Characterization of Partially Purified Lipoxygenase of Mackerel Gill

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Lipoxygenase of mackerel gill exhibited the highest reactivity toward eicosapentaenoic acid (EPA) followed by arachidonic acid, linoleic acid. The optimum pH were pH 4.5, 5.0 and 4.8 for EPA, arachidonic acid and linoleic acid, respectively. The enzyme was the most stable at pH 5.5. Optimum temperature was 25℃ for all substrate fatty acids. For linoleic and arachidonic acids the highest thermal stability was observed at 8℃; whereas, for (EPA) at 20℃. Optimum ionic strength was 0.22*M*. Sn^{2+}, vitamin E and catechin completely inhibited the enzyme at the concentration of 1.0*mM*. Molecular weight of the enzyme was 42,000 dalton.

Introduction

As fish and seafood products have increasingly been acknowledged as wholesome foods, the quality of these products has been the major issue of manufacturers as well as consumers. However, high degree of unsaturation of fish lipids easily causes the deterioration by oxidation through the unappropriate handling proccessary and processing. Fish lipids, in particular, are very unstable upon oxidation and this directly relates to the quality deterioration (McDonald *et al.*, 1979). To retard or minimize the quality deterioration, it would be better measure to control the initial stage of lipid oxidation.

Upon harvesting fish damage in tissue will lose its control over certain enzymes such as lipoxygenase of fish gill and skin (German and Kinsella, 1985, 1986), peroxidase of fish blood (Kanner and Kinsella, 1983), and microsomal NADH peroxidase of fish muscle (Slabyj and Hultin, 1984); thereby, the oxidation may be initiated. Especially lipoxygenase in fish gill and skin reportedly initiate lipid oxidation of polyunsaturated fatty acids to produce unstable hydroperoxides (German and Kinsella, 1985, 1986). These hydroperoxides as pontential precursors causing off-flavors and adversely affect taste and smell of fish (Josephson *et al.*, 1984).

The properties of lipoxygenase draws practical interest as they play potential role in generating oxidative off-flavors in fish and seafood products (Hsieh *et al.*, 1988). To alleviate this problem, lipoxygenase (E.C. 1.13.11.12: linoleate: oxygen oxidoreductase) present in fish should be characterized.

This present study was undertaken to determine the properties of lipoxygenase in gill of mackerel to extend the shelf life.

Materials and Methods

Partial purification of lipoxygenase in gill tissue

Samples of gill tissue were carefully excised from mackerel. In 4 volumes of 0.05*M* potassium phosphate buffer (pH 7.4), gill tissue was homogenized and centrifuged at 10,000*g* and 4℃ for 20*min*. The supernatant fraction was used as the crude enzyme source. Ammonium sulfate fractionation was carried out by 20% increment of saturation. After addition of ammonium sulfate, sample was

centrifuged at 10,000*g* and 4℃ for 20*min*. Precipitate was dissolved with 0.02*M* sodium acetate buffer (pH 5.5). Fractionation was undertaken succesively. Lipoxygenase (LOX) activity toward linoleic acid was determined for each fraction.

For second purification stepwise separation using DEAE-Sepharose was employed. DEAE-Sepharose (20*mL*) was suspended with 0.02*M* sodium acetate buffer (pH 5.5) and vortexed for 5*min* followed by centrifugation at 6,000*g* and 4℃ for 10*min*. Resultant supernatant was decanted and the same buffer was added. The same procedure was repeated for 10 times to equilibrate ion exchanger. Crude enzyme pool (1.0*mL*) was added to ion exchanger and mixed well. After 10*min* centrifugation, 0.02*M* sodium acetate buffer (pH 5.5) containing 0.1*M* NaCl was added to the vesin. Employing the same procedure supernatant was obtained at the increment of 0.1*M* NaCl. Each pool was assay for lipoxygenase activity.

Lipoxygenase assay

The activity of lipoxygenase was determined spectrophotometrically by the method of Kim and Lee (1987). Stock solution for substrate was 4.885 *mM* linoleic acid, arachidonic acid, or eicosapentaenoic acid (EPA) and working solution was prepared by mixing stock solution (5*mL*), 0.1*M* buffer (50*mL*), 20% Tween 20, deionized water (44*mL*). Working solution (3.0*mL*) in cuvet was aerated for 3 min and then enzyme (200$\mu\ell$) was added to initiate the reaction. Increase in absorbance at 234*nm* was recorded as a function of reaction time with double-beam UV/visible spectrophotometer. Enzyme activity was calculated from the linear portion of the recorded kinetic curves. One unit (U) corresponed to 0.001 O.D unit/min (Kim and Lee, 1987).

