Reaction of Autoxidizing Linoleate with Coho Salmon Myosin

R.J. BRADDOCK[2] and L.R. DUGAN, JR., Department of Food Science, Michigan State University, East Lansing, Michigan 48823

ABSTRACT

Reactions that contribute to denaturation, destruction and quality changes of fish muscle lipids and protein were studied. Compounds with characteristic fluorescence spectra were isolated by extraction and thin layer chromatography from an autoxidizing system consisting of sodium linoleate, Coho salmon myosin and buffer. Similar compounds were also present in extracts from freeze-dried salmon steaks and salmon kept frozen at -20 C for 1 year. TBA values and oxygen uptake of the autoxidizing system showed initial rapid increases with time followed by a significant decrease in TBA values and gradual leveling off of oxygen uptake upon prolonged oxidation. IR spectra before and after borohydride reduction and UV, visible and fluorescence spectra indicated the presence of C=N functional groups in extracts from the various samples. These compounds were not extractable from the control myosin solutions allowed to oxidize without addition of linoleate. Amino acid analyses of the myosin from the autoxidizing system, when compared with nonoxidized myosin-linoleate systems, indicated significant decreases in the amounts of histidine, lysine and methionine following oxidation. These reactions apparently contribute to the denaturation and destruction of the lipids and protein in the model system, as well as in a frozen or freeze-dried product.

INTRODUCTION

Autoxidation in foods containing significant quantities of the polyunsaturated fatty acids (PUFA) can be very extensive and may result in severe impairment of quality, particularly in the case of seafoods and fishery products. One study has reported that decomposition products of oxidized lipid in freeze-dried salmon probably reacted with the amino groups of the protein to increase browning (12). In fish tissue, where reactive constituents are formed from PUFA oxidation, many reactions may take place because of the biologically active compounds capable of reacting under stress with the oxidizing lipids (4). One of these reactive constituents, malonaldehyde, has been implicated in some instances in protein-lipid breakdown product reactions, and has been shown to participate in reactions involving free-amino groups of proteins and amino acids (3,9).

It is possible that other carbonyl compounds besides malonaldehyde may be probable reactants in protein-lipid oxidation product interactions. The spectrum of fatty acids in Coho salmon has been shown to be very complex (2); and it would be expected that the oxidation products from these fatty acids, which could be available for reaction with other tissue constituents such as proteins, should be many and complex.

The salmon studied here contained significant proportions of oleic, linoleic, linolenic and arachidonic acids (2), all of which have been shown to produce malonaldehyde or malonaldehyde-like products under oxidative conditions (18). These malonaldehyde-like products, in the presence of water, have been postulated to exist mainly as nonvolatile enolate ions, and as such they can react with amino acids, proteins, glycogen and other food constituents to produce a bound complex hydrolyzable by acid or heat (11). In addition, it was reported that browning of some food systems containing oxidizable lipids and proteins may also involve complex lipid-protein interactions and reactions implicating other constituents (10).

This research presents new evidence indicating that production of C=N compounds occurs as a result of reactions between autoxidation products of the PUFA and amino groups present in a biological system, and should contribute to a better understanding of autoxidative mechanisms in foods that are susceptible to such deteriorations.

EXPERIMENTAL PROCEDURES

Systems

Sodium linoleate: Buffered solutions containing either sodium linoleate (L), Coho salmon myosin (M) or sodium linoleate-myosin (LM) were prepared for each experiment. The buffers used consisted of either 0.45 M KCl-0.001 M tetrasodium pyrophosphate or of 0.04 M tetrasodium pyrophosphate, pH 7.5, both of which proved suitable for reaction purposes. Sodium linoleate solutions (7.5×10^{-3} M) were prepared by rapidly stirring 0.5 ml *cis*-linoleic acid (Sigma Chemical Co.) into 10 ml buffer, pH 9.0, to form a lasting emulsion. Then one to three NaOH pellets were added and dissolved until a clear solution was obtained. This solution was brought to a final volume of 200 ml with pH 9.0 buffer, adjusting to the desired pH with concentrated HCl.

