Effects of Protector and Hydroxyapatite Partial Purification on Stability of Lipoxygenase from Gray Mullet Gill

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Three lipoxygenase (LOX, EC 1.13.11.12) isozymes, 5-, 12-, and 15-LOX, were found in the gill of cultured gray mullet (*Mugil cephalus*). The major LOX in mullet gill was the 12-isozyme followed by the 15-isozyme. Crude mullet gill extract (using 0.05 M potassium phosphate buffer, pH 7.0) catalyzed oxidation of arachidonic acid at an oxygen consumption rate of 30.6 ± 7.1 nmol min^{-1} (mg of protein)$^{-1}$ at optimal pH 7.0 and 25 °C. The half-life of LOX was 7–80 h at 4 °C depending on the postmortem time of the mullet. The mullet gill LOX was a very labile protein and lost activity during or after purification. Adding imidazole, histidine, glutathione, and trimethylamine increased the stability of LOX significantly. Optimal quantity of imidazole was 2.5 mmol/g of protein. Hydroxyapatite purification resulted in a 10.75-fold specific activity and maintained >80% activity at 4 °C for 3 days.

INTRODUCTION

Lipoxygenase (LOX) (EC 1.13.11.12) catalyzes the incorporation of oxygen into polyunsaturated fatty acids to form hydroperoxide derivatives. Lipoxygenase contains an unusual non-heme iron atom (Boyington et al., 1993a,b). The active form of the enzyme contains iron in the ferric state (Funk et al., 1990). In aquatic animals, LOX activity was identified in coral (Bundy et al., 1986), in skin and gill of trout (Hiseh et al., 1988; German and Creverling, 1990), ayu (Zhang et al., 1992a), sardine (Mohri et al., 1990, 1992), and freshwater mussel (Hagar et al., 1989), in eggs of starfish (Meijer et al., 1986) and sea urchin (Hawkins and Brash, 1987), and in hemolymph of shrimp (Kuo and Pan, 1992; Kuo et al., 1994).

The LOX activity was known to be "suicidal" (Hartel et al., 1982; Yamamoto and Ishimura, 1991). The lipoxygenase enzymes from land and aquatic animal tissues have been widely recognized as unstable during purification. Most column steps resulted in extremely poor yields (German and Creveling, 1990). LOX from human platelet (Sigal, 1991), rabbit reticulocytes (Schewe et al., 1981), trout gill extract (Hsieh et al., 1988), shrimp hemolymph (Kuo and Pan, 1992), and ayu skin (Zhang et al., 1992a) showed similar patterns.

The objective of the present work is to minimize loss of lipoxygenase activity during purification by using an enzyme protector to prevent suicidal inactivation of the enzyme isolated from aquatic animals for further purification and characterization. In addition, lipoxygenase activities were identified in seafood waste, e.g., shrimp hemolymph, and gray mullet gills. They were able to produce seafood aroma upon reaction with polyunsaturated fatty acids (Pan and Kuo, 1994). A stable and purified LOX may enhance its application recovered from seafood waste for aroma development.

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MATERIALS AND METHODS

Preparation of Gill Tissue and Crude Gill Extract. Cultured gray mullet (*Mugil cephalus*), averaging 1.5 kg per fish, were harvested in November when the size of the ovary (gonadosomatic index) was maximal. For each preparation, gill tissue from three to four fish was excised from freshly killed gray mullets and was stored in liquid nitrogen until assayed. Gill tissue was homogenized in 4 volumes of cold 0.05 M potassium phosphate buffer, pH 7.0, containing 1 mM reduced glutathione. The homogenate was centrifuged at 4 °C for 30 min at 20000g to spin down debris, organelles, and membrane fractions. This supernatant was used as crude enzyme extract similar to the studies done on trout gill (Hsieh et al., 1988) and shrimp hemolymph (Kuo and Pan, 1992).

