脱脂凍結乾燥魚肉を用いた食品モデル系におけるエイコサペンタエン酸の酸化

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Autoxidation of Ethyl Eicosapentaenoate in a Defatted Fish Dry Model System

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A model of fish meats was prepared by combining several defatted freeze-dried tissues of sardine with ethyl eicosapentaenoate (EPA). The effects of fish tissues on EPA oxidation were compared quantitatively with those of casein and defatted soybean meal by oxygen uptake, conjugated diene formation, composition of oxidation products and reactions with proteins and other constituents, *i.e.* browning and fluorescence formation. Furthermore, to observe the involvement of enzymes in the oxidation, raw fish tissue preparations were also incubated with ethyl EPA.

The prooxidant activity of skin, among various sardine tissues was the strongest. The prooxidants in the skin were assumed to be enzymes labile to heat. Hydroperoxide determination (peroxide value) is less reliable as a quantitative measure of oxidation in fish and fishery products because of the instability of hydroperoxides in fish model systems. Thus, the determination of relatively stable secondary products or termination products such as polymers, fluorescent materials and browning matter are thought to be more reliable in evaluating oxidative deterioration in fish and fishery products.

Lipids in marine foods are known to be characteristically abundant in long chain highly unsaturated fatty acids (HUFA) such as eicosapentaenoate (EPA) and decosahexaenoate (DHA).[1] HUFA are known to be labile and easily oxidized causing an off-flavor. We reported previously that EPA and DHA esters were oxidized rapidly even at 5°C and polymers were found to be the major secondary products of these polyenoic esters. In addition, the studies indicated that the ratios of OOH-oxygen determined by peroxide value (POV) measurement to total absorbed oxygen were less than 50% during autoxidation of HUFA because of the instability of hydroperoxides (HPOs) in these HUFA.[2,3] In unsaturated fatty acids, it has been suggested that HPOs are formed as primary oxidation products by free radical mechanisms.[4,5] The HPO of unsaturated fatty acids is fruther oxidized to a complex mixture of various oxidized products, which are generally termed secondary autoxidation products.[4,6] Oxidative deterioration and off-flavor in foods are caused by these products. The autoxidation products readily react with proteins and decrease bioavailability.[7,8] Marine foods particularly contain an abundance of HUFA as well as various prooxidants such as heme proteins and free transition metals. Thus, although it has been suggested that HPOs in fish and fishery products are decomposed very rapidly, the details of the oxidation processes in marine foods are not understood completely.

In the present studies, in order to understand the effect of various constituents in fish meat on the oxidative deterioration process of HUFA, changes in HUFA together with proteins in fish model systems were followed during storage for comparison with soybean meal and casein. Furthermore, damage to the proteins in the fish model system were evaluated by elementary analysis and by amino acids composition. To observe the involvement of enzymes in the oxidation, preheated fish tissue preparates were also incubated with ethyl EPA.

Materials and Methods

Preparation of Freeze-Dried Fish Tissues

Ordinary and dark meats and skin were excised from fresh sardine *Sardinops melanosticta* or

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Alaska pollack *Theragra chalcogramma*, both of which are purchased from a fish market. Each tissue was chopped and then freeze-dried. The freeze-dried samples were extracted 3 times with a mixture of $CHCl_3$-methanol (2: 1, v/v) to remove lipids. Defatted soybean meal was prepared from unheated soybeans (Variety: Raiden) as described above. Casein was also defatted similarly. In order to inactivate enzymes in the fish tissues, soybean meal and casein, samples were autoclaved at 120°C for 1 h prior to incubation with ethyl EPA.

Preparation and Storage of Fish Model System

Each raw or cooked freeze-dried fish tissue was mixed with ethyl EPA in hexane mixture at the concentration of 10% (w/w), and dried under a reduced pressure using a rotary evaporator. Aliquots (2 g) of the mixture were put into an erlenmyer flask (100 m*l*) with a rubber cap and stored at 5°C in the dark.

Analytical Methods

After storage each sample was extracted 3 times with a mixture of $CHCl_3$-methanol (2: 1, v/v). The resulting lipid (ethyl EPA) fraction and residue (fish tissues etc.) were subjected to the following analyses.