Myosin: Myosin (M) solutions were prepared from frozen Lake Michigan Coho salmon according to the procedure of Richards et al. (16), who prepared myosin from frozen yellowfin tuna. Prepared myosin solutions, shown to be free of actomyosin by the light scattering test of Rice et al. (15), were stored at -20 C in 50% glycerol-buffer. When needed, the myosin was precipitated by the addition of 9 volumes of distilled water, collected by centrifugation and dissolved in the desired buffer (7). Final concentrations of myosin solutions used in this experiment were ca. 1 mg/ml, as determined by a biuret test. Aggregation of the myosin preparations during storage at -20 C did not affect the results of this experiment.

Linoleate-myosin: Equal volumes of L and M (1:1) were mixed in the prescribed buffer, placed in a closed flask under oxygen atmosphere in a 50 C water bath and shaken vigorously at periodic intervals. Experiments were performed using duplicate samples consisting of L and buffer, M and buffer, and LM in sufficient volume to perform several analyses.

Significant amounts of myosin were rendered insoluble by increasing the concentration of linoleate in LM mixtures. The decreased solubility of protein in this system became apparent at very dilute linoleate concentrations ($<0.1 \times 10^{-3}$ M). Concentrations of linoleate, which after mixing (1:1 v/v) with 1 mg/ml myosin solutions would precipitate almost all of the myosin, were found to be greater than 1.5×10^{-3} M. Preparation of sodium linoleate solutions of greater than 7.5×10^{-3} M concentration was not possible in the pH 7.5 buffers used for this study.

Fish: Lake Michigan Coho salmon were obtained from

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[1]Michigan Agricultural Experiment Station Journal Article 6304.

[2]Present address: University of Florida Agricultural Research and Education Center, P.O.B. 1088, Lake Alfred, Fla. 33850.

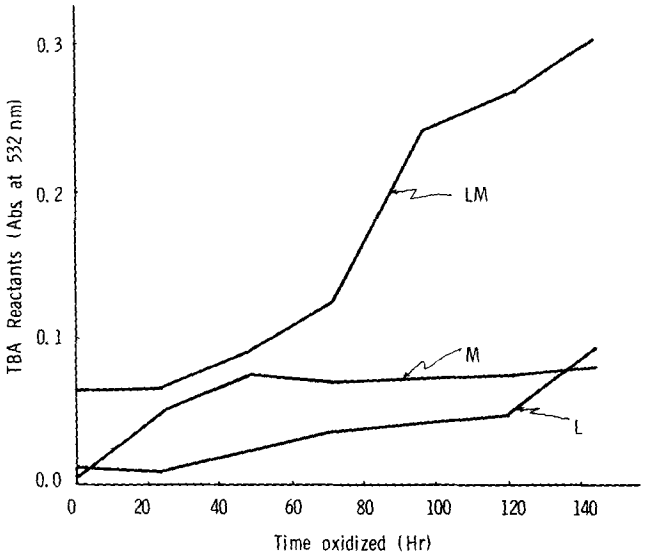

FIG. 1. Amount of TBA-reactive compounds produced in oxidizing linoleate, myosin and linoleate-myosin systems at 50 C. Relative concentration of linoleate = 2.5 x 10^{-3} M; myosin = 1.0 mg/ml; linoleate and myosin mixed 1:1 v/v with each other or with buffer.

the Michigan State Department of Natural Resources in September 1968 and in July 1969. These fish were kept on ice for ca. 15 hr, and then frozen and stored whole in a blast freezer at -20 C. Fish used for analyses were kept in separate polyethylene bags, and when needed steaks were sawed vertically from the dorsal fin region of the frozen fish (2) and the skin, belly flap and dark muscle discarded. The fish were not differentiated on the basis of sex.

Chemical Analyses

Oxygen absorption: Peroxide values were determined iodometrically and were converted to moles of oxygen absorbed per gram of linoleate or myosin, assuming conversion of 1 mol oxygen to 1 mol peroxide. In some instances, absorbed oxygen was also measured with a Gilson respirometer. The two methods showed close agreement for the first 12-15 hr, after which time the respirometer readings decreased considerably, while the peroxide values only leveled off.

TBA tests: TBA reactive substances were determined in the oxidizing L, M and LM systems by periodically removing 1 ml samples, adding 1 ml 0.02 M 2-thiobarbituric acid in 90% glacial acetic acid, holding in a boiling water bath for 20 min, cooling, adding 0.5 ml distilled acetone to relieve turbidity, and measuring the absorbance at 532 nm. A 10 min centrifugation at 3000 x g was necessary to obtain a clear solution in some cases. TBA tests were performed on frozen and freeze-dried salmon using a distillation procedure (17).