Assay of Lipoxygenase Activity. The oxygen consumption of crude gill extract catalyzed arachidonic acid oxidation was measured by polarographic analysis (Hsieh et al., 1988). Crude mullet gill LOX, 5–10 mg, was equilibrated in 0.05 M potassium phosphate buffer, pH 7.0. Reaction was initiated by the addition of arachidonic acid (400 μM), and the oxygen concentration was measured in a thermostatic incubation cell (20 mm × 68 mm) at 25 °C by a biological oxygen monitor (YSI 5300, Yellow Springs, OH) equipped with a YSI Clark oxygen probe. A data acquisition system (Notebook for IBM PC model) was used to record the change of oxygen concentration.

Five milliliters of bovine hemoglobin (Sigma, St. Louis, MO) in 0.05 M potassium phosphate buffer, pH 7.0 (10 mg/mL), was mixed with arachidonic acid to 400 μM. The mixture was reacted at 25 °C for 10 min. Oxygen consumption was measured to determine the catalytic effect of hemoglobin on autoxidation of arachidonic acid to distinguish whether the presence of hemoglobin interfered with LOX-catalyzed oxidation of arachidonic acid.

Lipoxygenase activity was also assayed spectrophotometrically according to the method of Surrey (1964). The assay mixture contained 0.05 M potassium phosphate buffer, pH 7.0, 200 μM arachidonic acid, Tween 20 at 0.12% (w/v), and 0.2 mL of enzyme solution (0.5–1 mg of protein/mL) in a final volume of 1 mL. The activity was expressed as the increase of the absorbance at 234 nm ($A_{234 \text{ nm}}$) of the enzyme solution at 20 °C.

Protein concentration of mullet gill LOX was determined according to the method of Bradford (1976) with bovine serum albumin (Sigma) as standard.

Identification of Isozymes. The gill extract was incubated with arachidonic acid (200 μM) at 25 °C for 5 min. The

Table 1. Oxygen Consumption Rate during Oxidation of Arachidonic Acid Catalyzed by Mullet Gill Extract

	O$_2$ consumption rate [nmol min^{-1} (mg of protein)$^{-1}$]	rel act. (%)	half-life (h)
mullet gill extract[a]	30.6 ± 7.1	100	7–80
trout gill extract[b]	20.0	65.36	1.5
shrimp hemolymph[c]	1.3	4.25	12

[a] Oxygen consumption rate and half-life, the time lapse at 4 °C that 50% of LOX activity was inactivated, were calculated from Figures 2–6. [b] Hsieh et al. (1988). Extracted with phosphate buffer (0.05 M, pH 7.4) and determined by polarography using arachidonic acid as substrate at 25 °C. [c] Kuo et al. (1994). Shrimp hemolymph was collected into 0.9% NaCl containing the antico-agulant 0.02 M EDTA. Determined by polarography using arachi-donic acid as substrate at 25 °C.

reaction was terminated by adjusting the pH to 3.0 with 6 N HCl. The reaction products were extracted with 2 volumes of ethyl acetate and methylated with diazomethane (Ayorinde et al., 1989). The methylated compounds were absorbed on a solid phase extraction column (J&W Scientific, Folsom, CA) (German and Berger, 1990). The HETE products were eluted with a hexane/ether mixture (75:25 v/v) and were subjected to HPLC analysis.

High-pressure liquid chromatographic analyses were per-formed on a Lichrospher 100 RP-18 column (25 cm × 4 mm, 5 μm) equipped with a pump (Waters, Model 510, Milford, MA) and a UV detector (Waters, Model 490E), monitored at 235 nm. The compounds were eluted using a solvent system of methanol/water (75:25 v/v) buffered with 5 mM ammonium acetate containing 0.5 mM EDTA to an apparent pH of 5.7. Retention times were compared and standardized with 5(*S*)-, 12(*S*)-, and 15(*S*)-HETE (Cayman Chemical Co., Ann Arbor, MI).

Hydroxyapatite (HPT) Chromatography. The crude gill extract was applied on a HPT (Macro-Prep Ceramic, Bio-Rad, Hercules, CA) column (50 mm × 10 cm) pre-equilibrated with 0.08 M potassium phosphate buffer, pH 7.0, with 1 mM reduced glutathione. After sample application, the column was washed with 480 mL of the equilibrating buffer. Then a linear gradient elution was performed ranging from 0.08–0.24 M and from 0.24–0.4 M. The flow rate of the column was maintained at 1.20 mL/min. Fractions of 6.0 mL were collected during the sample application and the subsequent elution. The enzyme was eluted at a buffer concentration of approximately 0.14 M.