Oxygen absorption in the head space of an erlenmyer flask was measured by gas chromatography with a thermo-conductivity detector as described previously.[2] Conjugated diene content, peroxide value (POV) and the distribution of oxidized products in the extracted lipid fraction were also determined as described previously.[2,3] Browning degree of the lipid fraction was determined by measuring the absorbance at 420 nm of a 2% alcohol solution. The fluorescence intensity was measured with a Hitachi 204 Fluorescence Spectrophotometer with excitation at 380 nm and emission at 440 nm, and the intensity was expressed as the value relative to that of quinine sulfate (1 ppm in 0.1 N H_2SO_4).

Changes in amino acid composition in the stored fish meat were determined after extraction with chloroform/methanol by the method of Spackman *et al.*[9] using an amino acid autoanalyzer (Kyowa K-101 type) after hydrolysis in 6 N HCl for 24 h at 110°C. Elementary composition (C, H, N) of the same sample was analyzed at the Instrumental Analysis Center, Faculty of Science, Tohoku University.

Results and Discussion

The time course of oxygen absorption during storage of ethyl EPA with various fish tissues and other proteins at 5°C in the dark is shown in Fig. 1. No particular difference in prooxidant activities could be observed between ordinary and dark meats of sardine on the basis of oxygen absorption rate in these dry model systems. The prooxidant activity of heme compounds extraordinarily rich in dark meat was not remarkable in the dried condition.[8] However, the prooxidant activities of these fish tissues, not only from sardine

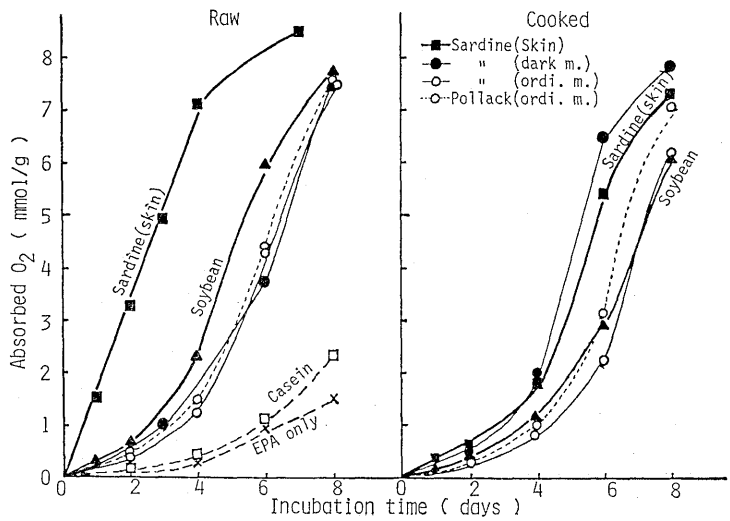

Fig. 1. Time course of oxygen absorption during autoxidation of ethyl EPA with various proteins at 5°C in the dark.

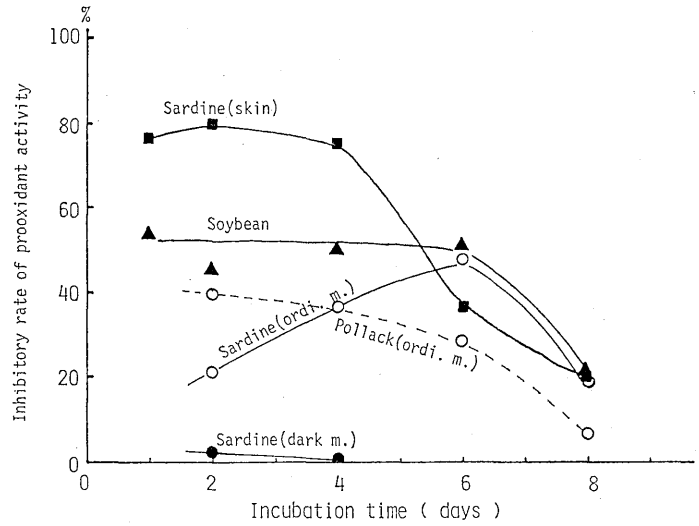

Fig. 2. Inhibitory rates of prooxidant activities of various proteins by cooking at 120°C for 1 h.