Effect of pH

The effect of pH on lipoxygenase activity was determined by carrying out the assay using 0.1*M* sodium acetate buffer (pH 3.8~5.5) and sodium phosphate buffer (pH 6.0~8.0). The enzyme assay proceeded using linoleic acid, arachidonic acid, or EPA at 25℃ and analysis of product formation was performed as stated in lipoxygenase assay. Stability of lipoxygenase to pH was determined at different pHs using the buffer solutions described above. Enzyme(1.0*mL*) was dissolved in buffer solution (1.0*mL*) and incubated at 5℃ for 20h. Lipoxygenase activity was determined as the above.

Effect of temperature

To determine the optimum reaction temperature, the same assay method was employed at temperatures from 20 to 40℃. Thermal stability was tested by incubating the enzyme solution at temperatures from 8 to 60℃ for 30*min* followed by lipoxygenase assay.

Effect of metal ions and inhibitors

The effects of metal ions and inhibitors were examined by mixing them with enzyme preparations. The standard assay method was used under the optimum conditions.

Molecular weight determination

Partially purified lipoxygenase was separated by HPLC using Protein PAK 125 gel filtration column. Flow rate and mobile phase were 1.0*mL/min* and 0.2*M* Tris buffer (pH 7.5), respectively. Each peak was assay for lipoxygenase activity and molecular weight standard was also applied to determine the molecular weight of lipoxygenase.

Results and Discussion

Partial purification of lipoxygenase

Extreme care was required to separate and purify lipoxygenase from gill tissue of mackerel because the enzyme easily inactivated when exposed to air. Conventional column chromatography could not be employed due to rapid inactivation of the enzyme. Thus batch separation using DEAE-Sepharose was followed by ammonium sulfate fractionation. As shown Table 1, most activity was obtained from the fraction with saturation of 40~60%. Pre-

Table 1. Ammonium sulfate fractionation of lipoxygenase in gill tissue of mackerel at 4℃

Saturation	Activity(U/*mg* protein)
0~ 20	1.74
20~ 40	2.78
40~ 60	69.79
60~ 80	3.47
80~100	0.00

cipitate of the above fraction was diluted with 0.02 *M* sodium acetate buffer(pH 5.5) and dialyzed against the same buffer. The dialyzate was used for further purification. Most enzyme activity was eluted by the same buffer containing 0.4*M* or 0.5*M* NaCl (Table 2). Therefore, both fraction were pooled and used for this study.

Table 2. Stepwise separation of lipoxygenase fractions ammonium sulfate fractionation using 0.02*M* sodium acetate buffer(pH 5.5) containing NaCl of different concentration at 4℃

NaCl(M)	Activity(U/*mg* protein)
0.1	0.00
0.2	0.00
0.3	0.35
0.4	26.04
0.5	45.14

Effect of pH on the activity and stability of lipoxygenase

Mackerel lipoxygenase showed optimum activity at 4.5, 4.8 and 5.0 toward EPA, linoleic acid and arachidonic acid, respectively (Fig. 1). The enzyme was less sensitive to pH change in linoleic acid than other substrates. Activity toward linoleic acid was seldom studied with fish lipoxygenase. However, a similar optimum pH was observed among lipoxygenase from potato tubers(Kim *et al.*, 1987; Hong and Song, 1992). Unlike trout gill lipoxygenase the enzyme showed optimum pH of pH 5.0 toward arachidonic acid. In addition activity toward arachidonic acid was reduced considerably at pH 6.0 or over and 52.9% of activity was observed at pH 4.0. With EPA the enzyme showed a similar pattern but pH was shifted from pH 5.0 to pH 4.5 and over pH 6.0 any significant activity was not observed. Considering gill lipoxygenase of rainbow trout showed optimum pH of pH 7.5 using arachidonic acid, EPA and docosahexaenoic acid (Heish *et al.*, 1988). Mackerel gill lipoxygenase definitely had different

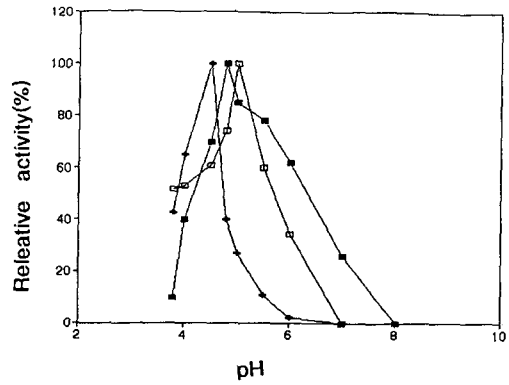

Fig. 1. Effect of pH on the activity of lipoxygenase purified partially from mackerel gill at 25℃.
(■, linoleic acid; □, arachidonic acid; +, eicosapentaenoic acid)

characteristics. EPA exerted the narrowest range of pH optimum followed by arachidonic acid and linoleic acid. This result implied the possible relationship between the degree of unsaturation and lipoxygenase activity.