RESULTS AND DISCUSSION

Identification of Isozymes. LOX activity was found in the gill of gray mullet, the extract of which catalyzed the oxidation of arachidonic acid at an oxygen consumption rate of 30.6 ± 7.1 nmol min^{-1} (mg of protein)$^{-1}$ (Table 1). Five preparations of gill LOX extract resulted in oxygen consumption rates ranging from 20 to 37 nmol min^{-1} (mg of protein)$^{-1}$ (Table 1), shown as the control curves in Figures 2–6. The mullet gills were sampled from cultured fish of the same age from the same pond during the same harvest. There-fore, the postmortem time is probably the major factor causing the variation in gill LOX activity.

By the same extraction method, activity of trout gill LOX was 20 nmol min^{-1} (mg of protein)$^{-1}$ (Hsieh et al., 1988) and that of shrimp hemolymph LOX was 1.32 nmol min^{-1} (mg of protein)$^{-1}$ (Kuo and Pan, 1992; Kuo et al., 1994). The catalytic rate of mullet gill LOX was about 1.53 and 23.2 times that of the crude extract of trout gill 12-LOX and shrimp hemolymph 12-LOX (Table 1).

The oxidation products were 5-, 12-, and 15-HETE (hydroxyeicosatetraenoic acid) on the basis of retention time in HPLC analysis (Figure 1). According to the
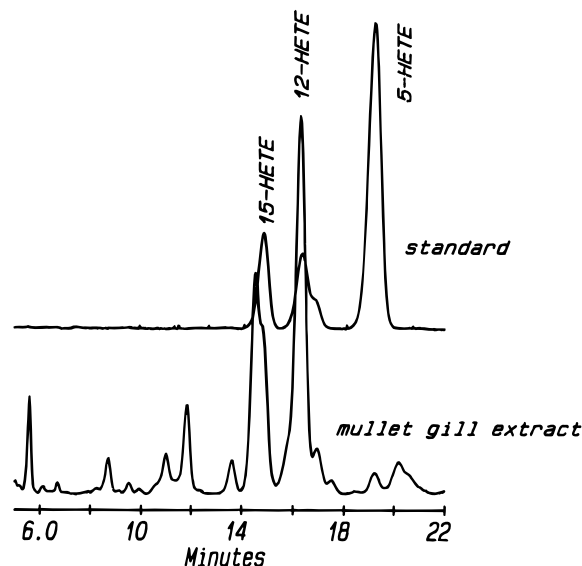

Figure 1. HPLC chromatograms of hydroxy fatty acids formed by gray mullet gill extract. Arachidonic acid (100 μM) was used as substrate.
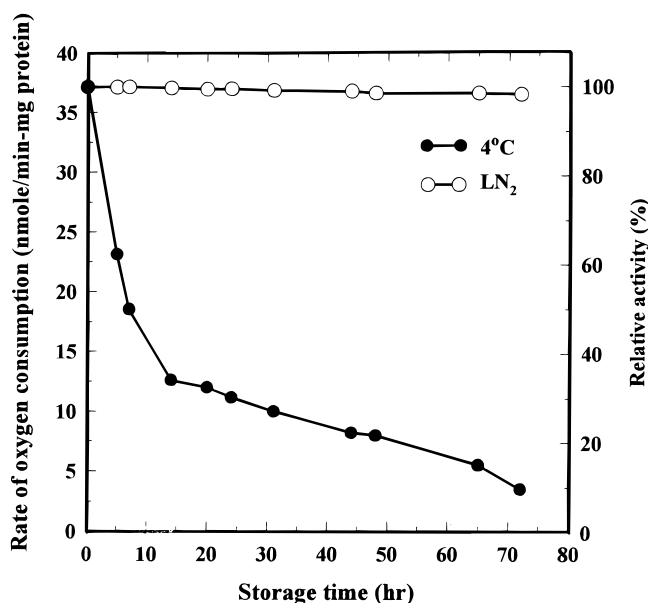

Figure 2. Storage stability of mullet gill lipoxygenase extract during storage at 4 °C and in liquid nitrogen. The activity was assayed at 25 °C.

peak height, the major LOX activity in mullet gill was the 12-isozyme followed by 15-LOX (64% of the 12-LOX activity), and the minor activity was from 5-LOX (4% of the 12-LOX) and possibly other isozymes.

