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Evaluation of Biochemical Properties of Burnt and Normal Meat in Pacific Bluefin Tuna (*Thunnus orientalis*)

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Abstract: Burnt meat is one of the unusual meats often occurring in tuna species. In order to reveal the biochemical properties of burnt meat in tuna, burnt meat and normal meat samples were collected to examine the quality parameter including pH, color parameters, lactic acid content, the activities of antioxidant enzymes, and the levels of thiobarbituric acid reactive substances (TBARS) and scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The results showed pH was significantly lower in burnt meat than in normal meat, and the lactic acid content was generally higher in burnt meat. In color parameters, the L* and b* values were higher in burnt meat. Besides, the activities of antioxidant enzymes, namely, superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT) in normal meat were higher than those in burnt meat. TBARS levels were significantly higher in burnt meat than in normal meat. It is thus likely that strong struggle of fish throughout catch and high water temperature induce partial inactivation of these antioxidant enzymes, resulting in free radical formation which triggers the occurrence of burnt meat. This fact could be beneficial for aquaculture and livestock industry of tunas to prevent burnt meat.

Keywords: Burnt Meat, Bluefin Tuna, Antioxidant Activity, Lipid Oxidation

1. Introduction

Burnt meat is one of the unusual meats often taking place in yellowfin (*Thunnus albacores*), bigeye (*T. obesus*) and bluefin tunas (*T. thynnus*). It has been also reported in other species like yellowtail (*Seriola quinqueradiata*) [1, 2]. Burnt meat, as its naming suggests, looks like cooked meat. The meat color is obviously pale and the elasticity is low [3, 4]. Besides, it tastes a little more sour and rancid than normal meat. The occurrence of burnt meat deteriorates the commercial values of fresh tunas, and thus causes a marked economical damage to fishermen and processing industry. The "Sik-Bah" is a Taiwanese word for unusual tuna meat. We have previously reported that "Sik-Bah" is burnt meat [5]. Although the problem of burnt meat has been existing in Taiwan from a long time ago, the mechanisms involved are still not clear.

Burnt meat has been reported in some studies as follows. Konno and Konno [6] demonstrated that burnt meat occurring in the central part of tuna body is followed by protein denaturation, suggesting that insufficient cooling is responsible for burnt meat. Davie and Sparksman, by comparing the meat quality for tuna caught by a long line with that by a handline, concluded that longer capture time generates less burnt meat [7]. Cramer et al. suggested that high body temperature of tuna accelerates the anaerobic metabolism of glycogen and induces the decrease of pH quickly [3]. These two factors are probably the causes of the occurrence of burnt meat. In addition, Ochiai suggested that myoglobin is not largely involved in discoloration of burnt portions [4]. It is thus likely that the main reason for the burnt meat of tuna is strong struggling during catching. The elevated body temperature and accelerated anaerobic glycolysis may cause lactic acid accumulation, resulting in the drop of muscle pH.

The bluefin tuna meat is rich in polyunsaturated fatty acid such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). It is thus suspected that free radicals production or lipid peroxidation may be another reason of tuna burn meat. In order to prove the hypothesis, the changes in free radical level are needed to be analyzed.

Reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are chemically reactive molecules. ROS may cause significant damages to cell structures. Cumulatively, this is known as an oxidative stress. Jenkins indicated that strenuous physical exercise increased ROS more than 10-fold of the resting level [8]. Minyi et al. also reported that anaerobic exercise may induce prolonged ROS generation [9]. Thus, tuna may generate a large amount of ROS in the muscle by struggling. In addition, ROS generated by extreme exercise may attack polyunsaturated fatty acids in cell membranes and lead to lipid peroxidation [10]. Oxidative stress also causes deterioration of meat by lipid oxidation.

