CHAPTER IV
STUDY ON LIPOXYGENASE ACTIVTY IN TROFICAL FISH AND SURIMI: PARTIAL PURIFICATION AND CHARACTERIZATION OF LIZARDFISH (*Saurida tumbil*) GILL

4.1 Abstract

LOX activity of major tropical fish used for surimi production, including threadfin bream, lizardfish, and goatfish were followed in gill, skin, and muscle. The highest LOX activity of 376.56 U/mg protein was found in lizardfish gill, whereas the highest LOX activity in skin and muscle was found in threadfin bream of 60.67 and 137.04 U/mg protein, respectively. Among tropical fish, threadfin bream showed the highest amount of peroxide value. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the main polyunsaturated fatty acids (PUFA) in all tissues. Lizardfish surimi contained high amount of DHA and EPA. Residual LOX activity of 57.85 U/g sample remained after washing, indicating that LOX would contribute to oxidative off-flavor of surimi. LOX was purified from lizardfish gill was purified by two successive chromatographic steps of Sephacryl S-200 and DEAE-Sepharose, resulting in 3.52 % yield and 22.43-fold increase in purity. Optimum activity was found at 25 $^{\circ}$ C and pH 7.5 with pH stability at 6.0 - 8.5. At 15 $^{\circ}$ C, residual LOX activity remained 80%, suggesting that the enzyme might contribute to fish lipid oxidation during refrigerated storage. The enzyme was thermally inactivated at 50 $^{\circ}$ C. The most preference substrate was 2.5 mM EPA. The enzyme was inhibited by 1 mM ethylenediaminetetraacetic acid (EDTA) and activated by 1 mM Fe^{2+}, Na^{2+}, and Ca^{2+}.

Keywords: Lipoxygenase, Purification, Lizardfish, Lipid oxidation, Surimi

4.2 Introduction

Oxidation of lipids deteriorates fish quality, resulting in off-odors/flavors, color problems, texture defects, and safety concerns (Wu, Richards, and Undeland, 2022). Fish contain high amounts of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic (DHA), eicosapentaenoic acids (EPA), linoleic acid (LA), and alpha-linolenic acid (ALA) (Strobel, Jahreis, and Kuhnt, 2012). Lipoxygenase (LOX, 1inoleate: oxygen oxidoreductase, EC 1.13.11.12), is a dioxygenase that oxygenates polyunsaturated fatty acids (PUFA) containing a cis, cis- 1,4 pentadiene structure to form conjugated unsaturated fatty acid hydroperoxides (Grechkin, 1998). Hydroperoxy fatty acids can be further metabolized to produce alcohols, aldehydes, ketones and hydrocarbons, lowering fish quality (Josephson et al., 1983). Decanal, nonanal, octanal, and (E)-2-nonenal, exhibiting green and fatty note, were the most potent contributors to the aroma of the raw tuna fish (Zhang, Ma, and Dai, 2019). Iglesias et al. (2009) reported that Z-4-heptenal is an important contributor to off-flavors in gilthead sea bream, which is produced by the LOX-catalyzed oxidation of EPA. Moreover, strong fishy odor of silver carp is caused by 2, 4-heptadienal (E, E) resulted from LA oxidation catalyzed by LOX (Fu et al., 2009). LOX has been studied in muscle, skin, and gill tissue of various aquatic species, including trout, rockfish, mackerel, gray mullet, grass carp, sardine (German and Creveling, 1990; Banerjee, Khokhar, and Apenten, 2002; Hong, Shim, and Byun, 1994; Hsu and Pan, 1996; Wang et al., 2012; Mohri, Cho, Endo, and Fujimoto, 1992). However, few reports have been studied on LOX in tropical fish used for surimi production.

Goatfish (*Mullidae spp.*), lizardfish (*Saurida spp.*), and threadfin bream (*Nemipteridae spp*.) are raw materials for tropical surimi production. Typically, surimi has bland taste and less fishy note than its respective mince. However, tropical surimi is known to have strong fishy odor and sometime is considered as “off-odor”. This is likely due to poor post-harvest handlings of raw materials. Although washing can remove odorous compounds, the extent of strong off-note is much higher than cold water species surimi (An, Qian, Alcazar Magana, Xiong, and Qian, 2020). Membrane phospholipids rich in PUFA are difficult to remove by washing and are very sensitive to oxidation (Eymard, Baron, and Jacobsen, 2009). These phospholipids can serve as a

substrate of LOX, causing off-flavors. However, it is not known how much LOX residual activity remains after washing. Understanding LOX characteristics of tropical fish would lead to better control strategies for surimi qualities. Therefore, the objectives from this study were to prepare a partially purified and characterize LOX from lizardfish gill. The effects of industrial surimi washing process on LOX activity and fatty acid composition were also studied.

4.3 Materials and methods

4.3.1 Chemicals and reagents

Bovine serum albumin (BSA), 2-thiobarbituric acid, cumene hydroperoxide, guanidine hydrochloride, trichloroacetic and ferrous chloride were purchased from Fluka (Buchs, Switzerland). Diethylaminoethyl (DEAE)-Sepharose and Sephacryl-200 were purchased from GE Healthare (Uppsala, Sweden). Oleic acid (≥98%), Arachidonic acid (≥99%), Linolenic acid (≥99%), Eicosapentaenoic acid (EPA) (≥99%), Docosahexaenoic acid (DHA) (≥99%), heptadecanoic acid (≥99%), tween-20, L-glutathione reduced (≥98.0) and cyclohexanol were purchased from Sigma-Aldrich (Oakville, Canada). Other chemicals and reagents were of analytical grade.

4.3.2 Sample preparation

Goatfish (*Mullidae spp.*), lizardfish (*Saurida spp.*), and threadfin bream (*Nemipteridae spp.*) were obtained from Andaman Surimi Industries company (Samut Sakhon, Thailand). Fish were caught and off-loaded approximately 7-10 days after capture. Fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to Suranaree University of Technology within 5 h. Whole fish were immediately washed and kept in ice with a fish/ice ratio of 1:2 (w/w). Skins, gill, and flesh were manually separated and immediately stored at -70 $^{\circ}$C until use. Mince fish, the first washed mince, the second washed mince, the third washed mince, mince from refiner, screw press and surimi of all 3 species were also collected at the surimi production facility of Andaman Surimi Industries. Surimi samples were either used immediately or stored at -70 $^{\circ}$C until use.

4.3.3 Total lipid content

Total lipids content of gill, skin, and muscle of 3 tropical fish and surimi samples were analyzed according to Bligh & Dyer method (Bligh and Dyer, 1959). Samples (12 g) was homogenized with 60 ml chloroform and methanol solution (2:1, v/v) at 8000 rpm for 2 min at 4 ° C using an IKA homogenizer (IKA Works Asia, Bhd, Selangor, Malaysia). The homogenates were filtered through Whatman No. 1 filter paper into a separating funnel. Thereafter, 24 ml of chloroform, 24 ml of distilled water and 0.58% (v/v) sodium chloride solution was added. The mixture was gently shaken. After separation, chloroform phase was drained off into a 125 ml Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulfate and was shaken well to remove any traces of water that might be present in the chloroform phase. The solution was filtered through Whatman No. 1 filter paper into a pre-weighed 125 ml Erlenmeyer flask and solvent was removed by flushing with nitrogen. To allow for further analysis of phospholipids and fatty acid, the extracted lipid was dissolved in 10 mL chloroform and stored at -80 ° C.