The enzyme was the most stable at pH 5.5 regardless of substrate used. In case of linoleic acid and arachidonic acid, residual activities were 70% and 63%, respectively. However, only residual

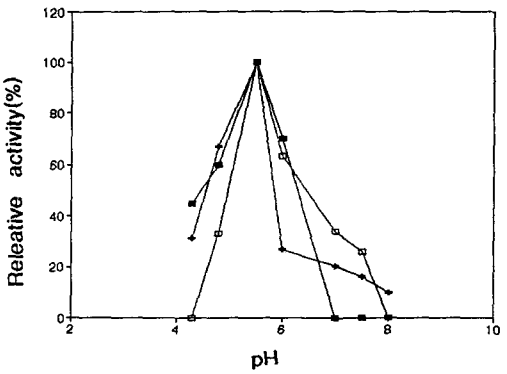

Fig. 2. The pH stability of lipoxygenase from purified partially from mackerel gill at 25℃.
(■, linoleic acid; □, arachidonic acid; +, eicosapentaenoic acid)

activity of 27% was observed for EPA (Fig. 1). In addition activities were completely lost over pH 7.0 for linoleic acid and pH 4.3 and 8.0 for arachidonic acid. Broad pH stability was observed when using EPA as a substrate. This result could be utilized for purification and storage of the enzyme.

Effect of temperature on the activity and stability of lipoxygenase

Because of the potential role of lipoxygenase in causing off-flavors, practical methods to minimize lipoxygenase activity are of interest. Therefore, the effects of temperature on lipoxygenase were studied. For all substrates used the optimum temperature was 25℃ (Fig. 3). This coincided with the result of trout gill lipoxygenase using EPA(German *et al.*, 1986). However, this comparison was not accurately made because of insufficient published data. Complete inactivation was observed over 35℃ for linoleic acid and arachidonic acid. In contrast, residual activity for EPA was 29% at 40℃, indicating double bonds of EPA easily lead oxygenation by lipoxygenase compared to linoleic acid or arachidonic acid. Lipoxygenase activity was completely lost by heating at 50℃ or over for 30*min* (Fig. 4). There were 78% of lipoxygenase activity for EPA and 100% for linoleic acid and arachidonic acid at 8℃. The high activity of mackerel lipoxygenase at near refrigeration condition suggests that the inhibition of fish lipid oxidation may occur rapidly under refrigeration. The instability of gill lipoxygenase above 50℃ may provide an approach for controlling enzyme activity and help improve flavor stability and the quality of fish and sea food products.

Effect of ionic strength on the activity of lipoxygenase

The ionic strength of buffer solution often affects the activity of enzymes. To determine optimum ionic strength buffer solution of different concentrations were used for the enzyme assay under optimum pH and temperature (Fig. 5). Regardless of substrate used, optimum ionic strength was 0.22*M* and abrupt decline in activity occurred at 0.50*M*. Activity toward EPA, in particular, was lost completely at 0.97*M*, providing the importance of ionic strength during enzyme activity measurement. Low ionic strength did not affect the enzyme activity considerably compared to high ionic strength. Ions around the active site of the enzyme are likely to provide microenvironment for the catalytic reactions. Furthermore, few researches were carried out concerning the effect of ionic strength on lipoxygenase. This result strongly suggest that study on ionic strength should be carried out.

Substrate specificity

EPA is abundant in fish lipids; the lipoxy-

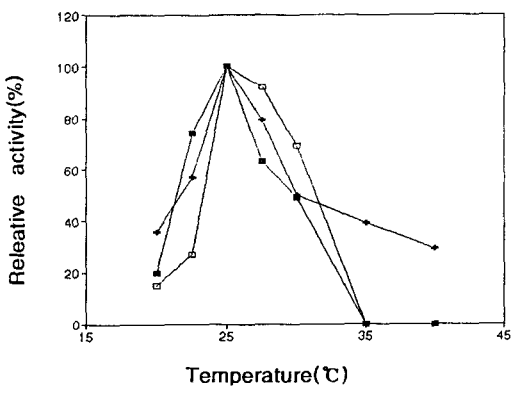

Fig. 3. Effect of temperature on the activity of lipoxygenase purified partially from gill of mackerel. (■, linoleic acid; □, arachidonic acid; +, eicosapentaenoic acid)