Storage Stability of LOX. The LOX activities decreased rapidly first and then leveled off when the rate of oxygen consumption reached approximately below 15 nmol min^{-1} (mg of protein)$^{-1}$ (Figures 2–6). When mullet gill extracts were stored at 4 °C, about 40% of the total activity was lost within 5 h (Figure 2). The half-life was determined as the time lapse that 50% of the LOX activity was inactivated, being 7–80 h (Table 1) as calculated from Figures 2–6. Longer half-life seemed to be associated with lower initial activity.

Although mullet gill LOX activity was completely preserved when the gill tissue was stored in liquid nitrogen, the crude LOX extract was found to be unstable. Various protective compounds including imi-dazole, histidine, glutathione, and trimethylamine were
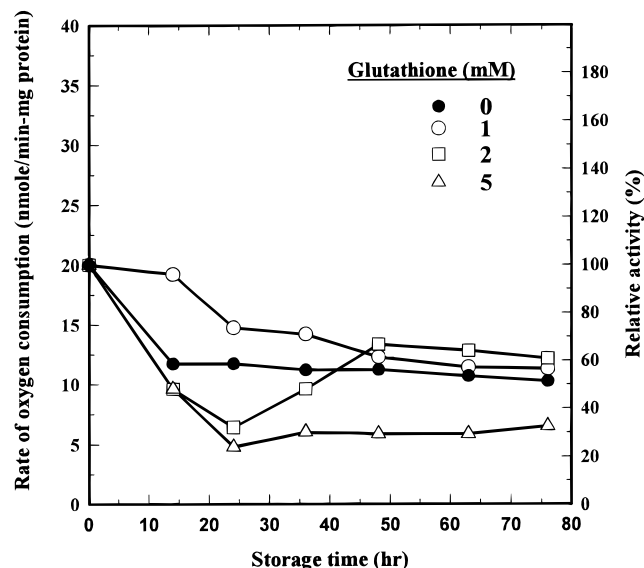
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Figure 3. Effects of glutathione on storage stability of mullet gill lipoxygenase during storage at 4 °C. The activity was assayed at 25 °C.

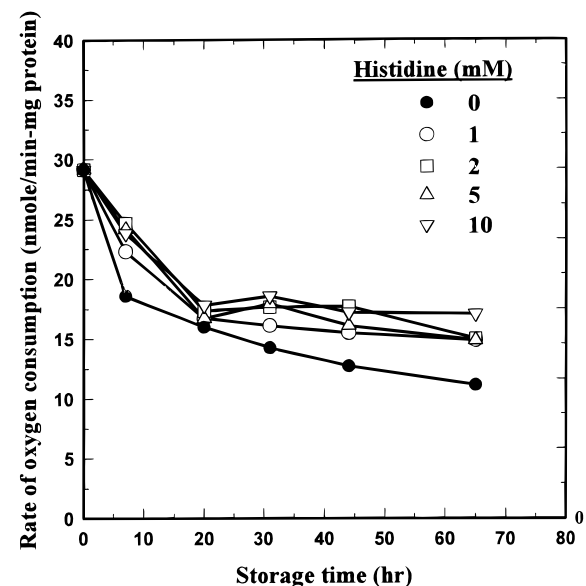**Figure 5.** Effects of imidazole on storage stability of mullet gill lipoxygenase during storage at 4 °C. The activity was assayed at 25 °C.

Figure 4. Effects of histidine on storage stability of mullet gill lipoxygenase during storage at 4 °C. The activity was assayed at 25 °C.