4.3.4 Fatty acid profile

Fatty acid profiles of extracted muscle were determined as fatty acid methyl esters (FAMEs) using gas chromatography (GC). Aliquots of the lipids extracted were used to prepare the FAME according to the method of AOAC (2000). In brief, the sample was mixed with 0.5 M methnolic solution (2% sodium hydroxide in methanol), heated at 85 ° C for 30 min, cooled and extracted with 2,2,4-trimethylpentane (Isooctane). Heptadecanoic acid (C17:0) was used as an internal standard. The FAME samples were injected, separated and identified on an Agilent/HP 7890 gas chromatograph (Agilent, Palo Alto, USA) with flame ionization detector (FID) equipped with an A fused silica capillary column (100 m ×0.25 mm × 0.2 μm film thickness) (Supelco, Bellefonte, PA, USA), using helium as the carrier gas at a flow rate of 1 ml/min. The analytical conditions were: injection port temperature of 250 ° C and detector temperature of 270 ° C. Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated,

based on the peak area of internal standard and expressed as mg fatty acid/100 g dry sample.

4.3.5 Peroxide value

Peroxide value (PV) of gill, skin, and muscle of 3 tropical fish and surimi samples was determined according to Richards and Hultin (2002). Samples (1 g) were homogenized in 11 ml of chloroform/methanol (2:1, v/v). Homogenates were then filtered through Whatman No. 1 filter paper. Two milliliters of 0.5% NaCl were added to 7 ml of the filtrate. The mixtures were vortexed and then was centrifuged to separate the sample into two phases. To 3 ml of lower phase, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride was added to the mixture. The reaction mixture was kept for 20 min at room temperature prior to reading the absorbance at 500 nm. Blanks were prepared in the same manner, except that deionized water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5–2 ppm. PV was expressed as mg cumene hydroperoxide/kg sample after blank subtraction.

4.3.6 Enzyme extraction and partial purification

Lizardfish gill were homogenized in 0.05 M phosphate buffer (pH 6.5) with 2 mM reduced L-glutathione (≥98.0%) and 0.04% Tween-20 at ratio of 1:4 (w/v) using an IKA homogenizer (IKA Works Asia, Bhd, Selangor, Malaysia). The homogenate was centrifuged at 15,000×g (Sorvall Legend MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold, Germany) for 20 min. Supernatant was collected and fractionated by 40-70% ammonium sulfate. Precipitated proteins were collected by centrifugation at 15,000× g for 30 min at 4 ° C. The precipitates were dissolved in 0.05 M phosphate buffer (pH 6.5) with 2 mM L-glutathione reduced (≥98.0%) and 0.04% Tween-20 and applied to Sephacryl-200 column equilibrated with 50 mM phosphate buffer (pH 6.5) with 2 mM reduced L-glutathione and 0.04% Tween-20 and then eluted with the same buffer. Five-ml fractions were collected at a flow rate of 1 ml/min. Fraction containing lipoxygenase activity were pooled and concentrated using 10-kDa MWCO ultrafiltration membrane (Amicon, Billerica, MA, USA). The concentrated sample was applied to

Sephacryl-200 column and eluted at a flow rate of 0.3 ml/min. The concentrated sample was applied to DEAE-Sepharose column equilibrated with 50 mM phosphate buffer, pH 6.5 and eluted using a linear gradient of 0-2.0 M NaCl, 50 mM phosphate buffer, pH 6.5. Two-ml fractions were collected at flow rate of 1 ml/min. Fractions containing lipoxygenase activity were pooled and subsequently dialyzed overnight against the 50 mM phosphate buffer, pH 6.5 with two changes of the buffer using SnakeSkinTM pleated dialysis tubing with 10 kDa molecular weight cut-off (MWCO) (Pierce Chemical Co., Rockford, IL, USA). The dialysates were concentrated using 10-kDa MWCO ultrafiltration membrane and used for LOX characterization. Protein content was carried out by the modified Bradford method using BSA as a standard (Bradford, 1976).

4.3.7 Lipoxygenase assay

LOX activity was measured by a UV spectrophotometer at 234 nm, which monitored the formation of conjugated dienes. Linoleie acid was used as a substrate and prepared according to method of Patel, Patel, and Thakkar (2015). LA of 45 mg was mixed with 90 mg of Tween-20 in 1 ml of DI water and emulsifying in 150 µl of 1 N NaOH. Final volume was brought to 20 ml with 0.05 M phosphate buffer (pH 6.5). The reaction mixture was prepared by adding 1.3 ml 50 mM phosphate buffer, pH 6.5, containing 1 mM glutathione and 0.04% Tween-20 and 100 µl crude extract. The reaction mixture was initiated by adding 100 µl of substrate. Absorbance at 234 nm of the mixture were recorded in 3 min. One unit of LOX activity [U] was defined as an increase in absorbance of 0.001 at 234 nm in 1 min under the specified condition.

4.3.8 Biochemical characterization

4.3.8.1. Temperature and pH optima

The effect of temperature on the partially-purified LOX activity was investigated in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.01%Tween-20 using LA as a substrate at 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 $^{\circ}$ C, for 5 min. The optimum pH was investigated at 25 $^{\circ}$ C at various pH levels of pH 3, 4,

5, 5.5 using 50 mM sodium acetate; pH 5.5, 6.0, 6.5, 7, 7.5, 8 using 50 mM phosphate buffer; pH 8, 8.5, 9 using 50 mM Tris-HCl; and pH 9, 10, 11 using 50 mM borate buffer.

4.3.8.2 pH stability

pH stability was determined by pre-incubating the partially purified LOX at various pH levels including pH 3 -6 in sodium acetate, pH 6 -7.5 in phosphate buffer, 9.5 in Tris-HCl, at final concentration of 50 mM. The mixture was pre-incubated at 25 ° C for 30 min. When preincubation time was reached, LOX activity was determined. Control samples at each pH value were run in the same manner except that their activities were determined immediately without preincubation.

4.3.8.3 Thermal stability.

Thermal stability of the partially-purified LOXwas determined by preincubating the enzyme at various temperatures (10 - 80 ° C) for 30 min. When preincubation time was attained, samples were rapidly cooled in ice water for 20 min. Some precipitates were noticed at high temperatures which was removed by centrifugation. Lipoxygenase activity was determined as described above. Activities of the control sampleswithout preincubation were determined at 25 ° C and pH 7.5.

4.3.8.4 Substrate specificity.

Substrate specificity of the partially-purified LOX was determined using various substrates, including oleic acid, linoleic acid, linolenic acid, arachidonic acid, EPA, and DHA at 2.5 mM. LOX activity was determined as described above.

4.3.8.5 Effect of inhibitors and ions on activity.

The effect of various inhibitors on LOX activity was determined using various substances, namely, nordihydroguaiaretic acid (NDGA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbic acid, and N-propyl gallate, at final concentration of 1 mM. The effect of metal ions ($CuCl_2$, $FeCl_3$, $MnCl_2$, $HgCl_2$, and $ZnSO_4$) and mono- and divalent cations (NaCl, KCl, $MgCl_2$, and $CaCl_2$) at 1 mM was also investigated.

4.3.9 Statistical analyses

All experiments were conducted in triplicate, and results were analyzed by one-way analysis of variance (ANOVA). Significant differences in mean values were analyzed by Duncan's multiple range mean comparison test within the 95% confidence interval using. All data were analyses with the SPSS 17.0 statistical package (SPSS Ltd., Working, Surrey, UK).

4.4 Results and discussion

4.4.1 Lipid composition in various tissues

Total lipid contents and amounts of each fatty acid of gill, skin, and muscle of 3 tropical fish are listed in Table 4.1. Gill of lizardfish contained the highest total lipid content, which was about 5 and 29 times higher than that of goatfish and threadfin bream, respectively. Total lipid contents of skin and muscle of all 3 species ranged 20.6-37.0 g/kg dry weight. All tissues of tropical fish showed higher saturated fatty acid (∑SFA) content than polyunsaturated fatty acid (∑PUFA) and monounsaturated fatty acid (∑MUFA), while gill contained the highest of ∑SFA, ∑MUFA, and ∑PUFA. Wu, Forghani, Abdollahi, and Undeland (2022) reported that ∑SFA and ∑MUFA of head were higher than fillet, whereas ∑PUFA was the highest in fillet. Twenty-two marine fish species from the Pearl River Estuary contain high contents of SFA and low contents of PUFA, which might be associated with their differed dietary composition (Zhang et al., 2020). Among 3 species, all tissues of lizardfish showed the lowest ∑SFA and ∑PUFA, whereas all tissues of threadfin bream exhibited the highest ∑MUFA. Predominant SFA and MUFA in all tissues of all species was palmitic and oleic acid, respectively, whereas DHA and EPA are the main PUFA. Goncalves et al. 2021 reported that PUFAs of muscle were major fatty acids in twelve of fourteen marine fish species from Northeast coast of Brazil and EPA and DHA were major fatty acids in most species studied. n-3 PUFAs in marine fish are mostly found in phospholipids (Burri, Hoem, Banni, and Berge, 2012). Our results indicated that amount of total lipid and fatty acid contents of 3 tropical species differed among speices and tissues.