To prevent the damage by free radicals, the organisms have a defense system against antioxidants. Several endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), can reduce free radical levels to prevent lipid peroxidation [11, 12]. Fish species with its high content of unsaturated fatty acids in muscle are highly susceptive to attacks by free radicals, causing a marked lipid peroxidation [13]. Such rancid development of fish meat can lead to undesirable changes in flavor, texture and color after slaughter [14]. Bluefin tuna is rich in polyunsaturated fatty acids in its body. However, limited information is so far available on the effects of the activities of antioxidant enzymes and lipid peroxidation on burnt meat.

Meat color is the most important factor affecting not only purchasing motivation of consumers but also the criteria for burnt meat. The International Commission on Illumination (French name, Commission internationale de L'éclairage) LAB (CIELAB) color system is a common standard to quantify color value [15]. The major pigments in tuna muscle are myoglobin (Mb) and hemoglobin (Hb). After proper slaughtering and bleeding, Mb becomes the major pigment of meat color. In tissues, a reduced form of Mb (deoxymyoglobin) exists in equilibrium with its oxygenated form of oxymyoglobin (oxyMb) which is vivid red. In brownish meat, oxyMb seems to have changed to metmyoglobin (metMb) through the oxidation of heme iron. Baron and Andersen demonstrated the proportion of the Mb redox forms was fundamentally influenced by ROS [16].

It is hypothesized that struggling of tuna during capture leads to elevation of body temperature and the accelerated anaerobic glycolysis accompanied by ROS generation deteriorates meat color and quality of tuna meat, finally resulting in burnt meat. The objective of this study was to compare the biochemical properties between burnt and normal meat of Pacific bluefin tuna caught off Taiwan.

2. Materials and Methods

2.1. Materials

The burnt meats of Pacific bluefin tuna were collected at Dong Gang Fishing Harbor, Ping Tung County, Taiwan. Burnt and normal meats were sampled from the same fish. The samples were collected from April to July, 2014. A total of 30 individuals (15 individuals each having burnt and normal meat) were sampled. Only the dorsal ordinary muscles were excised. The collected samples were immediately put in vinyl bags, manually deaerated, kept in ice and analyzed for meat color. The remainder was then stored at -60°C until used.

2.2. pH Measurement and Color Determination

pH was measured using a pH meter (PH100, Extech Instruments Corporation, Waltham, MA, USA). The weighed muscle was mixed with 5 volumes of ice-cold deionized water by using a homogenizer (780CL-04 Tissue-Tearor, BioSpace Product. Inc., Racine, WI, USA) at 4°C. The homogenate was subjected to pH measurement.

Color measurements were carried out by Lab color space (L*, lightness, a*, redness and b*, yellowness) using a sphere spectrophotometer (SP 60, X-rite, Grand Rapids, MI, USA) after calibration with black and white tiles according to manufacturer's instructions. Each meat was measured at three different locations.

2.3. Lactic Acid Content

Lactic acid content was measured as described by Zhu et al. [17]. Meat was homogenized with 10 volumes of 1 M perchloric acid (PCA) at 4°C. Then, the homogenate was centrifuged at 5,000 ×g at 4°C for 10 min. The analysis was conducted using a reversed phase C18 column (Waters Corporation, Milford, MA, USA) with a 1.6 mm particle size, 2.1 mm in inner diameter, and 100 mm in length. The Ultra Performance Liquid Chromatography (UPLC, Waters Corporation, Milford, MA, USA) system consisted of a photodiode array (PDA) wavelength detector. Data acquisition, analysis, and reporting were performed using the Empower chromatography software.

Chromatographic analyses were performed in an isocratic mode with a mobile phase consisting of 10 mM ammonium hydrogen phosphate (NH_4HPO_4) (pH 2.4) and 10 mM NH_4HPO_4 (pH 2.4)/methanol (95:5, v/v) for 5 min at a flow rate of 0.2 ml/min. The sample injection volume was 10 μl, and the PDA was set at 210 nm. The total run time was for 5.1 min at 40°C.