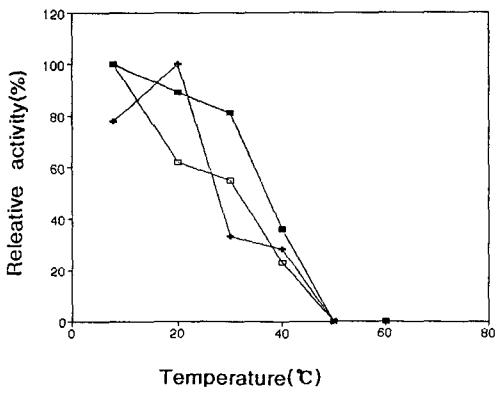

Fig. 4. Thermal stability of lipoxygenase from purified partially from gill of mackerel. (■, linoleic acid; □, arachidonic acid; +, eicosapentaenoic acid)

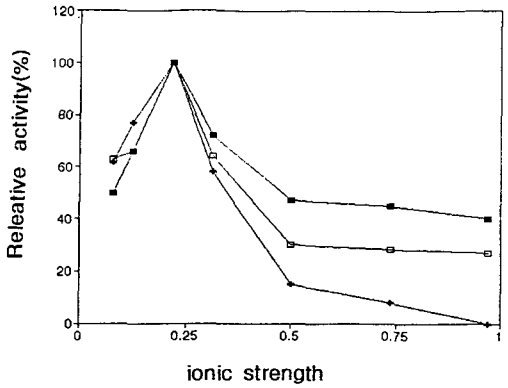

Fig. 5. Effect of ionic strength on the activity of lipoxygenase purified partially from gill of mackerel. (■, linoleic acid; □, arachidonic acid; +, eicosapentaenoic acid)

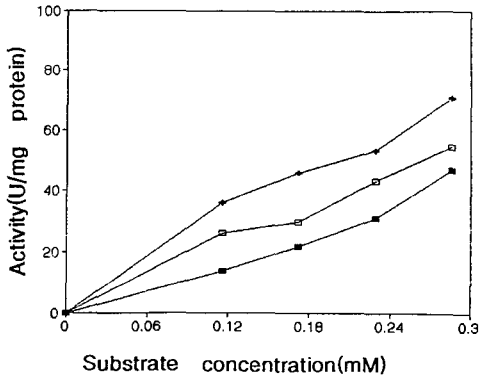

Fig. 6. Substrate specificity of lipoxygenase purified partially from gill of mackerel. (■, linoleic acid; □, arachidonic acid; +, eicosapentaenoic acid)

genase of mackerel gill exhibited significant selectivity toward substrate types (Fig. 6). The enzyme showed the highest reactivity toward EPA followed by arachidonic acid and linoleic aicd. This result was quite different from trout gill lipoxygenase which exhibited no selectivity for EPA and arachidonic acid (Heish *et al.*, 1988). However, the enzyme also showed much less activity toward linoleic acid. In case of 12-lipoxygenase from rat lung, little activity was shown toward linoleic acid (Yokoyama *et al.*, 1983). Therefore, high unsaturation may enhance the activity of mackerel gill lipoxygenase.

Effect of metal ions on lipoxygenase activity

The enzyme activity was significantly increased by the addition of Ca^{2+} and Fe^{2+} for all substrates; it was completely inhibited by Sn^{2+} (Table 3). Active site of the enzyme might be considerably affected by Sn^{2+}. This is similar to human platelet lipoxygenase (Wallach and Brown, 1981). Fe^{2+} caused an dramatic increase in lipoxygenase activity which was apparently due to Fe^{2+}-catalyzed autoxidation. However, Cu^{2+} decreased the activity considerably. Na^{2+} did not affect the activity; in con-

Table 3. The effect of metal ions on the activity of lipoxygenase toward different substrates.

Metal ions (1.0*mM*)	LA	AA	EPA
		Relative activity(%)	
control	100	100	100
Mg^{++}	106	117	109
Ca^{++}	137	136	128
Co^{++}	128	162	143
Mn^{++}	106	122	113
Zn^{++}	111	100	128
Cu^{++}	71	74	70
Fe^{++}	122	133	120
Ni^{++}	91	112	114
Sn^{++}	9	0	0
Na^{+}	99	98	100
K^{+}	118	121	133

LA : linoleic acid; AA : arachidonic acid; EPA : eicosapentaenoic acid

Table 4. The effect of various inhibitors on the activity of lipoxygenase toward different substrates