Figure 6. Effects of trimethylamine on storage stability of mullet gill lipoxygenase during storage at 4 °C. The activity was assayed at 25 °C.

tested to minimize inactivation of gill LOX extract during storage at 4 °C.

Glutathione as LOX Protector. Self-inactivation of LOX is likely caused by hydroperoxides generated from LOX when the hydroperoxides reached a concentration higher than 1 μM (Hartel et al., 1982; Hsieh et al., 1988). Glutathione reduces hydroperoxide to its stable hydroxy analogues and thus removes excessive hydroperoxides to keep the LOX active. The stability and net turnover of the LOX were significantly improved by the addition of glutathione (German and Kinsella, 1986). The half-life of trout gill 12-LOX at 4 °C was 1.5 h and increased to 4 h in the presence of 1 mM glutathione (Hsieh et al., 1988).

The effect of reduced glutathione on the storage stability of LOX at 4 °C was examined. Addition of 1 mM reduced glutathione significantly improved the stability of gill LOX (Figure 3). The protective effect was very pronounced, being 40% higher than the control within 15 h, and was still very significant up to 40 h.
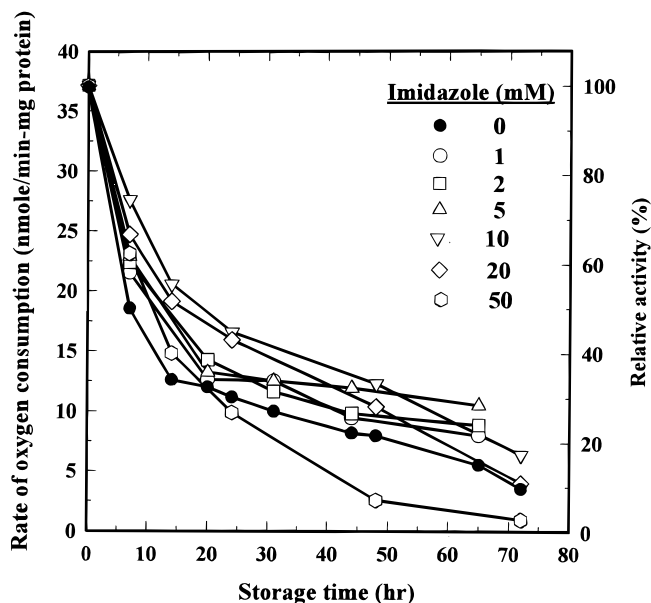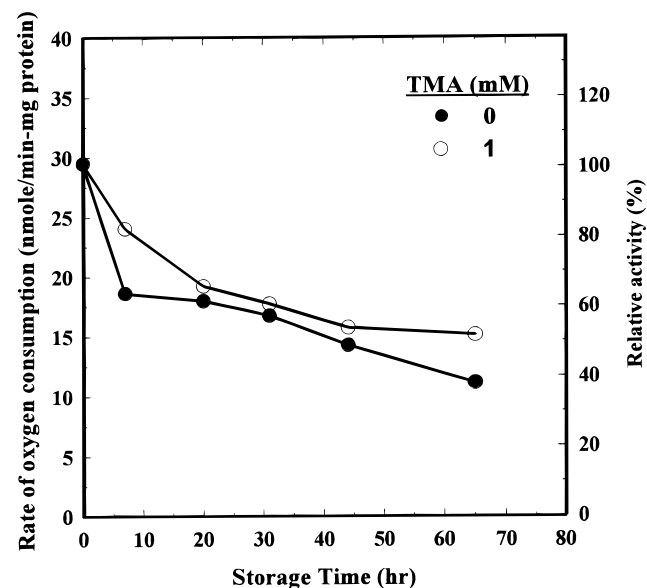
However, addition of reduced glutathione at ≥2 mM resulted in loss of protective effect.

Histidine and Imidazole as LOX Protectors. The active site of LOX consists of six histidines (Boyington et al., 1993a,b). Single amino acid replacement at three of the six conserved histidines in soybean LOX-1 (Steczko et al., 1992) or human 5-LOX (Zhang et al., 1992b) produced completely inactive enzymes. Since the structure of imidazole is similar to that of histidine, addition of histidine or imidazole during extraction may help to stabilize the active site structure of LOX.

