赤色魚類の体色変化に関する研究 VII

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Studies on the Discoloration of Red Fishes—VII.* Effect of Chemicals on Enzymatic Discoloration

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Fractionation and characterization of the enzyme responsible for the discoloration of the skins of red-colored marine fishes, was described in the previous paper.[1] Since the nature of the enzyme has been clarified to some degree, it was felt essential to study the effect of chemical agents on the enzyme activity in order to consider any preventive means for the discoloration of red fishes.

In the present report, a variety of antioxidants, reducing agents, enzyme inhibitors, neutral salts, and metallic salts were examined regarding their inhibitive or promotive effect on the enzymatic discoloration of the carotenoids present in the skin of red fishes.

Materials and Methods

Enzyme preparations: Crude enzyme preparations were made from two red fishes; gurnard, *Chelidonichthys kumu*, and rockfish, *Sebastes thompsoni*, by using ammonium sulfate fractionation as reported in the previous paper,[1] and the vacuum-dried specimens thus prepared were dissolved in 200-fold volumes of water.

Substrates and carotenoids: Methyl linoleate was used as a substrate for fish skin lipoxidase active substance. Astacene and tunaxanthin prepared from red fishes, were used as test carotenoids.

Determination of carotenoid discoloration: Most test reagents as listed in Tables 1～5 were dissolved in M/50 phosphate buffer solution of pH 6.90, while phosphate buffer-insoluble chemicals were dissolved in small amounts of acetone and diluted with 20-fold volumes of the phosphate buffer. Since some metallic compounds produced precipitates when they were added to phosphate buffer solution, distilled water was substituted in these cases. A series of different concentrations of these reagents was prepared, and added to the reaction mixtures containing 2 mg of enzyme, 4 mg of methyl linoleate, and 20～25 μg of carotenoid. Enzyme preparations used in experiments of Tables 1～3 was obtained from rockfish, and those in Tables 4～5 from obtained from gurnard. The reaction was performed for 60 min. at 25～30°C. Details of the other experimental conditions were the same as in the preceding paper.[1]

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Thus the minimum concentration to inhibit or to promote the discoloration activity was determined for each of these chemicals. All the determination were performed on duplicate samples and their average values were taken. Since the values for the same sample showed a certain range of deviation, the degree of the effect of these reagents on the enzyme activity was relatively expressed as follows: the discoloration of carotenoid in the run where water was substituted for enzyme solution was taken as 0% discoloration on one hand, and the discoloration by the enzyme without test reagent was taken as 100% on the other. If the discoloration with a given reagent was smaller than 5%, the author recorded the reagent to have shown a complete inhibition, and seven grades of score as indicated below were employed to discriminate the inhibitory effects of reagents on the basis of percentage discoloration.

Score of inhibition	Percentage of discoloration
5	0～ 5%
4	5～ 25%
3	25～ 50%
2	50～ 75%
1	75～100%
−1	100～125%
−2	more than 125%*

Remark: * indicates an enhancement effect for discoloration

Results and Discussion

Fat-soluble antioxidants: Butylhydroxyanisol (BHA), butylhydroxytoluene (BHT), n-propyl gallate (PG), iso-amyl gallate (IAG), and α-naphthylamine (NA) were tested. As indicated in Table 1, BHA and BHT revealed remarkable effects of inhibition on the discoloration and a concentration of 5×10^{-4}M was found sufficient to stop the enzyme action and even at a level of 5×10^{-6}M their inhibitory effects still remained. Since a satisfactory effect of inhibition was also noted in PG, IAG, and NA, these fat-soluble antioxidants seem to be effective anti-discoloration agents. According to RHEE's observations[2], antioxidants belonging to phenol group, in particular BHA and PG, exerted a strong inhibition on lipoxidase activity, while TAPPEL et al.[3] and SIDDIQI et al.[4] reported the strongest inhibition by nordihydroguaiaretic acid (NDGA) and PG next to NDGA.

The role of fat-soluble antioxidants may be interpreted by their action to retard the oxidation of unsaturated fatty acids, which in turn prevents the carotenoids from oxidative discoloration.

Water-soluble antioxidants and reducing agents: In Table 2, results of similar experiments using ascorbic acid, erythorbic acid, and their sodium salts, araboascorbic acid,

Table 1. Effects of fat-soluble antioxidants on the activity of lipoxidase-like enzyme from the skin of rockfish.
Results are given in terms of the score of inhibition as defined in the text.

Chemicals*	Concentration (Mol)		
	5×10^{-4}	5×10^{-5}	5×10^{-6}
IAG	4	3	2−1
PG	3	2	2
BHT	5	4	3
BHA	5	4	3
NA	4	2	2−1

* IAG: iso-Amyl gallate
PG: *n*-Propyl gallate
BHT: Butyl hydroxytoluene
BHA: Butyl hydroxyanisol
NA: α-Naphthylamine

Table 2. Effects of ascorbic acid, erythorbic acid, and their sodium salts, araboascorbic acid, and reducing agents on the activity of lipoxidase-like enzyme from the skin of rockfish.
Refer to Table 1 for expression of results.