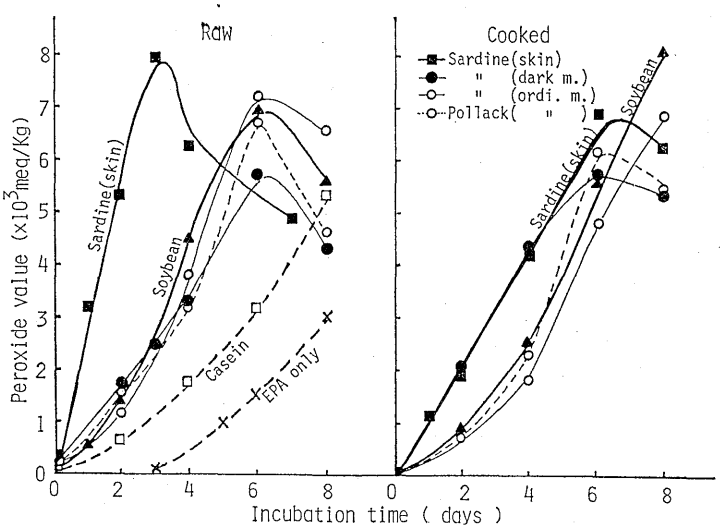

Fig. 3. Formation of conjugated diene in ethyl EPA during autoxidation with various proteins at 5°C in the dark.

but also from pollack, were much higher than that of casein. On the other hand, the activity of soybeans, which are known to contain lipoxygenase, was comparable with fish meats. Of all the samples examined, the prooxidant activity of sardine skin was most prominent and much higher than that of dark meats. The prooxidant activities of soybean and sardine skin were 50 and 80% inhibited by pre-cooking at 120°C for 1 h, respectively (Fig. 2), while oxidation with sardine and pollack ordinary meats was not significantly inhibited by cooking. On the contrary, oxidation with sardine dark meat was accelerated by cooking, compared with that of raw meat (Fig. 2). Hence, the lipid oxidation in sardine dark meat was shown to be catalyzed non-enzymatically by heme protein, because the prooxidant activity of heme protein was shown to increase by denaturation, which exposes the heme from the center of the inert heme protein.[10] Furthermore, the occurrence of the prooxidant enzymes in sardine skin was strongly suggested. The activity of enzymes in sardine skin was equivalent to or stronger than those in soybean, which is known to contain lipoxygenase with very high activity.[11,12]

The preferential oxidation of lipids in the skins of both lean and fatty fish has been previously

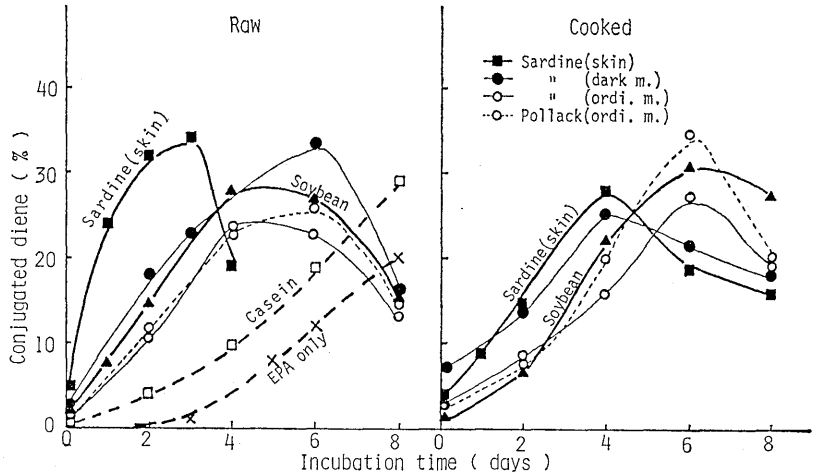

Fig. 4. Time course of peroxide value of ethyl EPA during autoxidation with various proteins at 5°C in the dark.

