Sardine Oil Triacylglycerols (TAGs). The TAGs of sardine oil (6 g) were separated from free fatty acids, carotenoids, cholesterol, and α-tocopherol by column (25 mm × 450 mm) chromatography using silicic acid/Celite (4:1 w/w, 50 g) (Takagi et al., 1979). After application of the sardine oil onto the silicic acid/Celite, the column was washed with 250 mL of hexane. TAGs were then eluted with 500 mL of hexane/diethyl ether (19:1 v/v). Isolated TAG produced only one spot upon Whatman K5 silica gel thin layer chromatography using hexane/diethyl ether/acetic acid (80:30:1 v/v) as the mobile phase.

Oxidation of Sardine TAG Emulsions. Emulsions were prepared using purified sardine triacylglycerols (0.25 g), Tween 20 (2.5 g), and 50 mM, pH 7.4 phosphate buffer (100 mL) which were sonicated on ice using a Tomy UD-200 Ultrasonic Disrupter (Tokyo, Japan) at a setting of 80 W for 3 min. Oxidation studies were performed in a 10 mL model system which contained 2.5 mg of emulsified sardine triacylglycerol per milliliter, the salmon spermary fractions (0−25%), or purified antioxidants, lipid oxidation catalysts, and 50 mM phosphate buffer. Control samples contained all components except for the salmon spermary fractions or purified antioxidants. The pH of the system was 7.4 except in pH studies where the pH ranged from 4.0 to 8.0. Autoxidation of the emulsion was performed at 37 °C in the dark. Accelerated lipid oxidation reactions were catalyzed using 15 μM ferric chloride and 100 μM ascorbate (iron/ascorbate) (Decker and Faraji, 1990) or 2.0 mM AAPH (Niki, 1990). Lipid oxidation was also catalyzed using AMVN where AMVN was dissolved in 2.0 mL of chloroform along with 0.25 g of sardine TAG. Chloroform was then evaporated using nitrogen; 2.5 g of Tween 20 and 100 mL of cold (4 °C) phosphate buffer were added, and the emulsion was sonicated as described above. All catalyzed oxidations were performed at 37 °C in a shaking water bath for 90 min except for pH experiments which were carried out for 120 min due to slower lipid oxidation rates at low pH. Lipid oxidation was measured by the thiobarbituric acid (TBA) method described by McDonald and Hultin (1987) using 1.0 mL of emulsion and the thiocyanate lipid peroxide method described by Inatani et al. (1983) using 0.25 mL of emulsion.

Analysis of the LMW Fraction. The LMW fraction for further analysis was prepared as described above except that distilled water was used in place of phosphate buffer. The solid content of 10 mL of the LMW fraction was determined by freeze-drying at 23 °C in a Labconco Lyph-Lock 6 Freeze Drier (Kansas City, MO) for 24 h. Ash content of 50 mL of the LMW fraction was determined by drying at 90 °C for 5 h followed by 110 °C for 10 h and ashing at 550 °C for 24 h.

Glutathione concentrations in the LMW fraction containing 5% trichloroacetic acid and 20 mM EDTA were determined enzymically as described by Tietze (1969). Polyamines and nucleotides in the LMW fraction were quantitated using a Hitachi liquid chromatograph (Tokyo, Japan) consisting of a Model L-6200 intelligent pump, a Model 7125 manual injector, Model 5020 column heater (35 °C), a Model L-4250 UV-vis detector, and Model D-2000 integrator, and a 4.6 × 250 mm ODS-2 column (GL Science, Tokyo, Japan). Concentrations of phenylthiocarbamyl (PTIC) derivatives (Kuwano et al., 1987) of spermine, spermidine, and putrescine were determined by reverse phase HPLC using a modified method of Yen and Hsieh (1991). Separation of the polyamines was accomplished using a linear mobile phase gradient consisting of solvent A (methanol/water, 55:45 v/v) and solvent B (methanol/water, 88:22 v/v). The mobile phase (1.2 mL/min) initially consisted of 100% solvent A with the concentrations of solvent B increasing linearly for 6 min until the mobile phase was 100% solvent B. The flow rate was increased to 1.3 mL/min for 9 min and then decreased to 1.1 mL/min for the final 10 min. Nucleotide concentrations in the LMW fraction were determined using the method of Tsuchimoto et al. (1985) using a 50 mM, pH 6.0 phosphate buffer isocratic mobile phase at 1.0 mL/min. Nucleotides were identified at 254 nm by comparing the retention times of authentic nucleotide samples. Concentrations of both polyamines and nucleotides were determined using peak areas and appropriate standard curves.