Table 4.1 Total extracted lipids and amount of each fatty acid of various tissues of 3 tropical species used in surimi production.

	Gill			Skin			Muscle		
	Goatfish	**Lizard**	**Threadfin beam**	**Goatfish**	**Lizard**	**Threadfin beam**	**Goatfish**	**Lizardfish**	**Threadfin bream**
Total lipid (g/kg dry weight)	30.80 ± 1.80c	162.34 ± 7.04d	5.66 ± 0.32a	22.77 ± 0.32b	22.52 ± 0.24b	37.04 ± 4.40c	26.41 ± 0.50b	23.53 ± 0.58b	20.67 ± 0.18b
FA content (mg/100 g dry weight)									
C12 : 0	8.75 ± 0.38	1.94 ± 0.22	9.77 ± 0.73	4.58 ± 0.50	1.12 ± 0.11	6.51 ± 0.31	1.75± 0.12	ND	1.85 ± 0.16
C13 : 0	4.66 ± 0.18	0.81 ± 0.09	3.40 ± 0.37	3.09 ± 0.48	0.48 ± 0.00	2.31 ± 0.05	1.06 ± 0.21	ND	0.78 ± 0.07
C14 : 0	222.15 ± 9.17	84.74 ± 6.90	177.27 ± 16.72	129.47 ± 16.04	65.73 ± 0.18	116.34 ± 14.44	45.59 ± 6.17	19.37 ± 1.91	36.63 ± 3.01
C15 : 0	ND	20.03 ± 1.61	50.92 ± 0.46	36.65 ± 4.05	12.55 ± 0.45	37.79 ± 0.46	15.23 ± 0.02	6.34 ± 0.63	12.85 ± 0.04
C16 : 0 (Palmitic acid)	1052.97 ± 41.24	485.86 ± 39.65	1244.95 ± 23.62	529.67 ± 18.21	327.12± 1.62	877.67 ± 108.41	332.28 ± 1.55	230.01 ± 22.72	406.47 ± 17.18
C17 : 0	410.08 ± 10.44	187.56 ± 12.50	432.75 ± 11.68	217.33 ± 8.99	117.75 ± 2.60	245.83 ± 28.89	140.14 ± 8.50	106.54 ± 10.52	136.14 ± 3.00
C18 : 0	516.79 ± 20.16	196.04 ± 20.08	611.57 ± 19.39	286.76 ± 8.93	116.35 ± 2.04	393.63 ± 45.34	162.62 ± 1.80	81.61 ± 8.06	171.02 ± 10.66
C22 : 0	6.84 ± 0.32	3.65 ± 0.41	9.16 ± 0.45	4.36 ± 0.13	2.78 ± 0.03	7.09 ± 0.36	2.57 ± 0.08	1.81 ± 0.18	3.94 ± 0.37
C23 : 0	ND	ND	0.05 ± 0.00	2.70 ± 0.44	4.18 ± 0.03	8.24 ± 0.85	1.97 ± 0.13	0.90 ± 0.09	2.15 ± 0.00
∑SFA	2222.23 ± 81.89b	980.61 ± 81.46a	2545.48 ± 73.69b	1214.62 ± 57.78ab	648.06 ± 7.06a	1695.40 ± 199.11b	705.21 ± 19.82b	446.58 ± 44.11a	771.82 ± 34.48b
C14 : 1	67.98 ± 2.70	ND	2.52 ± 0.37	0.63 ± 0.11	0.63 ± 0.00	1.83 ± 0.23	ND	ND	ND
C16 : 1	260.73± 10.69	102.09 ± 8.38	259.53 ± 31.37	133.81 ± 13.72	77.57 ± 0.14	195.14 ± 21.56	48.28 ± 6.17	32.70 ± 3.23	67.48 ± 3.21
C17 : 1	14.20 ± 0.59	ND	ND	7.23 ± 1.04	4.72 ± 0.04	9.21 ± 0.36	3.28 ± 0.75	ND	2.55 ± 0.05
C18 : 1n9t (Oleic acid)	361.95 ± 15.64	146.10 ± 13.24	554.58 ± 24.68	180.24 ± 13.52	89.27 ± 0.62	391.34 ± 41.93	80.95 ± 4.02	61.26 ± 6.05	170.05 ± 3.53
C18 : 1n9c	147.90 ± 6.16	65.94 ± 5.42	184.18 ± 15.66	73.57 ± 3.49	38.13 ± 0.10	120.45 ± 15.16	35.94 ± 1.86	25.61 ± 2.53	46.33 ± 0.30
C20 : 1	22.00 ± 1.00	6.49 ± 1.39	24.13 ± 0.70	5.99 ± 0.31	4.76 ± 0.07	10.44 ± 4.60	2.63 ± 0.21	1.77 ± 0.18	2.34 ± 0.10
C22 : 1n9	8.62 ± 0.37	ND	ND	ND	ND	ND	105.71 ± 6.92	ND	126.56 ± 13.38
C24 : 1	23.49 ± 14.83	2.69 ± 0.60	6.76 ± 0.31	2.30 ± 0.48	9.28 ± 0.28	46.90 ± 1.54	17.88 ± 0.17	5.37 ± 0.53	26.97 ± 3.67
∑MUFA	906.86 ± 51.98d	323.48 ± 29.28b	1031.71 ± 73.10d	403.76 ± 32.67c	224.38 ± 1.25b	775.31 ± 85.25cd	294.67 ± 20.10b	126.71 ± 12.52a	442.28 ± 24.23c
C18 : 2n6c (Linoleic acid)	64.81 ± 2.57	24.66 ± 2.73	53.23 ± 3.75	43.34 ± 3.83	17.52 ± 0.21	4.22 ± 0.39	22.54 ± 0.94	9.84 ± 0.97	18.99 ± 0.02
C18 : 3n6	6.71 ± 0.27	2.73 ± 0.25	4.89 ± 0.63	3.93 ± 0.28	2.34 ± 0.07	3.77 ± 0.16	1.79 ± 0.11	0.95 ± 0.09	1.74 ± 0.33
C18 : 3n3	13.16 ± 0.50	3.52 ± 0.35	15.62 ± 0.26	8.06 ± 0.63	2.50 ± 0.07	9.00 ± 0.92	2.91 ± 0.43	1.10 ± 0.11	2.73 ± 0.14
C20 : 2	18.94 ± 0.84	5.45 ± 0.48	22.49 ± 1.32	13.18 ± 1.14	3.49 ± 0.12	13.62 ± 0.91	6.18 ± 0.38	2.34 ± 0.23	5.79 ± 0.15
C20: 3n3	4.77 ± 0.21	4.04 ± 0.48	10.61 ± 1.37	4.85 ± 0.29	2.84 ± 0.06	6.30 ± 0.72	ND	48.33 ± 4.77	ND
C20 : 4n6 (Arachidonic acid)	183.51 ± 7.10	116.83 ± 8.72	244.31 ± 2.39	104.12 ± 3.41	67.47 ± 1.52	150.76 ± 16.15	2.43 ± 0.20	2.37 ± 0.23	3.97 ± 0.09
C22 : 2n2	6.43 ± 0.19	2.27± 0.81	0.05 ± 0.00	3.33 ± 0.16	1.12 ± 0.10	4.30 ± 0.06	ND	ND	ND
C20 : 5n3 (EPA)	291.45 ± 10.87	129.07 ± 13.42	290.29 ± 33.70	124.38 ± 4.72	96.97 ± 3.78	227.54 ± 34.53	92.35 ± 1.96	76.31 ± 7.54	107.56 ± 1.42
C22 : 6n3 (DHA)	877.34 ± 34.73	465.92 ± 37.05	705.48 ± 53.71	604.39 ± 20.19	393.35 ± 11.89	523.97 ± 95.06	570.04 ± 21.44	356.24 ± 35.19	374.61 ± 55.10
∑PUFA	1467.12 ± 57.29d	754.50 ± 64.29b	1353.96 ± 97.51d	909.58 ± 34.65c	587.60 ± 17.80a	943.48 ± 148.91c	698.25 ± 25.46b	497.49 ± 49.14a	515.39 ± 57.37a

*SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

[a-d] Mean values in the same column with different superscripts differ significantly ($P < 0.05$)

4.4.2 LOX activity

LOX activity and peroxide values of gill, skin, and muscle of tropical fish namely goatfish, lizardfish, and threadfin bream are listed in Table 4.2. LOX activities varied with tissues. Gill of lizardfish exhibited the highest LOX activity, which was about 2 times higher than threadfin bream muscle ($p < 0.05$). Gill of Baltic herring showed the lowest activity, which was comparable to muscle and skin tissue. (Stodolnik and Samson, 2000). Muscle of lake herring showed higher LOX activity than skin tissue (Wang, Miller, and Addis, 1991). Hsieh, German, and Kinsella (1988) reported that gill and skin tissues of freshwater fish species contained active LOX activity and there was no detectable LOX activity in muscle. Wu et al. (2022) reported that LOX activity of head was higher than fillet in sorted herring and this enzyme had a significant impact on its lipid oxidation.

PVs of gill tissue of all 3 species were the highest compared to other tissues with threadfin bream being the highest ($p < 0.05$). Lipid oxidation occurred during postmortem handlings and was influenced by tissue type, and age. Fresh fish is known to contain peroxide which is naturally produced and can react with biological molecules. Fu et al. (2009) reported LOX from silver carp muscle caused a faster initial reaction of lipid oxidation than hemoglobin but had lower lipid hydroperoxide value and severe fishy odor. Our results indicated that LOX activity was not related to PV.

Table 4.2 LOX activity and peroxide value of various tissues of 3 tropical species used in surimi production.

Tissues	species	LOX activity (U/mg protein)	Peroxide value (mg hydroperoxide/kg dry weight)
Gill			
	Goatfish	48.93 ± 0.87b	67.43 ± 2.54c
	Lizardfish	376.56 ± 22.0d	66.79 ± 3.94c
	Threadfin Bream	55.66 ± 0.32b	78.23 ± 4.87d
Skin			
	Goatfish	26.19 ± 1.57a	37.56 ± 2.4a
	Lizardfish	50.67 ± 1.08b	41.58 ± 3.52a
	Threadfin Bream	60.67 ± 0.18b	51.77 ± 3.22b
Muscle			
	Goatfish	30.53 ± 0.54a	32.54 ± 3.45a
	Lizardfish	75.62 ± 0.08b	42.57 ± 1.33a
	Threadfin Bream	137.04 ± 4.4c	48.11 ± 4.51b

[a-d] Mean values in the same column with different superscripts differ significantly ($P < 0.05$)

4.4.3 Changes of LOX activity during lizardfish surimi processing

The changes in LOX activities (units/g sample) during lizardfish surimi processing are shown in Figure 4.1. A significant decrease in LOX activities was detected throughout surimi processing ($p < 0.05$). A decrease in activity suggested that the enzyme can be removed by washing. There was approximately 57.5% reduction of lipoxygenase activity of lizardfish mince after washing. Washing not only removes fat and undesirable substances such as blood, pigments and odorous contents, but also increases insoluble proteins, including myofibrillar protein, connective tissue proteins, as well as membrane-bound cholesterol and lipids. Yamashita, Nakamura, Noguchi, Niki, and Kühn, (1999) reported LOX are capable of specially oxidizing phospholipids and cholesterol esters in membranes and lipoproteins. 15-LOX was localized in the cytosol but also bound to intracellular membranes (Brinckmann et al., 1988). Mince reached it minimum level of LOX activities by a dewatering with a 70% decrease. However, the enzyme residual activity in surimi remains after frozen storage (57.85 U/g sample). LOX activity has been reported in silver carp surimi products at setting stage LOX activity reached 4.19-4.81 U/min•g (An et al., 2022). The enzyme residual activity remaining in the surimi could partly contribute to lipid oxidation of surimi during frozen storage.

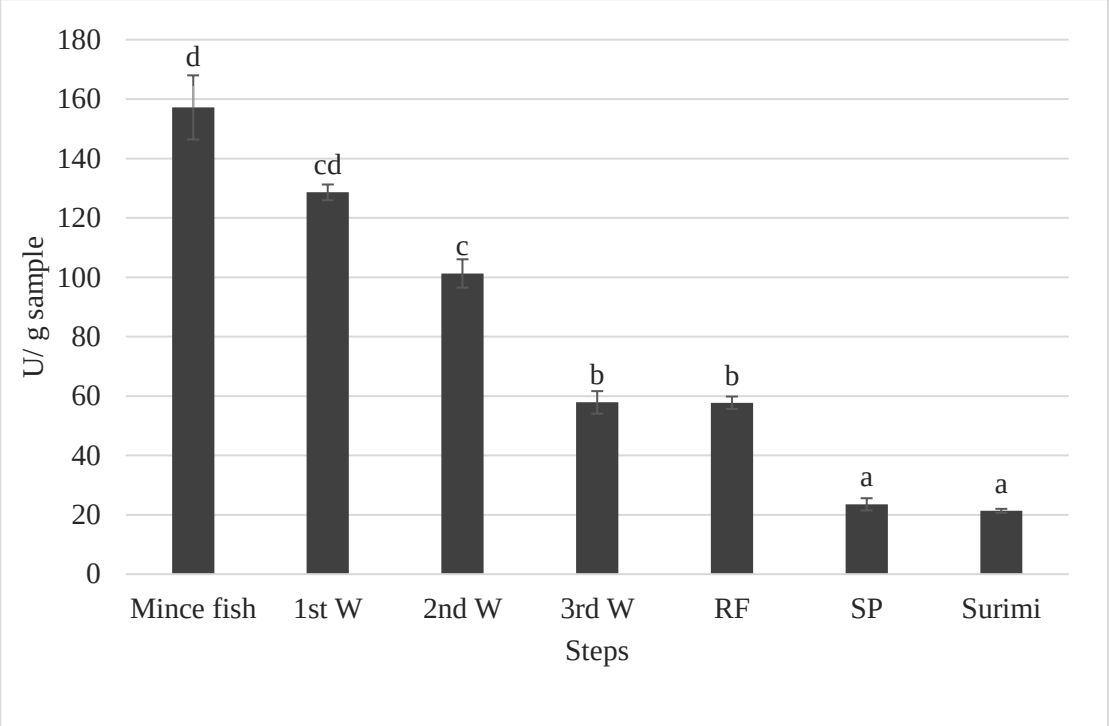

Fig. 4.1 Changes of LOX activity during lizardfish surimi processing (1st W = First washing, 2nd W = Second washing, 3rd W = Third washing, RF = Refining, SP = Screw pressing)