Fluorescent compounds: Blue-fluorescing compounds were extracted from oxidized LM solutions by shaking with chloroform or by freeze drying and extracting with chloroform-methanol 2:1. Blue-fluorescing compounds were also extracted with chloroform-methanol 2:1 from whole frozen salmon kept for 1 year at -20 C, freeze-dried salmon steaks stored at ambient temperatures for 6-8 months and salmon steaks maintained at -20 C under an oxygen atmosphere for 3 months.

Separation of three major fluorescing fractions was achieved by thin layer chromatography (TLC) on Silica Gel G. Extracts were pipetted in a thin band onto 20 cm x 20 cm glass plates and developed in hexane-diethyl ether 60:40. After drying the plate by evaporation under a stream of nitrogen, a second development was achieved in chloroform-methanol-water 65:25:4. Fluorescing bands could be visualized under UV radiation (3600Å) at R_f values of ca. 0.95, 0.7 and 0.35. Additional purification of each band was accomplished by TLC.

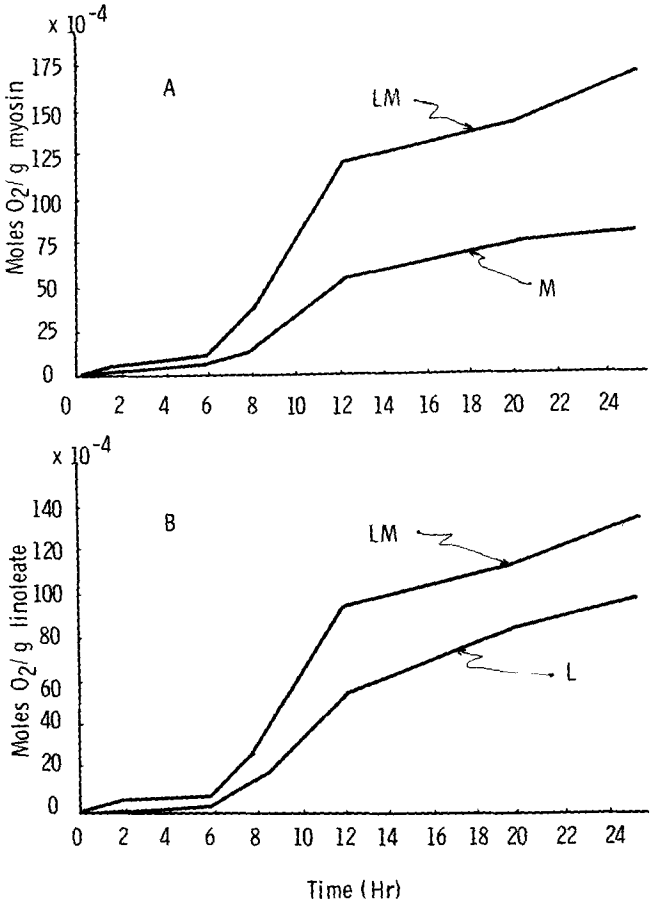

FIG. 2. Effect of sodium linoleate on amount of oxygen absorbed by linoleate, myosin and linoleate-myosin systems at 50 C. Relative concentration of linoleate = 2.5 x 10^{-3} M; myosin = 1.0 mg/ml; linoleate and myosin mixed 1:1 v/v with each other or with buffer. A. Moles oxygen absorbed per gram myosin. B. Moles oxygen absorbed per gram linoleate.

Spectrophotometric analyses of the fluorescing compounds were performed to determine the presence of C=N functional groups (5,6). Using a Beckman IR-12 spectrophotometer, IR analyses were performed before and after sodium borohydride reduction (5). Fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer, and UV spectra were obtained with a Beckman model DK-2A spectrophotometer.

Amino acid analysis: Amino acid analyses of precipitates from unoxidized and oxidized duplicate samples of LM were made with a Spinco Model 120-C amino acid analyzer. Hydrolysis of samples was performed in 6 N HCl at 110 C for 24 hr only; hence values were not extrapolated to zero time to correct for hydrolysis losses.