Carnosine and anserine concentrations in the LMW fraction were determined by separation of *o*-phthalaldehyde derivatives (Chan et al., 1994) using a Tosoh (Tokyo, Japan) HPLC system consisting of a CCPM pump, a CO 8011 manual injector and column oven (35 °C), an FS 8010 fluorescent detector (emission at 310 nm, excitation at 375 nm), SC 8010 integrator, and a 4.6 × 250 mm ODS-2 column (GL Science, Tokyo, Japan). The presence of carnosine and anserine were determined by the comparison of retention times using a Type B Amino Acid Standard Solution kit (Wako Pure Chemicals, Tokyo, Japan). The LMW fractions for all HPLC analyses were prepared by addition of 5% trichloroacetic acid followed by adjustment to pH 7.0 and centrifugation at 10000*g* for 10 min. The LMW fraction was passed through 0.45 μm filters prior to injection.

All lipid oxidation experiments were conducted on two separate trials consisting of triplicate samples. Compositional analysis of the LMW fraction was performed on triplicate samples.

RESULTS

Compared to that of the control (no additive) samples, autooxidation of the sardine TAG emulsion was initially (1 day) accelerated by 10% intact water-soluble and HMW fractions of salmon spermary as measured by lipid peroxides (Figure 1A) and thiobarbituric acid reactive substances (TBARS, Figure 1B). After 2 days of autoxidation, the intact and HMW fractions had lower peroxide concentrations than and TBARS concentrations similar to those of the controls. The LMW fraction (10%) of the salmon spermary inhibited the formation of both lipid peroxides (Figure 1A) and TBARS (Figure 1B). Intact and HMF fractions (10%) were also observed to increase iron-catalyzed oxidation of sardine oil emulsions as measured by TBARS (Figure 2). The LMW fraction (10%) inhibited iron-catalyzed lipid oxidation with TBARS ranging from 54 to 80% lower than the control. Since the LMW fraction exhibited only antioxidative activity in both the autooxidation and iron/ascorbate systems, subsequent experiments focused on its antioxidative properties.

The LMW fraction at concentrations ranging from 0.05 to 10% of the model system volume inhibited

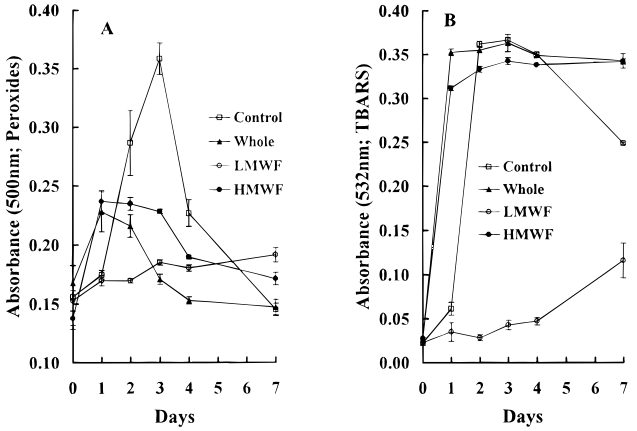

Figure 1. Inhibition of the autoxidation of a sardine triacylglycerol emulsion (pH 7.4, 37 °C) by 10% water-soluble (whole), low-molecular weight (LMWF), or high-molecular weight (HMWF) fractions of salmon spermary tissue. Lipid oxidation was determined by lipid peroxides (A) and thiobarbituric acid reactive substances (B, TBARS). Error bars represent standard deviations.