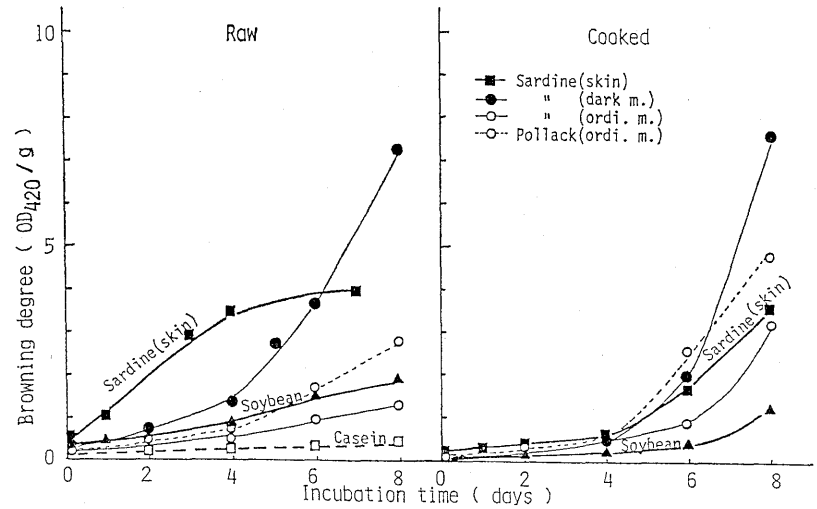

Fig. 5. Time course of browning degree of ethyl EPA during autoxidation with various proteins at 5°C in the dark.

recognized by several investigators.[13-15] Tsukuda[16] also demonstrated the occurence of a lipoxygenase-enzyme which involves in the discoloration of carotenoids in fish skin. Ke *et al.*[17] showed that one or more organic solvent-extractable prooxidants, alone or associated with trace metals, were present in mackerel skin lipids. Recently the occurrence of lipoxygenase-like enzymes which are involved in the initiation of lipid oxidation has been suggested in fish skin by Yamaguchi and Toyomizu[18] and in fish gill and skin by German and Kinsella.[19] However, the properties of the prooxidants in fish skin still remained to be elucidated.

Fig. 3 shows the time course of peroxide value (POV) of ethyl EPA during storage with various proteins at 5°C in the dark. The greatest increase in HPOs occurred with sardine skin, and the POV decreased very rapidly after reaching a maximum. Changes in POV of ethyl EPA stored with other fish tissues and soybean were very similar to each other, and were in accord with the data of oxygen uptake. Cooking treatment suppressed the POV, which was increased most remarkably in the sardine skin, although was somewhat suppressed in other tissues. The retardation of oxidation by cooking observed in the skin-ethyl EPA system was especially significant in the early stage of

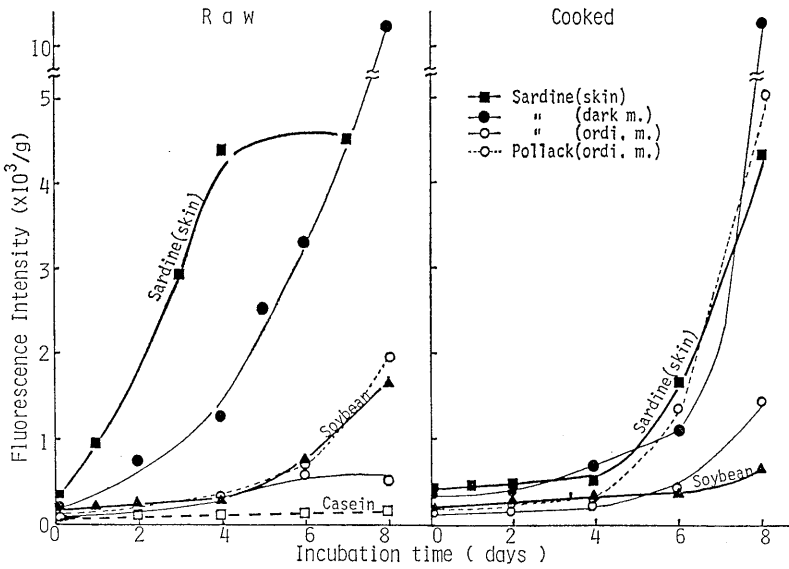

Fig. 6. Time course of fluorescence intensity of ethyl EPA during autoxidation with various proteins at 5°C in the dark.