RESULTS AND DISCUSSION

Oxidizing Systems

Evidence is strong that some of the deterioration of muscle protein from frozen fish and other meats is a consequence of reactions with carbonyl compounds produced by lipid autoxidation during storage. To facilitate study of this concept, model systems were employed that contained L, M and LM under oxidizing conditions. The systems were permitted to oxidize at 50 C under an oxygen atmosphere in order to increase rates of reaction.

TBA reactive components were produced during oxidation of L, M and LM mixtures. The amounts produced during an oxidation time of 6 days for L and M were much

less than produced in LM systems, which began to show appreciable increases after 3 hr (Fig. 1). The amount of oxygen absorbed per gram of either L or M was clearly greater for the LM system (Fig. 2), and while this amount may appear to be additive in terms of L and M, the rate of uptake during the interval from 6-12 hr was significantly greater for the LM system than for either the L or M systems. It was during this interval that fluorescence of the LM system under UV radiation began to increase notably.

An examination of Figures 1 and 2 supports the assumption that, as oxidation of the LM system proceeds, carbonyl compounds and TBA reactives increase and become involved in crosslinking between ϵ-amino groups of the protein (6,9,11). A continuing increase, with oxidation time, of TBA reactive substances was noted, even though they are involved in reactions with the protein. This would be possible if the extent of production of the TBA reactants by the oxidizing systems was much greater than their participation in crosslinking and other reactions with the protein. Also, reaction with the TBA reagent itself may not be specific for only free, unbound malonaldehyde (9).

Denaturation of the protein by linoleate and oxidation products in the LM systems may increase the efficiency of oxidation of the protein, accounting for the increased oxidation rate of the LM mixture. This is supported by the fact that serum albumin bound by linoleate hydroperoxide showed increased efficiency of thiol oxidation, probably due to steric changes resulting from the binding (13). On the other hand, binding of the fatty acid salts or oxidation products to the proteins in the LM systems may cause these fatty acids and products to become oriented around the protein in a manner that would make them susceptible to oxidation, still having the effect of increasing the oxidation rate of the LM system, relative to either L or M.

Prolonged oxidation of LM for 6 days at 50 C resulted in breakdown of the complex and increasing fluorescence in the reaction mixture. A consequence of this oxidation was a loss of certain amino acids from the protein in the LM system, as presented in Table I. Some slight changes can be observed when comparing amino acids from unoxidized and oxidized LM precipitates for such amino acids as threonine, serine, proline, alanine, leucine and phenylalanine (Table I). These changes are not within the allowable ±3% error inherent to the analysis procedure, but are close enough to consider that the differences may not be significant. An unknown peak, which was in approximately equal quantities in both the oxidized and unoxidized LM samples, was observed following arginine on the recorder chart. This unknown, which was present in all the samples hydrolyzed for 24 hr, disappeared when a 72 hr hydrolysis time was used, indicating an unhydrolyzed peptide was probably responsible for the peak. The most significant changes were in losses of methionine (58%), histidine (43%) and lysine (37%). These losses were obtained when amino acid analysis of 4000 x g precipitates of oxidized LM systems were compared with unoxidized LM precipitates. Losses of these amino acids compared favorably with the observations of Buttkus (3), who reacted malonaldehyde with trout myosin and amino acids. He was able to show that lysine, histidine, arginine, tyrosine and methionine participated preferentially in the reaction, depending on the reaction temperature. He postulated that histidine residues were more accessible for reaction when the molecule unfolded during denaturation, and noted that in frozen malonaldehyde-myosin systems some lysine residues formed a product stable to acid hydrolysis.

TABLE I

Amino Acid Analysis of Precipitates from Oxidized[a] and Unoxidized Linoleate-Myosin Systems

	Linoleate-myosin system, g residue wt/100 g sample	
Amino acid	Unoxidized	Oxidized
Lysine	2.69	1.71
Histidine	0.69	0.39
Arginine	1.98	2.00
Unknown peak	0.45	0.44
Aspartic acid	2.81	2.82
Threonine	1.30	1.57
Serine	1.03	1.19
Glutamic acid	3.88	3.93
Proline	0.84	0.93
Glycine	0.99	1.08
Alanine	1.78	1.63
Half cystine	tr	tr
Valine	1.51	1.57
Methionine	0.43	0.18
Isoleucine	1.64	1.68
Leucine	2.28	2.38
Tyrosine	1.06	1.06
Phenylalanine	1.06	1.31

[a]Oxidized for 6 days at 50 C.