The influences of histidine and imidazole on storage stability of gill LOX are shown in Figures 4 and 5, respectively. Histidine and imidazole increased storage stability of gill LOX. The optimal concentrations of both of those were 10 mM. At this concentration, the LOX at 4 °C increased by 20 and 25% within 25 h as compared to the unprotected. Addition of imidazole over 50 mM reduced the protective effect.
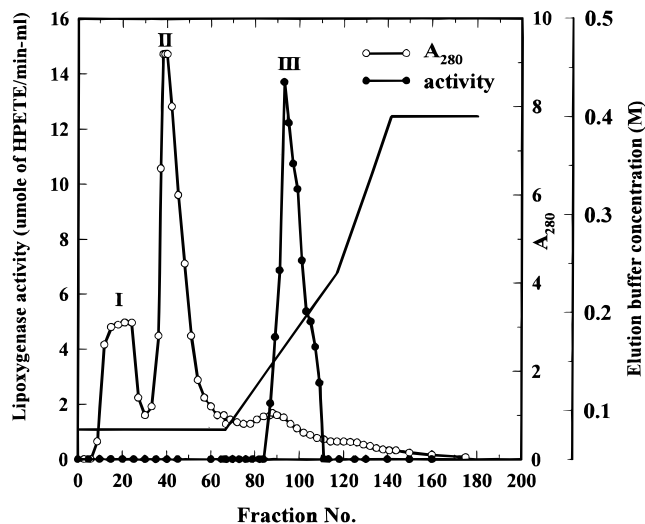
Figure 7. Elution of mullet gill lipoxygenase activities from hydroxyapatite. Fractions of 6.0 mL were collected. Lipoxygenase activity was measured spectrophotometrically using 200 μM arachidonic acid as substrate at 20 °C.

Trimethylamine (TMA) as LOX Protector. TMA is known to reduce lipid hydroperoxides to alcohols (Schwimmer, 1981) as follows:

$$(CH_3)_3N + LOOH \rightarrow (CH_3)N \rightarrow O + LOH$$
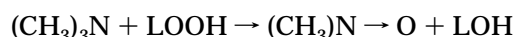
Hence, trimethylamine may serve as a protective agent to the enzyme which may be inactivated by hydroperoxides.

Addition of trimethylamine at 1 mM slightly increased storage stability of LOX by 20% within 15 h of storage at 4 °C (Figure 6). The protective effect became less significant after 15 h. The influence of TMA on 12-LOX activity from mullet platelets was also determined using polarography. In the presence of 2 mM TMA, the initial velocity of arachidonate oxidation catalyzed by mullet platelet LOX increased by 35% as compared with the unprotected (Haard et al., 1995).

Partial Purification of LOX. Chromatography of crude mullet gill extract on hydroxyapatite at a flow rate of 1.2 mL/min separated three protein peaks, each of which was tested for lipoxygenase activities (Figure 7). HPT chromatography resulted in the successful separation of lipoxygenase (peak III) from the contaminating proteins (peaks I and II). Protein peak II was red in color and showed a UV−visible spectrum similar to that of bovine hemoglobin. The SDS−PAGE of peak II indicated its molecular weight was close to that of hemoglobin (data not shown). Oxygen consumption measured for 10 min on the reaction mixture of arachidonic acid and either protein peak II (Figure 7) or authentic bovine hemoglobin was close to zero (data not shown). These observations indicated that the catalysis caused by the presence of hemoglobin was negligible as compared to the catalysis caused by LOX in the measurements.

A summary of the LOX purification experiments is presented in Table 2. HPT purification resulted in a 10.75-fold specific activity and 85.9% yield. HPT was used to purify LOX from trout gill (German and Creveling, 1990) and human leukocytes (Izumi et al., 1991). The purification recovery caculated for the former was 9.5-fold. The latter was purified 38.5-fold. The specific activity of this enzyme following HPT purification was significantly greater than that in the crude tissue preparation.