4.4.4 Changes of fatty acid during surimi processing

Total extracted lipids and fatty acid contents of lizardfish surimi processing is shown in Table 4.3. A decrease in total lipids was detected in mince from screw pressing and lizardfish surimi (LZ surimi) ($p<0.05$). Total lipids of mince from screw pressing and LZ surimi decreased by 45.81% and 56.7% as compared to minced fish. Proportions of fatty acid were similar in minced fish and in LZ surimi. Amount of monounsaturated fatty acid (∑MUFA) lower than saturated fatty acid (∑SFA) and polyunsaturated fatty acid (∑PUFA). A decrease in fatty acid contents were detected in screw pressing step and surimi ($p<0.05$). ∑SFA, ∑MUFA, and ∑PUFA decreased by 63.67%, 65.76%, and 62.79% at surimi step as compared to minced fish. A decrease in total lipids and fatty acid contents suggested that the lipid can be removed by dewatering by screw press. Surimi has a higher phospholipid content than minced could be explained by the membrane polar lipids, such as phospholipids, interacting

with proteins and consequently being less easily removed than neutral lipids (Eymard et al., 2005). These phospholipids are highly unsaturated and often in contact with muscle heme iron and very sensitive to oxidation. According to Clark et al. (2011) LOX may act on phospholipids in which fatty acids are esterified to the glycerol backbone. Omega-3 PUFA in marine fish are mostly found in phospholipids form (Burri et al., 2012). In our study, palmitic acid as the predominant SFA, oleic acid is highest MUFA, whereas DHA and EPA are the main PUFA in LZ surimi. These results indicating that PUFA in surimi can serve as a substrate of LOX. Eymard et al. (2005) reported that the last dewatering stage of mackerel surimi production can remove a major portion of lipid oxidation products. However, our findings indicated that PUFA and LOX were still present in surimi, which both substrate and enzyme could lead to oxidation of lipids during storage, which has not been reported.

Table 4.3 Total extracted lipids and amount of each fatty acid of the samples taken during surimi manufacturing.

	Mince fish	Mince from refining	Mince from screw pressing	Surimi
Total lipid (g/ kg dry sample)	18.71 ± 3.62b	17.94 ± 2.25b	10.14 ± 1.07a	8.22 ± 1.45a
FA content (mg/ 100 g dry sample)				
C14 : 0	42.33 ± 6.38	32.96 ± 32.96	19.89 ± 3.64	13.91 ± 0.80
C15 : 0	15.19 ± 2.30	12.25 ± 1.60	7.07 ± 1.26	5.16 ± 0.29
C16 : 0	351.74 ± 46.48	303.7 ± 40.69	162.27 ± 27.51	127.87 ± 6.99
C17 : 0	159.18 ± 13.23	153.55 ± 15.51	75.05 ± 7.14	62.2 ± 1.14
C18 : 0	137.65 ± 18.72	111.58 ± 15.15	61.75 ± 10.81	47.52 ± 2.63
C20 : 0	1.70 ± 0.16	1.37 ± 0.17	0.90 ± 0.05	0.53 ± 0.05
C21 : 0	1.64 ± 0.24	1.24 ± 0.16	0.77 ± 0.15	0.50 ± 0.06
C23 : 0	58.18 ± 7.14	49.25 ± 6.56	26.26 ± 4.36	20.54 ± 1.07
∑SFA	767.60 ± 94.7b	665.95 ± 84.5b	353.95 ± 54.92a	278.23 ± 13.03a
C16 : 1	57.67 ± 8.31	47.00 ± 644	27.36 ± 4.69	19.63 ± 1.11
C18 : 1n9t	0.51 ± 0.09	0.45 ± 0.12	0.23 ± 0.04	0.08 ± 0.00
C18 : 1n9c	106.3 13.51	87.55 ± 11.75	46.34 ± 8.00	36.95 ± 2.14
C20 : 1	6.00 ± 1.50	4.28 ± 0.59	2.51 ± 0.45	2.20 ± 0.04
C22 : 1n9	2.35 ± 0.33	1.81 ± 0.26	1.04 ± 0.18	0.88 ± 0.27
C24 : 1	1.40 ± 0.13	0.92 ± 0.11	0.60 ± 0.08	0.35 ± 0.02
∑MUFA	174.24 ± 23.87b	142.01 ± 10.27b	78.07 ± 13.45a	60.08 ± 3.65a
C18 : 3n6	1.77 ± 0.27	1.41 ± 0.19	0.81 ± 0.15	0.58 ± 0.09
C18 : 3n3	4.83 ± 3.97	1.87 ± 0.22	1.04 ± 0.18	2.84 ± 0.18
C20 : 2	4.45 ± 0.59	3.71 ± 0.49	1.99 ± 0.34	1.55 ± 0.08
C20 : 3n6	3.11 ± 0.39	2.71 ± 0.36	1.42 ± 0.23	1.14 ± 0.05
C20: 3n3	2.15 ± 0.35	1.6 ± 0.23	0.97 ± 0.19	0.27 ± 0.02
C20 : 4n6	85.9 ± 10.31	79.03 ± 10.73	38.87 ± 6.17	32.61 ± 1.68
C22 : 6n3	629.26 ± 71.65	599.18 ± 80.92	295.61 ± 46.61	233.42 ± 10.85
∑PUFA	731.50 ± 87.53b	689.52 ± 93.14b	340.71 ± 53.87a	272.41 ± 13.21a

[a] Different letter in the same column indicate difference at $P < 0.05$.

4.4.5 Partial purification of LOX from Lizardfish Gill.

Based on purification scheme, ammonium sulfate precipitation of 40-70% effectively removed contaminant proteins, rendering an increase of 4.55 folds of purity, and about 118% activity when compared to the crude extract (Table4.4). Wang et al. 2012 reported 40% saturated ammonium sulfate was effective for LOX fractionation in grass carp muscle. A Sephacryl S-200 column based on size exclusion chromatography was used in the second purification step. The recovery of LOX activity was 12.89 % with 12.01-fold purification (Figure 4.1). The active fraction from the first gel filtration was collected, concentrated, pooled and loaded again onto the Sephacryl S-200 column with a lower flow rate. Two protein peaks were well separated, and LOX activity was found in the minor protein peak. LOX recovery after the second gel filtration was 8.46 % with 15.85-fold purification (Table 4.4). Fractions giving LOX activity were pooled and loaded into a DEAE-Sepharose ion-exchange column. Thirty-five fractions were obtained from gradient elution and 5 fractions eluted at 0.8 M NaCl showing LOX activity (Fig. 4.3). An overall 22.43-fold purification was achieved with a yield of 3.52 %. The specific activity of pure LOX reached 1267.64 units mg^{-1} when linoleic acid was used as a substrate. LOXs have a single atom of non-heme iron essential for activity (Newcomer and Brash, 2015).

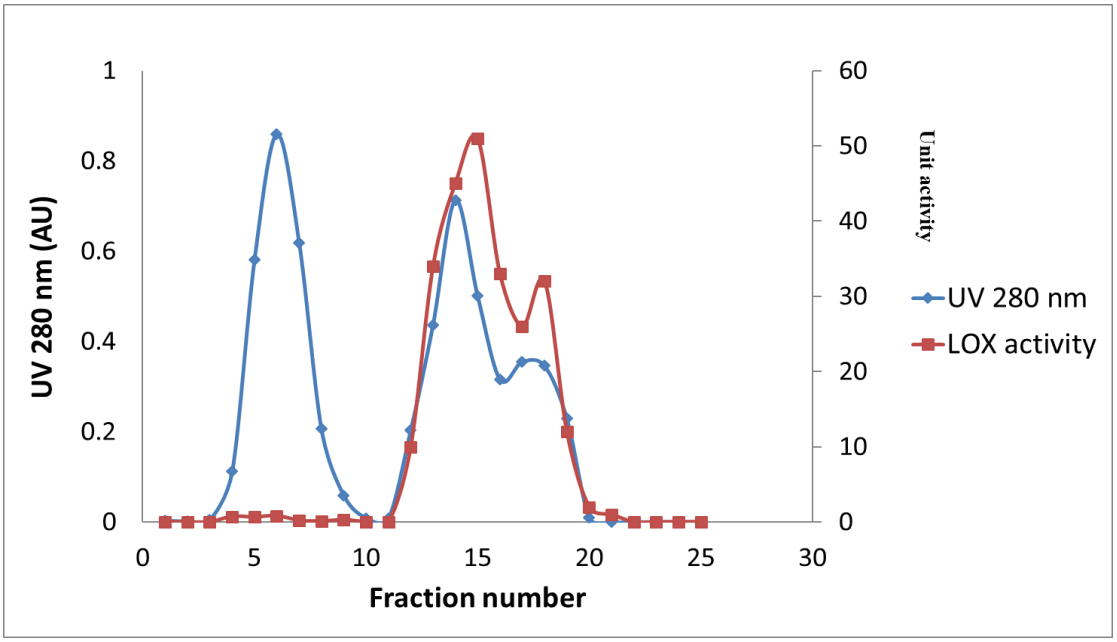

Fig. 4.2 Elution profile of lipoxygenase from lizardfish gill on a Sephacryl S-200 column. Fractions of 5 ml were collected at flow rate of 1 ml/min.