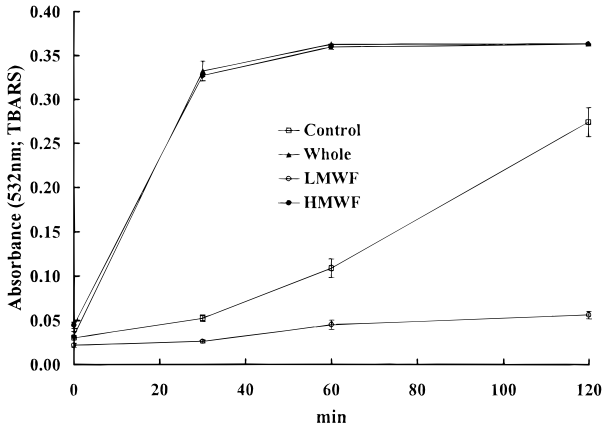

Figure 2. Inhibition of iron (15 μM)/ascorbate (100 μM)-catalyzed oxidation of sardine triacylglycerol emulsions (pH 7.4) by 10% water-soluble (whole), low-molecular weight (LMWF), or high-molecular weight (HMWF) fractions of salmon spermary tissue. Lipid oxidation was determined by thiobarbituric acid reactive substances (TBARS). Oxidation was performed at 37 °C for 90 min. Error bars represent standard deviations.

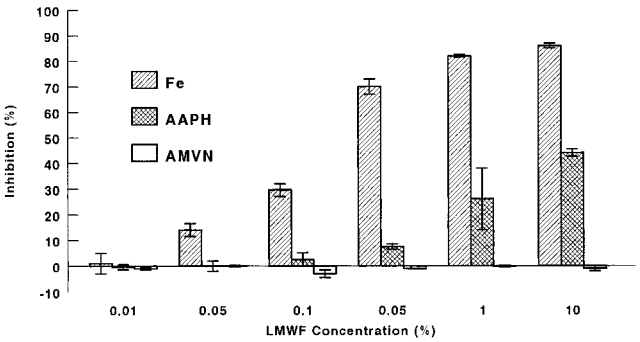

Figure 3. Inhibition of 15 μM iron/100 μM ascorbate (Fe)-, 2.0 mM 2,2′-azobis(amidinopropane) dihydrochloride (AAPH)-, and 2.0 mM 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-catalyzed oxidation of sardine triacylglycerol emulsions by varying the concentration of the salmon spermary low-molecular weight fraction (LMWF). Oxidation was performed at 37 °C for 90 min. Error bars represent standard deviations.

oxidation catalyzed by iron/ascorbate and the water-soluble peroxyl radical generator, AAPH (Figure 3). The LMW fraction did not inhibit lipid oxidation catalyzed by the lipid-soluble peroxyl radical generator, AMVN (Figure 3). The LMW fraction was more effective in the presence of the iron/ascorbate system than AAPH. Inhibition of TBARS formation in the presence of 1.0% LMW fraction was over 80% in the iron/ascorbate system but only 23.6% with AAPH.

The influence of pH on the antioxidant activity of the LMW fraction is shown in Figure 4A−C. To observe changes in antioxidant activity with pH, LMW fractions which inhibited approximately 50% of TBARS formation at pH 7.4 were chosen. The catalysis of oxidation of the sardine TAG emulsion by iron/ascorbate decreased with decreasing pH (4.0−8.0). The LMW fraction (0.25%) did not inhibit the formation of TBARS in the iron/ascorbate system at pH ≤6.0. Closer examination of the pH range of 5.6−7.2 (Figure 4B) shows the LMW fraction (0.25%) inhibited iron-catalyzed lipid oxidation 30 and 68% at pH 6.8 and 7.2, respectively, but did not inhibit TBARS formation at pH ≤6.4. The antioxidant activity of 10% LMW fraction was not strongly influenced by pH in the presence of AAPH with inhibition of TBARS being 55 and 42% lower than those of controls at pH 5.6 and 7.2, respectively (Figure 4C).

Table 1 shows the concentrations of total solids, ash, and selected antioxidants in the LMW fraction which had been isolated from spermary tissue using distilled water. Total solids and ash were 0.56 and 0.17% of the LMW fraction, respectively. Spermine and putrescine concentrations were 353.0 and 13.7 μg/mL, which represents approximately 141.2 and 55 mg per 100 g of spermary tissue (based on 4-fold dilution), respectively. Spermidine was not detected in salmon spermary tissue. Yamanaka et al. (1987) reported spermine concentrations in carp and rainbow trout spermary tissue to be 103 and 28 mg per 100 g of tissue, respectively. Spermidine and putrescine concentrations in carp and trout spermary tissue were significantly lower than spermine concentrations.