C=N Oxidation Products

Fluorescing components were extracted and isolated from LM systems that had been subjected to extensive oxidation. Extraction of the LM system with chloroform following a 3 day oxidation period yielded a fluorescing chloroform layer and an aqueous layer that contained a fluorescing particulate residue from the protein. This residue could be extracted with chloroform-methanol 2:1 to remove more of the fluorescing substances; however even extensive extraction with this solvent did not result in quenching the fluorescence of the particles when resuspended in water. Intra- and intermolecular crosslinking was probably the cause of this fluorescence in the oxidized LM mixtures (6).

TLC in petroleum ether-diethyl ether-acetic acid 70:30:1 of the fluorescing chloroform extract resulted in separation of several bands. One major band of interest, which moved slightly below the solvent front, contained carbonyl functional groups (2,4-DNP positive), was iodine reactive and fluoresced under UV radiation. A much better separation of the LM chloroform extract was achieved when plates were first developed in hexane-ethyl ether 60:40, causing a separation at the solvent front of the 2,4-DNP positive constituents, leaving all of the fluorescing substances at the origin. Development of this plate in chloroform-methanol-water 65:25:4 effected the separation of three fluorescing bands at R_f = 0.95, 0.70 and 0.35, respectively. Components of all three bands were reactive with iodine vapors. The band at R_f = 0.95 was very much more concentrated than the others. Purification and concentration of these components for further study was achieved by rechromatography of each band several times in the chloroform-methanol-water solvent.

When the LM system was freeze-dried and then extracted with chloroform-methanol 2:1, separation of additional fluorescing bands could be achieved. This study was confined primarily to an examination of the major fluorescing band (R_f = 0.95).

An examination of the UV-visible spectra of the fluorescing band (R_f = 0.95) revealed absorbance maxima at 240, 280, 375 and 431 nm (ref. solvent-methanol). Excitation and emission spectra (Fig. 3) indicated an excitation peak at 350 nm and an emission in the region of 450-475 nm. The peak at 700 nm is a second order fluorescence resulting from the excitation at 350 nm. These particular spectrophotometric properties have been shown to occur in Schiff base-type compounds classified as 1-amino-3-imino propenes, which were prepared by reacting amino acids with malonaldehyde (5). Earlier research reported chemical and spectral evidence indicating that malonaldehyde and glycine reacted to form the enamine, N-prop-2-enalamino-acetic acid (8); but these researchers precluded the presence

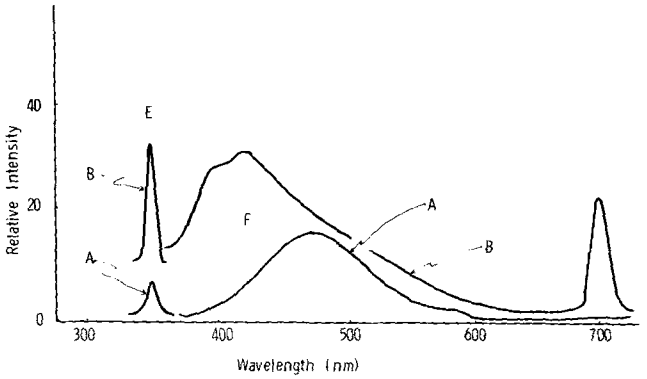

FIG. 3. Excitation (E) and emission (F) spectra of fluorescing compounds from oxidized linoleate-myosin systems. A. Band from thin layer chromatography (R_f = 0.95). B. Bands from thin layer chromatography (R_f = 0.7, 0.35). Solvents = carbon tetrachloride.

of an imine linkage in the molecule. However, when *n*-heptanal-tyrosine ethyl ester mixtures were reacted at 10, 20, 30 and 40 C, colored pigments were produced, suggesting an aldehyde-amine condensation reaction, since IR spectra showed a band at 1640 cm^{-1} for the imine linkage (14).