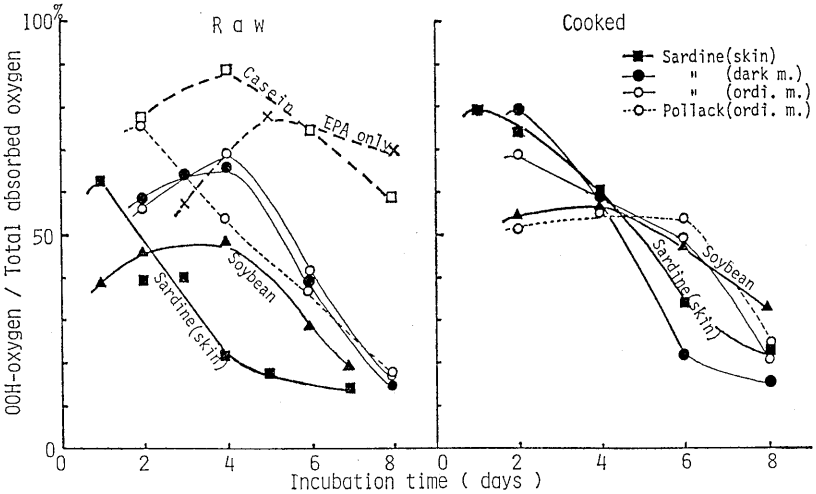

Fig. 7. Ratio of OOH-oxygen to total absorbed oxygen during autoxidation of ethyl EPA with various proteins at 5°C in the dark.

storage, indicating the involvement of heat-labile prooxidant substances, probably enzymes as mentioned previously.

The formation of conjugated diene in ethyl EPA during autoxidation with various proteins is shown in Fig. 4. The curves are very similar to those of POV shown in Fig. 3. However, the conjugated diene content in ethyl EPA oxidized with sardine skin on the 1st day represented only about 45% of the total absorbed oxygen, whereas that in ethyl EPA incubated with casein produced a higher ratio. A preferential formation of secondary products was observed in sardine skin.

The browning of fatty fish meat, often referred as rusting, has been shown to derive mainly from reactions between carbonyl compounds from lipid oxidation and various amino compounds.[20-22] Fig. 5 shows the increase in browning of ethyl EPA during storage with various proteins. The browning of ethyl EPA mixed with various fish proteins increased along with storage time, whereas POV and conjugated diene formation in fish protein sample turned to decrease in the middle phase of storage (Figs. 3 and 4). On the other

Table 1. Changes of elementary composition (%) in the sardine (ordinary meat) during incubation with ethyl EPA at 5°C in the dark

	Incubation days						
	0	2	4	6	8	10	12
C	47.1	49.0	48.5	49.1	47.9	49.1	48.3
H	7.1	7.5	7.4	7.4	7.3	7.5	7.1
N	15.2	15.2	15.2	15.2	15.2	15.2	15.2
Others	30.5	28.3	28.9	28.3	29.6	28.2	29.4

hand, it has been shown that fluorescent substances were formed in the reaction of aldehydes or oxidized lipids with various amino compounds.[23-25] Changes in fluorescence intensity of ethyl EPA during storage with various proteins are shown in Fig. 6. It is noteworthy that the formation of fluorescence during storage was highly correlated with browning. Davis,[26] Davis and Reece[27] reported that fluorescence of fish muscle during frozen storage occurred with lipid oxidation. The same phenomenon was obtained in this experiment. In sardine skin and dark meats the remarkable increases of browning and fluorescence formation occurred, and these reactions were suppressed significantly by cooking of the fish tissues (Figs. 5 and 6). Neither browning nor fluorescence formation was observed when ethyl EPA was stored without any proteinious preparation.