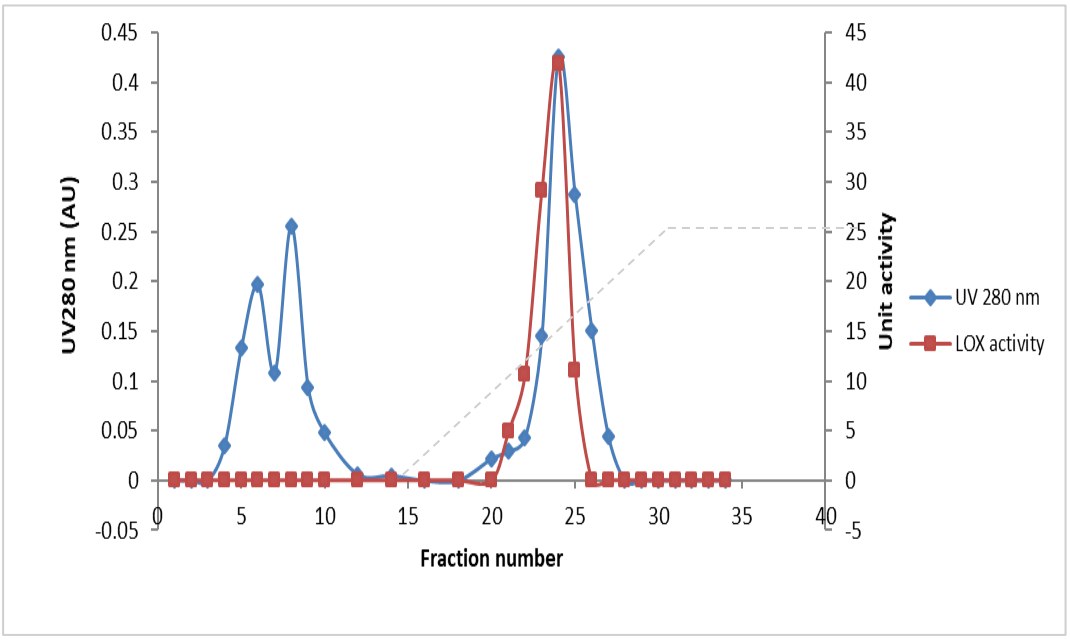

Fig. 4.3 Elution profile of lipoxygenase from lizardfish gill on a DEAE-Sepharose ion-exchange column.

Table 4.4 Purification table of lizardfish gill lipoxygenase

Step	Total protein (mg)	Total activity (Unit)*	Specific activity (Unit/mg)	Purity (fold)	Yield (%)
Crude extract	229.50	12973.21	56.53	1.00	100.00
$(NH_4)_2SO_4$ precipitation	59.49	15314.29	257.42	4.55	118.05
Sephacryl S-200 (Flow rate 1 ml/min)	2.46	1671.79	679.07	12.01	12.89
Sephacryl S-200 (Flow rate 0.3 ml/min)	1.22	1097.14	896.13	15.85	8.46
DEAE-Sepharose	0.36	456.35	1267.64	22.43	3.52

*One unit of enzyme activity is defined as an increase in absorbance at 234 nm of 0.001 after 1 minute under the specified condition.

4.4.6 Optimum temperature and pH.

Optimum temperature of the partially-purified LOX was at 25 ° C (Figure 4.4A). It showed high activity (82-98%) over a wide temperature range of 15-35 ° C. High activity of fish LOX at low temperatures suggests that it may contribute to initiation of fish lipid oxidation at refrigerated conditions. At 40 ° C, 60% of activity was retained. However, only minimal activity was observed at temperatures above 45 ° C. This was quite different from LOX purified from cold water species fish including mackerel and trout, which typically showed activity only at temperature of 20-25 ° (Hong et al., 1994; Hsieh et al.,1988). Enzymes from fish warm water habitat exhibited higher optimal temperature, indicating that it might be effect flavor quality much higher than cold water species.

Lizardfish had optimum activity around pH 7.5 with the activity rapidly declining below pH 6.0 and above pH 8.0 (Figure 4.4B). The enzyme was less sensitive to pH change in the acidic range than in the alkaline range. A decrease in pH from 7.5 to 6.0 resulted in the loss of 20% of the activity, while raising pH from 7.5 to 9.0 reduced the activity by 80%. A similar optimum pH was observed among LOX in teleost fishes and rainbow trout (German and Creveling,1990; Hsieh et al.,1988). LOX from mackerel muscle and gill showed optimum pH at 5.6 and 4.5 respectively (Banerjee et al., 2002; Hong et al., 1994), which was lower than the value observed in this study. The LOX distributed in gill and ovary of grey mullet showed optimum activity at pH 8 and pH 6.5, respectively (Cadwallader, 2000).