Inhibition of iron/ascorbate-catalyzed oxidation of the sardine TAG emulsion by 0.25% LMW fractions and spermine, putrescine, hypoxanthine, xanthine, reduced glutathione, and the combination of these antioxidants at concentrations equivalent to 0.25% LMW fraction is shown in Figure 5. Only spermine and putrescine were found to inhibit TBARS formation, while hypoxanthine, xanthine, glutathione, and the combination of antioxidants were found to be ineffective or slightly prooxidative.

DISCUSSION

Water-soluble fractions of biological tissues contain both prooxidants and antioxidants. Both the intact and HMW water-soluble fractions of salmon spermary tissue accelerated or had no influence on the rate of TBARS formation during either the autoxidation (Figure 1B) or iron/ascorbate-catalyzed oxidation (Figure 2) of sardine TAG emulsions. The intact and HMW fractions initially increased and then decreased the concentration of peroxides during autoxidation (Figure 1A). Potential HMW prooxidants which could be responsible for the increase in lipid oxidation by intact and HMW fractions include heme- and iron-containing proteins (Decker and Hultin, 1992) and enzymes such as lipoxygenase (German and Kinsella, 1985). The increase and subsequent decrease in peroxides could be due to the ability of iron- and heme-containing proteins to catalyze the decomposition of lipid peroxides or could be due to HMW antioxidants such as superoxide dismutase or catalase. The observed decrease in peroxides is unlikely due to

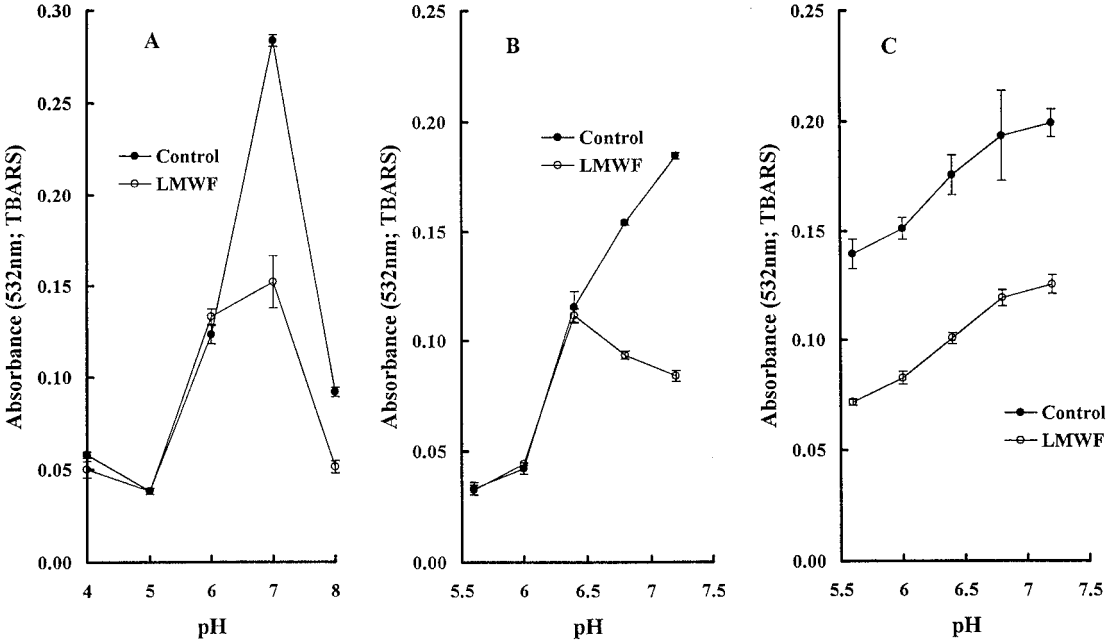

Figure 4. Ability of the low-molecular weight fraction (LMWF) of salmon spermary tissue to inhibit iron (15 μM)/ascorbate (100 μM) (A and B) and 2.0 mM 2,2′-azobis(amidinopropane) dihydrochloride (C)-catalyzed oxidation of sardine triacylglycerol emulsions as a function of pH. LMWF concentrations were 0.25% (A and B) or 10% (C). Oxidation was performed at 37 °C for 120 min. Error bars represent standard deviations.