Table 2. Partial Purification of Lipoxygenase from Mullet Gill

fraction	vol (mL)	total protein (mg)	total act.[a] (units)	sp act. (units/mg)	yield (%)	purifn (-fold)
supernatant (20000*g*)	50	234	10694	45.7	100	1
hydroxyapatite (>100K)	8	18.7	9185	491.2	85.9	10.75

[a] Lipoxygenase activity was conducted in 0.05 M potassium phosphate buffer, pH 7.0, at 25 °C for 5 min using arachidonic acid (400 μM) as substrate. One unit equals 1 nmol of oxygen consumption per minute.
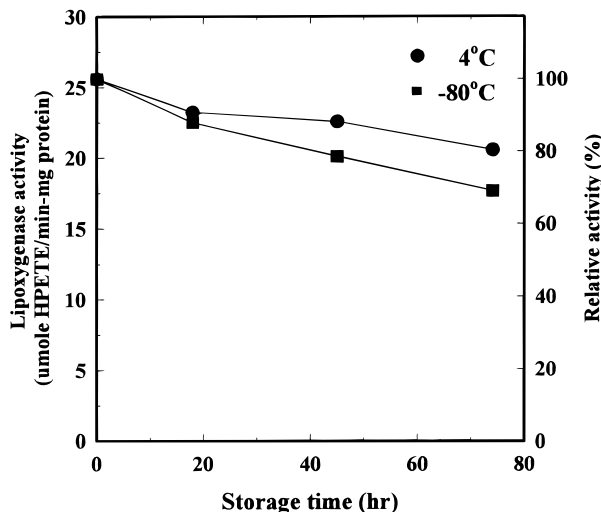

Figure 8. Storage stability of hydroxyapatite-chromatographed mullet gill lipoxygenase during storage at 4 and −80 °C.

Attempts to concentrate the purified LOX sample were tested using ultrafiltration membranes (a pM-100 and Amicon cell Model 8400, Beverly, MA). It was found to be effective in preliminary studies.

The hydroxyapatite-purified mullet gill LOX was stable for 3 days at 4 °C with only <20% loss in activity (Figure 8). It was more stable at 4 °C than at −80 °C, suggesting that freezing and thawing easily caused HPT-partial purified LOX unstable. Glycerol may be needed to improve the stability during freezing.

Conclusion. On the basis of polarographic analysis and HPLC separation, 5-, 12-, and 15-LOX activities were confirmed to exist in the gill of cultured gray mullet. The activities of 12- and 15-LOX were much higher than that of the 5-LOX in mullet gill. The physiological role of 12-LOX has not been as clear as that of the 5- and 15-isozymes of the sanguineous tissues of land animals. The unique pattern of the LOX isozymes, among which 12-LOX is the most active, in fish gill may be of special physiological implication.

The present results showed that adding glutathione, TMA, imidazole, and histidine or removing hemoglobin from crude gill extract could increase the stability of LOX significantly. It indicated that adding histidine, the same amino acid situated in the LOX active site, or adding the analogue of histidine, i.e. imidazole, prevented LOX inactivation, probably by stabilizing the LOX active site structure. HPT separation of LOX from hemoglobin increased LOX stability, suggesting that the contaminant hemoglobin (Fe^{2+}) and the active LOX (Fe^{+3}) may have undergone oxidation−reduction and resulted in the inactivation of LOX.

The gill tissue extract consisted of free and total polyunsaturated fatty acids (PUFA) at concentrations

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of 9.7 and 230 mg/g of oil, respectively (Tsai and Pan, 1995). The PUFA may be oxidized to the corresponding hydroperoxy derivatives by the LOX coexisting in the extract. The hydroperoxide products may cause self-inactivation of LOX (Hartel et al., 1982; Yamamoto and Ishimura, 1991). This may be the reason for the protective effects found in the reducing agents such as TMA and glutathione. However, at higher concentrations, TMA and glutathione showed inhibitory effects, probably due to additional reduction effect on the ferric ion involved in the active site.

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