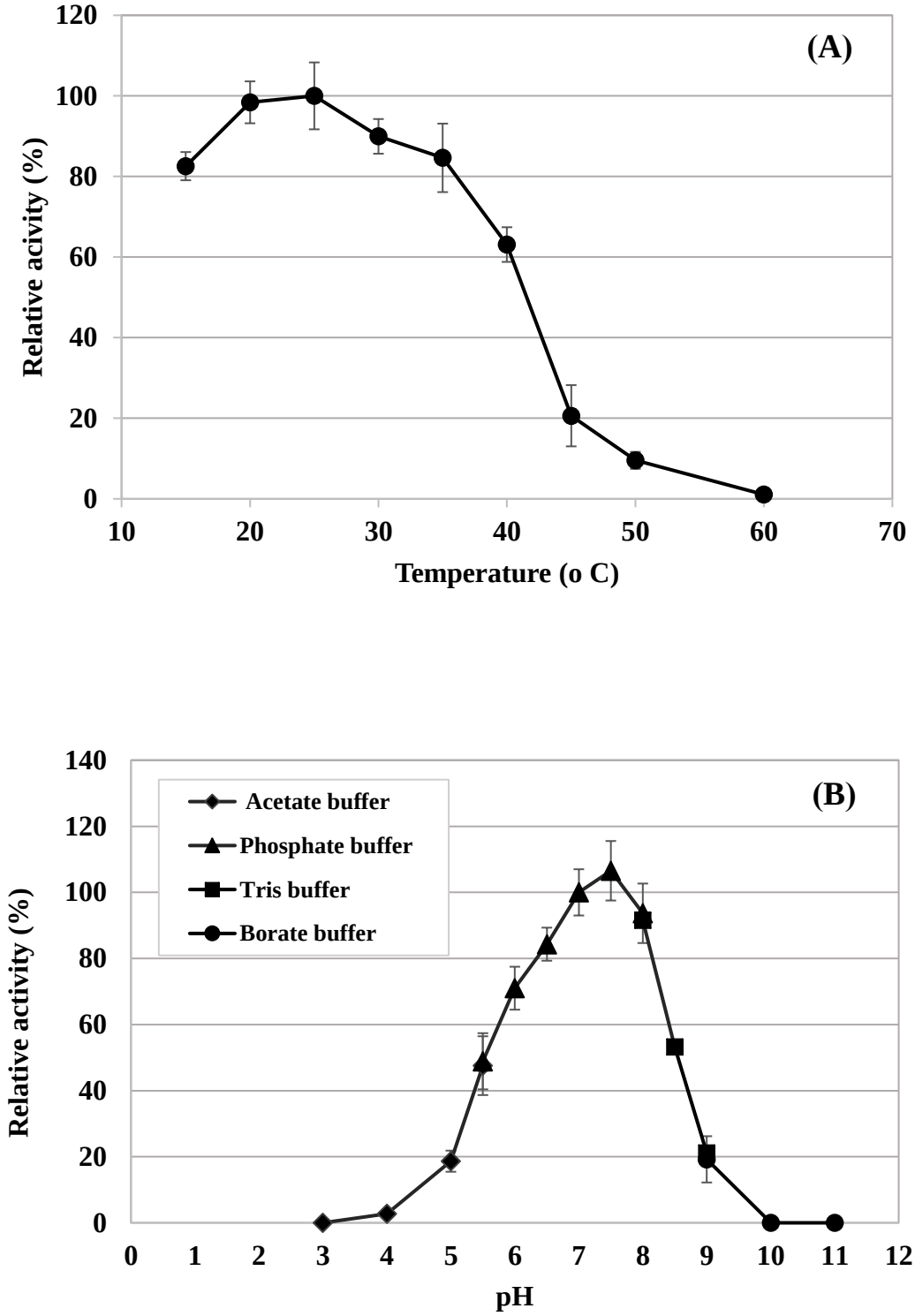

Fig. 4.4 Temperature (A) and pH profile (B) of partially-purified lipoxygenase from lizardfish gill.

4.4.7 Thermal stability and pH stability

The partially-purified lipoxygenase showed high stability at 10-50 ° C, and its stability rapidly decreased at temperature 60 ° C with 60% of the activity remaining (Figure 4.5A). Heating to 70 ° C and 80 ° C completely inactivated the enzyme. A decrease in activity is probably due to irreversible changes in the tertiary structure of the enzyme. The activity of LOX from mackerel muscle and trout decreased to 20% of the original activity, after incubation at 40 ° C for 10 min and over 80 % after incubation at 50 and 70 ° C for 10 min, respectively (Banerjee et al., 2002; Hsieh et al.,1988). An et al. (2022) reported LOX activity was high in the silver carp surimi gel at 40 ° C, and rapidly decreased at temperature 90 ° C for 10 min. Tolasa Yılmaz, Çaklı, Şen Yılmaz, Kırlangıç, and Lee (2018) found that increasing temperature (from 0 to 10 ° C) increased LOX activity significantly in fresh and frozen sardine fillets and mince. LOX activity in pork were completely inactivated at 50 ° C with high pressure 600 MPa (Huang, Wang, Wu, and Li, 2016). The instability of LOX above 50 ° C may provide an approach for controlling enzyme activity and help improve flavor quality of lizardfish.

The partially purified lipoxygenase was highly stable at a pH range of 6-8 (Figure 4.5B). The LOX from mackerel muscle and sardine were stable over broad pH ranges of 4-11 and 6-9, respectively (Banerjee et al., 2002; Mohri et al., 1992). The pH value of 6-8 falls in the postmortem pH of lizardfish, suggesting that enzyme likely remain active at postmortem during ice or refrigerated storage. Thiansilakul, Benjakul, and Richards, (2011) reported the highest lipid oxidation and off-odor were observed in washed Asian seabass mince at pH 6.0. Thus, lipid oxidation catalyzed by LOX likely occur at postharvest storage, which might be a main reason of distinct flavor of lizardfish meat.

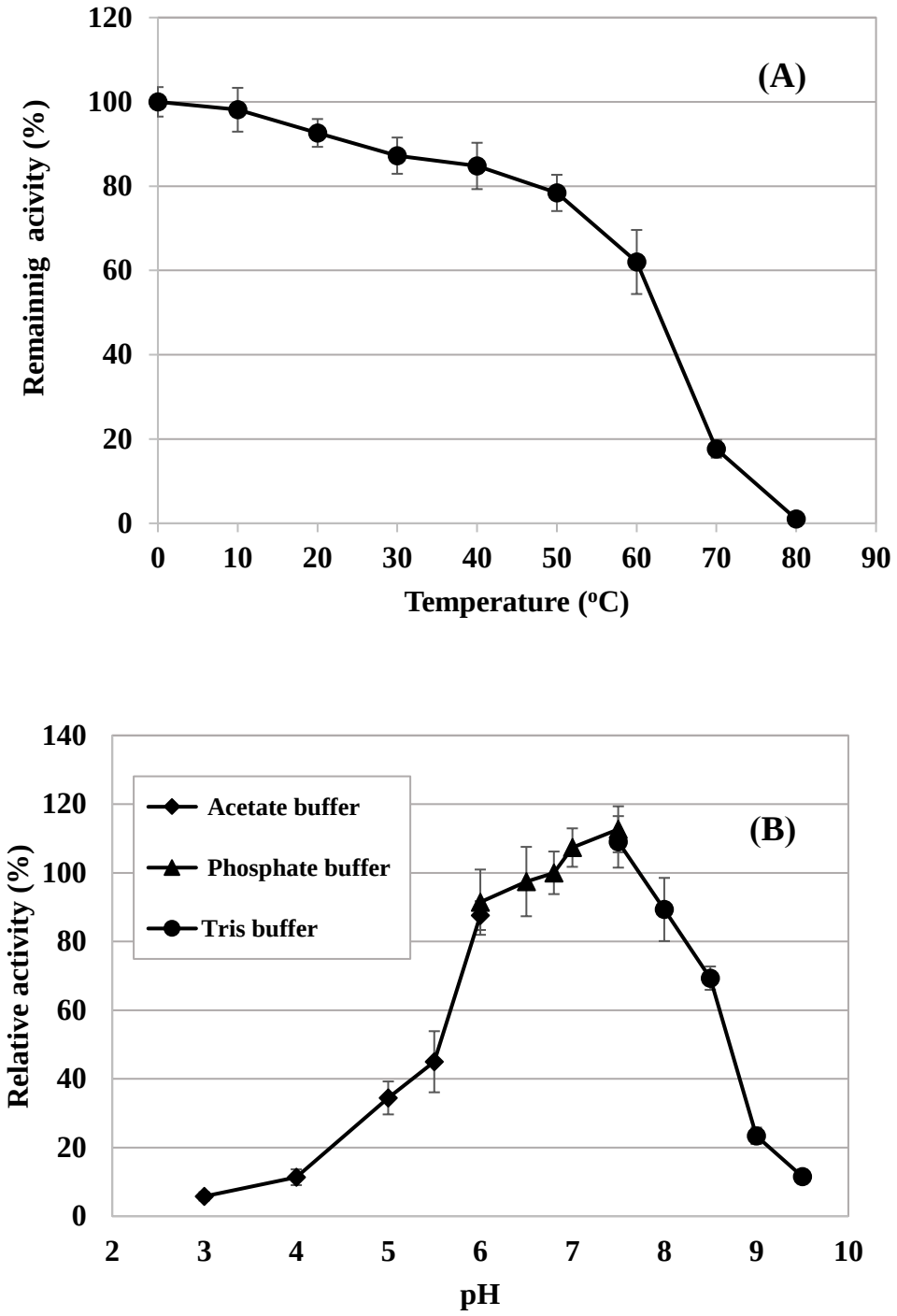

Fig. 4.5 Thermal (A) and pH stability (B) of the partially-purified lipoxygenase. Thermal stability was carried out by preincubating the enzyme at various temperature at pH 7.5 for 30 min, and pH stability was determined by preincubating the enzyme at varying pH values and determined activity at 25 °C for 30 min.