Table 1. Concentration of Total Solid, Ash, and Selected Components in Water-Soluble LMW Fraction of Salmon Spermary Tissue

solid content	5.6 ± 0.3 mg/mL LMW fraction
ash content	1.7 ± 0.1 mg/mL LMW fraction
spermine	3353.0 ± 33.3 μg/mL LMW fraction
putrescine	13.7 ± 2.9 μg/mL LMW fraction
spermidine	ND[a]
reduced glutatione	10.2 ± 0.3 μg/mL LMW fraction
hypoxanthine	49.5 ± 10.1 μg/mL LMW fraction
xanthine	20.3 ± 4.8 μg/mL LMW fraction
uric acid	ND
ATP	ND
ADP	ND
AMP	ND
anserine	ND
carnosine	ND

[a] ND represents not detected.

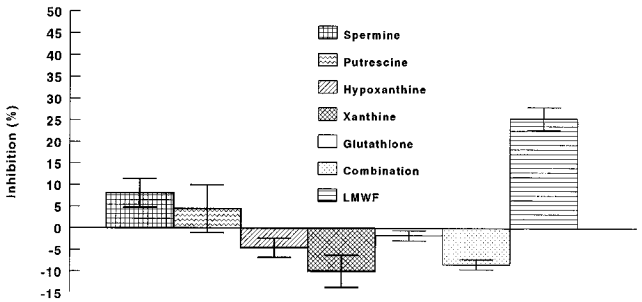

Figure 5. Ability of the 0.25% low-molecular weight fraction (LMWF) and spermine (0.88 μg/mL), putrescine (0.034 μg/mL), hypoxanthine (0.12 μg/mL), xanthine (0.051 μg/mL), and reduced glutathione (0.025 μg/mL) at concentrations equivalent to those found in 0.25% LMWF to inhibit iron/ascorbate-catalyzed oxidation of sardine triacylglycerol. Oxidation was performed at 37 °C for 90 min. Error bars represent standard deviations.

glutathione peroxidase since the HMW would not have contained the necessary cofactor, reduced glutathione.

The LMW fraction of the salmon spermary tissue inhibited lipid oxidation as measured by both TBARS and peroxides. The antioxidant components of the LMW fraction inhibited autooxidation as well as iron- and AAPH-catalyzed oxidation (Figures 1−3). Since the LMW fraction was extracted from the spermary tissue with water, it is not surprising that it was incapable of inhibiting the lipid-soluble peroxyl radical generator, AMVN (Figure 3). The ability of the LMW fraction to inhibit both iron/ascorbate and AAPH suggests that the antioxidant mechanism is not solely due to metal chelation. However, decreases in antioxidant activity with decreases in pH in the presence of iron/ascorbate but not AAPH (Figure 4A,B) suggest that interactions between the LMW compounds and iron or ascorbate are involved in the antioxidant activity. These interactions could help explain why the antioxidant activity of the LMW fraction was greater against iron/ascorbate than AAPH.

The ash content of the LMW fraction was 30% of the total solid content, indicating that the concentration of organic matter was approximately 3.9 mg/mL LMW fraction (Table 1). Potential organic LMW water-soluble antioxidants in the spermary tissue included carnosine, anserine, polyamines, nucleotides, ascorbic acid, and reduced glutathione (Decker and Chan, 1994; Yamanaka et al., 1987; Kanner et al., 1987; Matsushita et al., 1963). The antioxidant role of ascorbic acid in the LMW fraction was not determined since ascorbate acts as a prooxidant in the iron/ascorbate model system. Of these antioxidants spermine, putrescine, hypoxanthine, xanthine, and reduced glutathione were found in the LMW fraction. Their combined concentrations were approximately 11% of the LMW fraction organic matter. When these compounds were added, either individually or in combination, to the sardine TAG emulsion at concentrations equivalent to 0.25% LMW fraction, they exhibited lower antioxidant activity than the LMW fraction. The lower activity of these known antioxidants indicates that other unidentified antioxidants exist in the LMW fraction.

In conclusion, salmon spermary tissue contains LMW compounds which inhibit the autoxidation and iron/ascorbate- or AAPH-catalyzed oxidation of a sardine

TAG emulsion. The ability of the LMW antioxidants to inhibit both metallic- and nonmetallic-catalyzed lipid oxidation at the pH of many foods suggests that salmon spermary tissue could be used as a source of water-soluble food antioxidants. The presence of several LMW water-soluble antioxidants in the spermary tissue suggests that preparation of extracts by processes such as ultrafiltration may provide a more effective source of food antioxidants than processes which isolate individual antioxidants.

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