Chemicals	Concentration (Mol)			
	2×10^{-2}	1×10^{-2}	4×10^{-3}	1×10^{-3}
Ascorbic acid	1	1		
Sodium ascorbate	1	1		
Erythorbic acid	1	1		
Sodium erythorbate	1	1		
Araboascorbic acid	1	1		
$NaNO_2$	−1	1		
Hydroquinone		4	4	3
Pyrogallol		5	5	4

sodium nitrite, hydroquinone, pyrogallol are indicated. No appreciable effects of inhibition were observed with ascorbic acid, erythorbic acid, and their sodium salts, araboascorbic acid, and nitrite. However, both hydroquinone and pyrogallol, particularly pyrogallol, were effective at a level of 10^{-3}M. NAGAYAMA et al.[5] tested lipoxidase action of mackerel liver extract and recognized a marked inhibition by ascorbic acid and hydroquinone respectively, but their result with ascorbic acid disagreed with that of the present experiment.

Enzyme inhibitors: Inhibitors employed generally in the test for enzyme inactivation such as cyanide, mercuric salt, azide, fluoride, thiourea, and hydrosulfite were examined. Table 3 shows a strong inhibition by KCN at a concentration of 10^{-2}M, but a very weak inhibition at 10^{-3}M. Mercuric chloride revealed the effect at 10^{-2}M, while the other

Table 3. Effects of enzyme inhibitors on the activity of lipoxidase-like enzyme from the skin of rockfish.
Refer to Table 1 for expression of results.

Chemicals	Concentration (Mol)		
	1×10^{-1}	1×10^{-2}	1×10^{-3}
KCN	5	4	1−2
NaF	1	1	1
NaN_3	2	1	1
$HgCl_2$	4	3	1
$CS(NH_2)_2$	2	1	1
$NaHSO_3$	−2	1	1

Table 4. Effects of neutral salts on the activity of lipoxidase-like enzyme from the skin of gurnard.
Refer to Table 1 for expression of results.

Chemicals	Concentration (Mol)		
	5×10^{-1}	1×10^{-1}	1×10^{-2}
NaCl	3	2	2
KCl	3	2	2
$CaCl_2$	2	1−2	1−2
$MgSO_4$	2	1−2	1−2
$Na_4P_2O_7$	2	1−2	1

reagents showed poor to no effect. Hydrosulfite exhibited a rather promotive effect on the discoloration. NAGAYAMA et al.[5] found also a strong inhibition by NaCN in the experiment with liver extract of mackerel. On the other hand, KHAN[6] was not able to show the inhibitory effect in fluoride and azide, agreeing to the present findings. Since cyanide has been known to inhibit the catalysis of lipid oxidation by haemin compound, the author would like to confirm the fact by using more refined specimen of the enzyme in the future.

Neutral salts: In Table 4, the effects of salts, including NaCl, KCl, $CaCl_2$, $MgSO_4$, and $Na_4P_2O_7$ are indicated. These salts seem to have some suppressive effect on the discoloration, though the activity may not be high enough. A little more obvious effect was noticed with NaCl and KCl than with magnesium or calcium salts. RAMSEY et al.[7] reported that tripolyphosphate was effective, but pryophosphate in the present test did not show inhibitory action at a concentration of M/10.

Metallic salts: The most remarkable effect was observed with stannous chloride as seen in Table 5 and it showed a complete suppression of discoloration at 10^{-3}M, and retained a marked inhibition at a level of 10^{-4}M. Owing to the reducing action of stannous chloride, it may have a similar mechanism of inhibition to pyrogallol and hydro-

Table 5. Effects of metallic salts on the activity of lipoxidase-like enzyme from the skin of gurnard.
Refer to Table 1 for expression of results.

Chemicals	Concentration (Mol)			
	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
$ZnSO_4$	−1	−1	1	1
$SnCl_2$	5	5	4	2
$NiCl_2$	1	1	1	1
$CuSO_4$	−2	−2	−2	−2
$CuCl_2$	−2	−2	−2	−2
$FeCl_2$	−2	−2	1	1
$FeCl_3$	−2	−1	1	1
$FeSO_4(HN_4)_2SO_4$	−2	−2	−2	−1

quinone. Most metallic salts except $NiCl_2$, especially copper salts, revealed promotive effect at lower concentration. These results would suggest that minor contamination of these metallic ions could cause a promotion of the discoloration of red fishes in the practice of handling. It should be remarked that the control run in this experiment, to which no enzyme preparation was added, however, exhibited also the discoloration. So, under the experimental condition, promotive effect should have included both enzyme action and the action due to the presence of the metallic ions. Some additive effect by these two factors should be taken into consideration.

Summary

A series of experiments was conducted on the effect of chemical agents on the activity of enzyme preparation from the skins of red fishes.

1. A marked inhibitory effect was observed with fat-soluble antioxidants, particularly with BHA and BHT.

2. Almost none of effect was seen with ascorbic acid and its isomers or sodium salts. However, reducing agents such as pyrogallol and hydroquinone showed strong inhibition.

3. A weak effect of inhibition was observed with neutral salts at higher concentrations.

4. Potassium cyanide, followed by mercuric chloride, indicated strog inhibition among the enzyme inhibitors tested.

5. The most active inhibitor was stannous chloride in the metallic salts so far tested. But, copper and iron salts, in particular copper salts, gave a promotive effect.

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