4.4.8 Substrate specificity

The activity of lipoxygenase toward different fatty acids is shown in Figure 4.6. The highest activity was observed with eicosapentaenoic acid (EPA), followed by linoleic acid and docosahexaenoic acid (DHA). Substrate specificities of the enzyme appear to vary with sources of LOX. LOX from trout and mackerel efficiently oxidized n-3 fatty acids such as DHA and EPA (Hsieh et al., 1988; Harris and Tall 1994). LOX from carp gill preferred arachidonic acid (Iijima et al., 2000), while sardine skin LOX showed maximal activity toward linoleic acid (Mohri et al., 1990). Gill LOX of rainbow trout exhibited reactivity toward arachidonic, EPA, and DHA, but low reactivity toward linoleic acid (German and Creveling, 1990). Banerjee et al. (2002) reported that the highest activity of mackerel muscle was observed when linoleic acid was used as a substrate, followed by linolenic acid, DHA and arachidonic acid. Oleic acid with a single double bond is not a substrate for LOX, resulting in relatively low activity (Figure 4.5). Therefore, high unsaturation may enhance the activity of lizardfish LOX. In this study, related to Table 4.1. Gill, skin, and muscle of lizardfish are rich in DHA, EPA, and linoleic acid, which can serve as substrates for LOX.

4.4.9 Inhibitors and Metal Ions.

Ethylene diamine tetra-acetic acid (EDTA) at 1 mM completely inhibited LOX activity based on both substrates of LA and EPA (Table 4.5). LOX inhibition was also observed in the presence of BHA, BHT, ascorbic acid and N-propyl gallate. EDTA inhibits the enzyme by chelating iron. LOXs have a two-domain structure of β-barrel domain and α-helical catalytic domain containing a single atom of non-heme iron essential for activity. Moreover, Wang et al. (2019) found that EDTA can cause the content of α-helix and β-sheet inside the porcine 12-LOX molecule to decreased and result in LOX activity change. LOX catalyzes the peroxidation of cis, cis-1,4-pentadiene structures. In its oxidized state, LOX ($Fe^{3+)}$ can abstract a proton from a PUFA and reduce itself to LOX (Fe^{2+}), producing a fatty acid free radical. Antioxidants such as BHA, BHT, propyl gallate, and ascorbic acid can effectively scavenge free radicals by donating labile hydrogen to oxygen radicals derived from fatty acids. (Festjens et al., 2006). Propyl gallate inhibited LOX activity in soy milk more than BHA, BHT, and ascorbic acid (Vijayvaragiya and Pai, 1991).

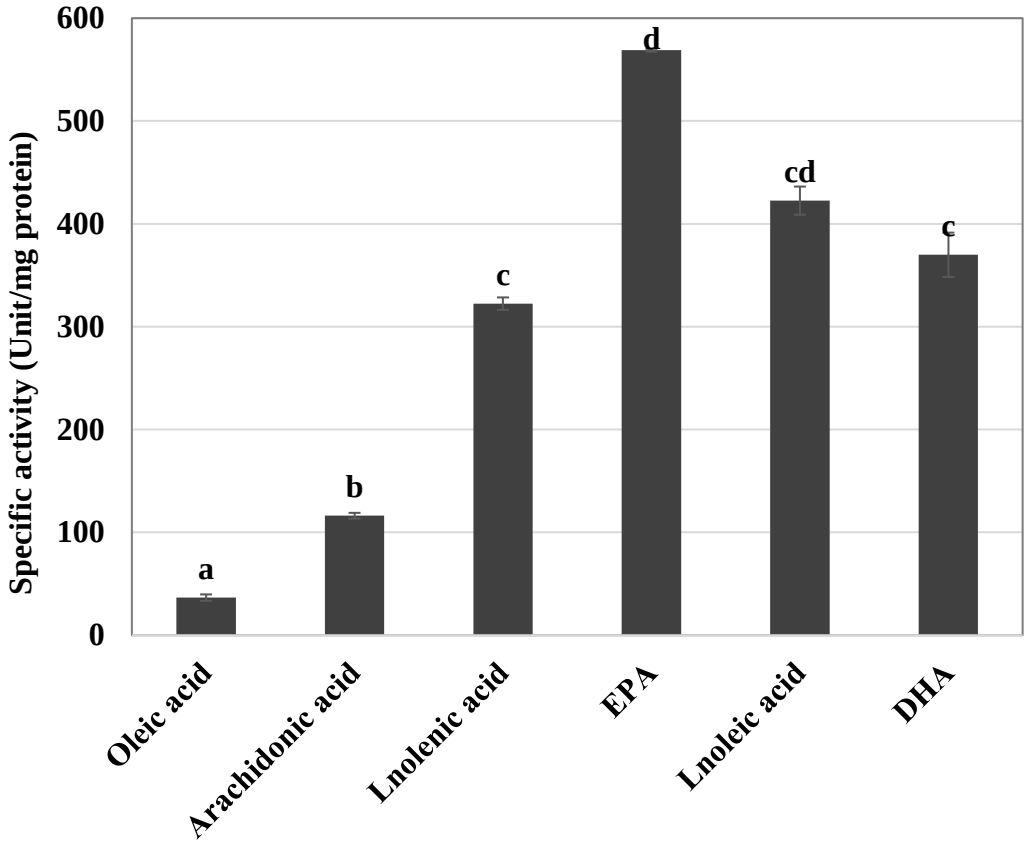

Fig. 4.6 Effect of different fatty acids on LOX activity (EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid)

Metal ions play an important role in the activity of fish lipoxygenases, acting as activators of an enzyme through its binding with a substrate or release of its active center. The enzyme activity was significantly increased by Fe^{2+}, Na^{+} and Ca^{2+} for both substrates but decreased in the presence of Cu^{2+} (Table 4.4). Fe^{2+} caused an increase in LOX activity, which was clearly due to Fe^{2+} catalyzed auto-oxidation. NaCl is an effective catalyzer of LOX in the muscle tissue and roe of Baltic herring (Samson and Stodolnik, 2001). Zhang et al. (2019) reported LOX activity increased at salting and resting stages in boneless dry-cured hams during processing might be attributed to the activation of salt. However, Activity of LOX from Mackerel gill was not affected by Na^{+} (Hong et al., 1994). The type of metal ion contained in food may have a major impact on the enzyme's activities.

Table 4.5 Effects of various chemicals on the activity of lizardfish LOX toward different

Substances (1.0 mM)	Residual activity (%)	
	Linoleic acid	EPA
Control	100	100
EDTA	0	0
BHA	95	0
BHT	85	0
Ascorbic acid	60	0
N-propyl gallate	77	0
Cu^{2+}	71	68
Fe^{2+}	120	138
Mn^{2+}	98	89
Hg^{2+}	89	92
Zn^{2+}	100	98
Na^{+}	137	128
K^{+}	106	109
Mg^{2+}	99	100
Ca^{2+}	118	133

4.5 Conclusion

In the manufacturing of tropical surimi, goatfish, lizardfish, and threadfin bream are the major raw materials. It contained a high concentration of PUFA susceptible to lipid oxidation, especially that catalyzed by LOX. DHA and EPA are the two most abundant polyunsaturated fatty acids (PUFA) in tropical fishes. Lizardfish gill exhibited the highest LOX activity as compared to gill, skin, and muscle of other tropical fish. Optimal activity of lizardfish gill LOX was at 25 ° C and pH 7.5. The enzyme was stable at temperatures below 50 ° C. Lizardfish gill LOX exhibited substrate specificity toward EPA. The enzyme was completely inhibited by EDTA but activated by metal ions. Lizardfish surimi showed highly unsaturated fatty acid contents and it can serve as a substrate of LOX. The enzyme residual activity remains after washing (57.85 U/g

sample). This study suggested that LOX activity could be one of intrinsic factors affecting lipid oxidation of surimi during frozen storage.

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