Fig. 7 shows the ratio of OOH-oxygen determined by POV measurement to total absorbed oxygen during storage of ethyl EPA with various proteins. Absorbed oxygen seems to be also consumed mainly for the formation of secondary oxidation products besides HPOs. HPOs formed in ethyl EPA stored with casein or alone were relatively stable, while HPOs formed in ethyl EPA during incubation with fish tissues or soybean were much more labile and decomposed rapidly to secondary products along with storage. The sardine skin among all these fish tissues decomposed HPOs most efficiently; consequently the ratio of HPOs to total oxygen-up-take reached a maximum after storage for 1 day and decreased quickly to 12% after the 7th day of storage. However, by cooking the sardine skin or soybeans, the decomposition of HPOs was somewhat suppressed. These results suggest that sardine skin shows "hydroperoxidase" activity together with prooxidant activity. Therefore, the present studies demonstrate that HPO determination is less reliable as a quantitative measurement of lipid oxidation in fish and fishery products. Thus, the determination of relatively stable secondary products or termination products such as polymers, fluorescence and browning are suggested to be preferable for evaluating the oxidative deterioration in fish and fishery products (Figs. 5 and 6).

Table 2. Changes of amino acids composition (%) in the sardine (ordinary meat) during incubation with ethyl EPA at 5°C in the dark

Amino acid	Incubation days				
	0	4	6	10	14
Asp	10.2	10.2	10.3	9.8	10.0
Thr	5.1	5.0	5.1	4.8	4.8
Ser	5.1	4.9	5.3	4.8	4.7
Glu	13.5	13.1	11.8	12.1	12.6
Gly	8.8	8.9	8.6	8.7	8.6
Ala	8.8	8.2	8.6	8.9	10.0
Val	5.8	5.6	5.8	5.9	6.0
Met	1.3	1.7	2.2	2.2	1.7
Ile	4.0	3.9	4.2	4.1	4.3
Leu	8.3	8.4	8.5	8.4	8.6
Tyr	2.6	2.7	2.8	2.7	2.7
Phe	3.4	3.4	3.9	3.3	3.3
Lys	10.0	10.9	10.1	10.5	9.8
His	3.7	3.7	3.7	3.9	3.8
Arg	4.5	4.6	4.7	5.1	4.6
Pro	4.8	4.9	4.5	4.9	4.6

As described above, ethyl EPA incubated with fish tissues was rapidly oxidized to secondary products via HPOs. To find out whether the secondary products from ethyl EPA reacted with protein to form complexes or not, elements and amino acids compositions in ordinary meat of sardine were examined after incubation with ethyl EPA at 5°C in the dark. The results are shown in Tables 1 and 2. C, H and N composition of ordinary meat remained unchanged after storage with ethyl EPA for 8 days (Table 1). This result suggests that even though a great amount of secondary oxidation products was formed during storage for 8 days, a notable quantity of oxidized lipids were not incorporated into the protein portions of the sample. This observation is also

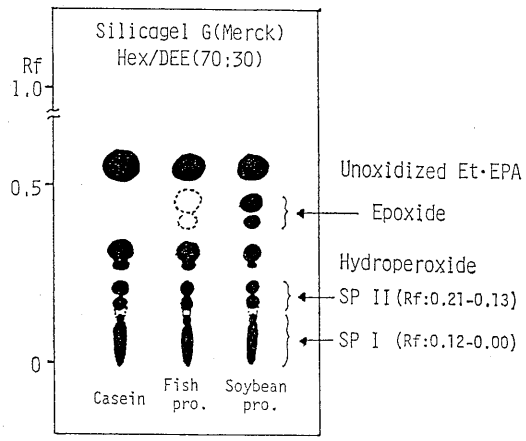

Fig. 8. Typical TLC patterns of ethyl EPA (Et·EPA) oxidized with various proteins at 5°C in the dark.

supported by the results of amino acid analysis of the meat after storage (Table 2), although more detail analysis on amino acid composition without acid hydrolysis will be also required, because acid hydrolysis is unsuitable for evaluating the oxidative damage of both methionine and tryptophan in proteins. These two amino acids, known to be modified or decomposed during acid hydrolysis,[28] have been reported to be susceptible to oxidative damage with oxidized lipids.[29] However, it may be possible that the secondary products of ethyl EPA react preferentially with free amino acids or amine (Figs. 5 and 6).