Further elucidation of the chemical properties of the fluorescent products is possible through examination of the IR spectra (Fig. 4). Changes in the IR spectra of the TLC isolate (R_f = 0.95) were noticeable following reduction with sodium borohydride. Bands at 1710 and 1665 cm^{-1} were greatly diminished, as exhibited by the spectrum of Figure 4B. The 1710 cm^{-1} peak represents a band of the C=N bond, while the 1665 cm^{-1} peak is a band of the C=C bond. It has been shown that sodium borohydride is effective in bringing about complete reduction of the double bonds in compounds of the structure, R-NH-CH=CHCH=N-R (5). The strong band at 1750 cm^{-1} due to carbonyl absorbance is resolved more completely following reduction of the C=N bond.

The evidence supports the presence of compounds containing C=N groups as a consequence of lipid oxidation in systems containing oxidizable fat and protein. Soluble C=N compounds were present in these systems, even though the protein-lipid complex itself was insoluble. These soluble Schiff bases may be produced when the oxidative reactions of the system cause bond cleavage and formation of new compounds. For example, the losses of lysine,

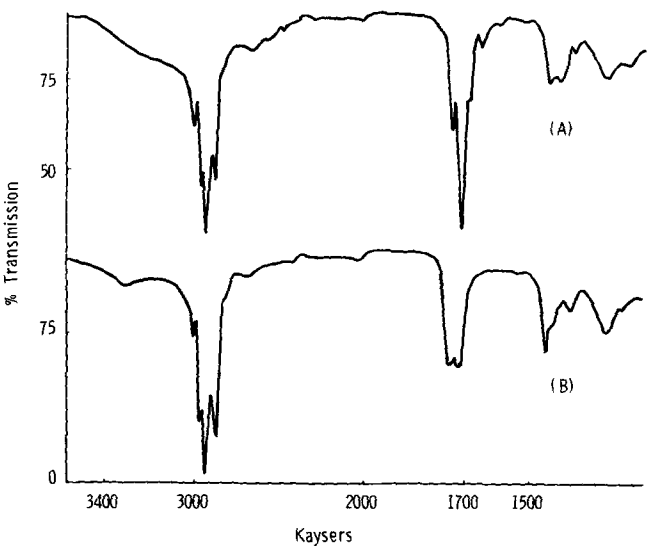

FIG. 4. IR spectra of fluorescing band from thin layer chromatography (R_f = 0.95) of extracts from oxidized linoleate-myosin systems. A. Before reduction with sodium borohydride. B. After reduction with sodium borohydride. Run as liquid, neat.

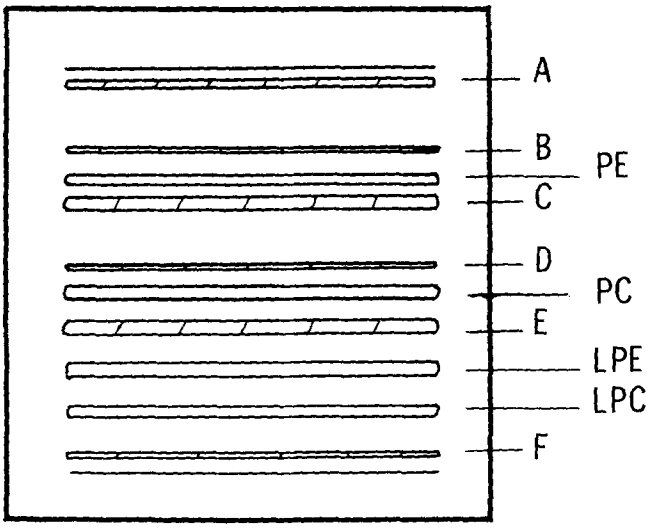

FIG. 5. Silica Gel G thin layer chromatography of fluorescing polar lipid fraction from frozen Coho salmon stored for 1 year at -20 C. Solvent system, $CHCl_3MeOH/H_2O$ 65:25:4. Bands A, B, C, D, E and F showed fluorescence under UV light.

histidine and methionine (Table I) may reflect a loss through cleavage of some functional group from the protein that combined with lipid autoxidation products to form a soluble compound. The fact that no similar components were found in buffered solutions of myosin oxidized in like manner supports the evidence that malonaldehyde-like compounds or other carbonyls produced through lipid oxidation react with amino nitrogen of proteins and amino acids to produce Schiff bases of various types. These compounds can then serve as intermediates leading to destructive reactions such as the nonenzymatic browning of foodstuffs. This was illustrated when LM solutions, allowed to react for many days, turned noticeably brown.