Inhibitors (1.0*mM*)	LA	AA	EPA
		Relative activity(%)	
control	100	100	100
Vitamin E	0	0	0
BHA	90	88	0
BHT	85	85	0
Catechin	0	0	0
Propyl gallate	77	79	0
Vitamin C	60	63	0
EDTA	0	56	78
Phosphatidylcholine	48	50	51

LA : linoleic acid; AA : arachidonic acid; EPA : eicosapentaenoic acid

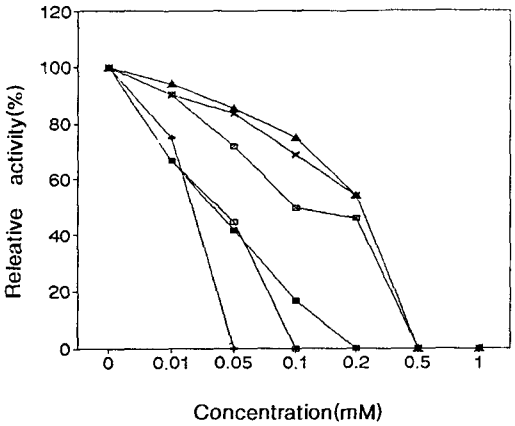

Fig. 7. Effect of catechin and Vitamin E on the partially purified mackerel gill lipoxygenase activity toward each substrate.
(■, ▲, linoleic acid; □, +, arachidonic acid; +, □ eicosapentaenoic acid)

strast, K^+ enhanced the activity considerably toward linoleic acid and arachidonic acid. Moreover, the type of metal ion present in food could affect the action of the enzyme significantly.

Effect of various inhibitors on lipoxygenase activity

Vitamin E and catechin completely inhibited the enzyme activity for all substrates. EPA was the most susceptible to the inhibitors. Any significant activities toward EPA were not observed upon addition of vitamin E, BHA, BHT, catechin, propyl gallate, vitamin C. EDTA alone was able to inhibit the activity in substantial amount; however the amount of inhibition was reduced as the degree of unsaturation increased. Phosphatidylcholine also exhibited the inhibition substantially.

Molecular weight determination

Lipoxygenase loses activity quite rapidly upon separation and purification. To determine the molecular weight of the enzyme protein PAK 125 HPLC column was employed because the short elution time would reduced the loss of activity remarkably. Lipoxygenase activity was measured for each separation fraction. The resulting lipoxygenase peak was compared with protein molecular weight markers and found to be 42,000 dalton. The lipoxygenase was very small compared to human platelet lipoxygenase of 100,000 dalton and 16.000 dalton (Siegel *et al.*, 1980), reticulocyte lipoxygenase of 78,000 dalton and potato lipoxygenase of 94,000 dalton (Lagutina *et al.*, 1990). Molecular weight of other fish lipoxygenase were not available.

Conclusion

These results demonstrate the potential role of mackerel gill lipoxygenase in initiating fatty acid

oxidation in fish tissue. Under postharvest conditions, the release of tissue lipoxygenase from endogenous constraints could result in generation of significant quantities of reactive lipid hydroperoxides. In the presence of metal ions such as ferrous, calcium and cobalt ions, hydroperoxides could serve as potential sources of free radical species (Kanner *et al.*, 1987) which in turn further catalyze autoxidation and generate off-flavors. Further these product could cause color and quality deterioration in fish and seafood products (Simic and Karel, 1980). Proper harvest method avoiding injury, blanching and control of pH may alleviate the loss of quality. The efficient measure to keep the high quality, inhibitors such as vitamin E and catechin could be applied in appropriate methods.

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고등어 아가미에서 추출한 Lipoxygenase의 특성

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고등어 아가미에서 추출한 리폭시게나아제는 에이코사펜타엔산에 대해서 활성이 가장 높았으며, 그 다음으로 아라키돈산, 리놀산순으로 활성을 나타내었다.

적정 pH는 에이코사펜타엔산, 아라키돈산, 리놀산에 대하여 각각 pH 4.5, 5.0, 4.8이었다. 또한 이 효소는 모든 기질에 대하여 pH 5.5에서 가장 안정하였다. 적정온도는 기질의 종류에 상관없이 25℃였으며, 리놀산, 아라키돈산의 경우 8℃에서 열안정성이 가장 높았다. 반면, 에이코사펜타엔산의 경우, 20℃에서 안정성이 가장 높았다. 적정이온강도는 모든 기질에 대하여 0.22*M*이었다. Sn^{2+}, vitamin E, catechin은 1.0*mM*에서 이 효소의 활성을 완전히 저해시켰다. 이 효소의 분자량은 42,000달톤이었다.