Fig. 8 shows the TLC patterns of ethyl EPA stored with various proteins at 5°C in the dark. Two spots appeared between unoxidized ethyl EPA and HPO are presumed to be epoxide from the Rf value[30] and the negative-coloring by 2,4-dinitrophenyl hydrazone reagent. SP I and II in Fig. 8 represent secondary products corresponding with hydroperoxy cyclic peroxides, and the mixture of polymers and polar monomers, respectively as described previously for oxidation in bulk.[3] Ethyl EPA incubated with soybean formed a great deal of epoxy compounds from the early stage of autoxidation, while in case of ethyl EPA oxidized with casein, the epoxy compounds were not detected in that stage of autoxidation. On the other hand, ethyl EPA oxidized with fish proteins formed little of the epoxy compounds from the early stage of autoxidation.

The time courses of oxidation products distribution of ethyl EPA stored with various proteins are shown in Fig. 9. Soybean remarkably promoted epoxide formation, the rate of was significantly suppressed by inactivating the enzymes by heat treatment. The catalytic conversion of linoleic acid hydroperoxides to epoxides was

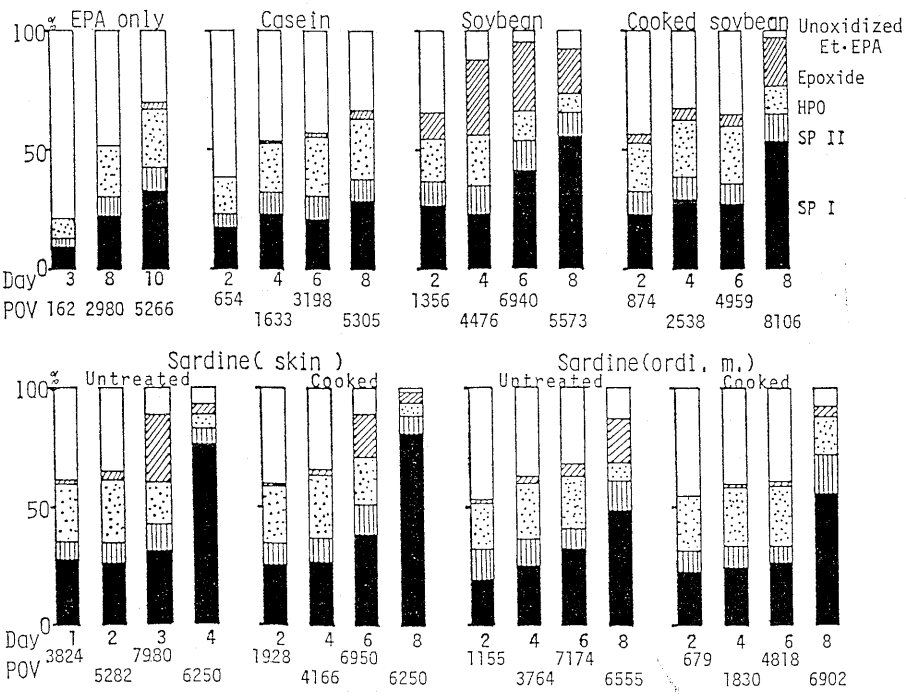

Fig. 9. Quantitative analysis of ethyl EPA autoxidized with various proteins at 5°C in the dark.

demonstrated in soybean lipoxygenase together with non-enzymic catalysts such as hemoglobin.[31] However, although the occurrence of the lipoxygenase-like prooxidant enzyme(s) is suggested in sardine skin (Figs. 1–4), no particular difference in epoxide formation was observed between raw and cooked sardine skins. Thus, the property of the assumed prooxidant enzyme in sardine skin is suggested to differ from that of lipoxygenase in soybean.

The prooxidant activity of skin, among the various sardine tissues examined, was the strongest. Prooxidants in the skin were assumed to be enzymes, labile to heat. HPO determination is less reliable as a quantitative measure of oxidation in fish and fishery products because of the instability of HPOs in fish model systems. On the other hand, the formation of secondary products or termination products such as polymers, fluorescent materials and browning matter were highly correlated with the oxidative deterioration of fish and fishery products. Further work is underway in our laboratory to clarify the structure and chemical characteristics of these compounds.

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