Frozen and Freeze-Dried Coho Salmon

Oxidative Changes: Reactions of the type mentioned above have strong significance in consideration of quality of food products. Because of the susceptibility of frozen and freeze-dried fish to lipid-related deteriorations, an investigation was conducted that revealed the presence of compounds containing C=N in solvent extracts from these two products. Other researchers have documented the possible existence of such autoxidation products in deteriorating frozen fish. The presence of unidentified dark spots was observed at or near the solvent front on thin layer chromatograms of total lipids extracted from stored whitefish muscle (1). It was suggested, but not confirmed, that the unidentified spots, increasing as muscle storage time increased, were probably autoxidation products.

Extracts from frozen whole Coho salmon stored at -20 C for over 1 year, frozen salmon steaks kept under an oxygen atmosphere at -20 C for 2 months and freeze-dried salmon steaks exposed to air at room temperature for 6 months were separated into neutral and polar lipid fractions by TLC as described above. The more polar fraction contained fluorescing bands, which exhibited C=N and the same spectral characteristics previously described for the extracts from the LM system.

Three major fluorescing bands (A, C and E [Fig. 5]) with R_f values similar to the bands from the oxidized LM system were obtained from the frozen stored fish. There were also several other minor bands (B, D and F), which fluoresced similarly under UV light, but no studies were made on these. One of the major bands (E, R_f = 0.3) showed a positive test for phosphorus by a molybdate spray test and also exhibited C=N bonding in the IR spectrum. This band moved just ahead of a band identified as LPE and may be a reaction product between a carbonyl compound

and the PE amino group to give a Schiff base more polar than the PE fraction, resulting in migration between the PE and the LPE. In addition, since LPE is a component of the lipids present in the frozen stored fish, reactions involving carbonyl compounds and the amino group of this compound may also lead to the production of Schiff bases with mobilities in the TLC solvent systems used in this experiment. Considering the probable complex nature of the autoxidation products that could react with the amino groups of PE and LPE in the frozen fish, it would be no simple task to establish the identity of these reaction products. Extracts from fresh fish treated in a similar manner did not contain these fluorescing products.

The stored frozen fish contained large amounts of TBA-reactive compounds as evidenced by a TBA test (17) that resulted in values as high as 35 mg malonaldehyde per kilogram flesh; whereas the oxidized freeze-dried fish, which had a very rancid odor, only gave TBA values in the range 0.5-3.5, based on the wet weight of the flesh.

The task of clarifying reactions involving lipid oxidations is formidable when the complexity of the lipids and proteins contained in frozen fish or of the LM system is considered. The extraction of compounds containing C=N from autoxidizing food systems consisting of lipids and proteins is proof that significant reactions can occur, which change the chemical nature of the original constituents. The fact that addition to and oxidation of linoleate in myosin solutions caused insolubilization of the protein is evidence for the occurrence of physical damage to the protein in such systems. These reactions and interactions could be very important to the final quality of stored food products and formulations for certain foodstuffs.

This study contains results that will aid in evaluation of some of the complex changes in food systems and organizes much of the recent research that implicates malonaldehyde-like compounds as playing a leading role in lipid deterioration reactions. The fact that malonaldehyde and other carbonyl compounds will interact with food constituents during autoxidation may account for some of the discrepancies associated with the use of TBA tests for quality evaluation of many lipid-containing foods. Also, it has not been firmly established that compounds resulting from carbonyl-amine reactions do not give positive TBA reactions. It has been shown, however, that other fatty acid oxidation products, besides malonaldehyde, react slowly with TBA reagent to give compounds absorbing at 450 nm, in addition to the malonaldehyde-TBA complex absorbing at 538 nm (18).

The development of rancidity in fish muscle from fatty species such as the salmon can be rapid, even at frozen storage temperatures, and is governed by complex factors common to many biological systems, such as metal ions, natural antioxidants, kind and amount of fatty acids, age, season of harvest and storage conditions before and following processing. Reactions involving oxidation of PUFA leading to the production of reactive free radicals, peroxides, aldehydes and ketones occur during this development of rancidity. These oxidation products may be very complex in type and quantity, depending on the conditions of oxidation and the degree of involvement with other constituents in a system, and play an important role in autoxidizing food systems, especially animal tissues.

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