2.4. Protein Concentration

Protein concentration was determined according to Lowry et al. [18], using bovine serum albumin (BSA) as a standard. The absorbance was measured at 660 nm with a spectrophotometer (DU 730, Beckman Coulter, Inc., Indianapolis, IN, USA).

2.5. SOD Activity

The SOD activity was assayed following Marklund and

Marklund [19], and by referring to the way of Shih et al. [20] to prepare the samples. Meat was homogenized with 9 volumes of 0.32 M sucrose buffer (pH 7.4) containing 1 mM EDTA and 10 mM Tris-HCl. Then, the homogenate was centrifuged at 13,600 ×g for 30 min. The reaction mixture was 1 ml in total containing 50 µl of supernatant in 100 µl of 50 mM Tris-HCl (pH 8.2) containing 1 mM EDTA, 880 µl of water, and 20 µl of 10 mM pyrogallol in 10 mM HCl, The absorbance decrease of pyrogallol was measured at 420 nm for 5 min with a DU 730 spectrophotometer. SOD activity was expressed as the amount of enzyme required to inhibit the increase of pyrogallol by 50%, where 1 unit of enzyme gave 50% inhibition of the reaction rate. The activity was expressed as units/mg protein.

2.6. GR Activity

The GR activity was assayed following the method of Carlberg and Mannervik [21]. Meat was homogenized with 20 volumes of 0.25 M sucrose containing 1 mM EDTA (pH adjusted to 7.4 with 1 M Tris). Afterwards, the homogenate was centrifuged at 14,000 × g for 45 min. The reaction mixture contained 20 µl of supernatant in 0.5 ml of 0.2 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA with 50 µl of 2 mM NADPH in 10 mM Tris-HCl buffer (pH 7.0), 50 µl of oxidized glutathione (GSSG), and 380 µl of water in total of 1 ml. The total reaction time was 3 min and reaction temperature was 30°C. The absorbance at 340 nm was measured for 3 min at 30°C. The GR activity was calculated based on the decrease of NADPH and expressed as nmol NADPH/min·mg protein.

2.7. CAT Activity

The CAT activity was assayed following the method of Aebi [22]. Meat was homogenized with 9 volumes of 10 mM sodium phosphate buffer (pH 7.4) at 4°C. The homogenate was subsequently centrifuged at 700 ×g and 4°C for 10 min. To 50 µl of supernatant was added 950 µl of 50 mM phosphate buffer (pH 7.0) containing 30 mM H_2O_2. The changes in absorbance at 240 nm were recorded for 2 min at 25°C. CAT activity was calculated based on the decline of H_2O_2 and expressed as mmole/min·mg protein.

2.8. Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured following the method of Uchiyama and Mihara [23]. Meat was homogenized with 9 volumes of 1.15% KCl. To 0.5 ml of homogenate was added 3 ml of 1% phosphoric acid and 1 ml of 0.67% thiobarbituric acid (TBA), and the mixture was subsequently incubated at 95°C for 45 min and cooled down to 25°C. 4 ml of n-butanol was then added, followed by centrifugation at 1570 ×g for 10 min. The TBARS value was determined based on the difference in absorbance at 535 and 520 nm using 1,1,3,3-tetraethoxypropane as a standard.

2.9. Radicals

The amount of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was determined according to the method of Hatono et al. [24] and Li et al. [25]. Meat was homogenized with 20 volumes of 0.25 M sucrose (pH 7.4) containing 1 mM EDTA-2Na. The homogenate was then centrifuged at 800 ×g, whereas 600 µl of the resulting supernatant was mixed with an equal volume of 0.2 M DPPH in methanol and incubated at 25°C for 30 min. The level of scavenging DPPH radicals was determined, according to the equation: [1- (ABS sample/ABS blank)] ×100%, where ABS is the absorbance at 517 nm.

2.10. Statistical Analyses

Statistical analyses were carried out using Sigma Plot software (Sigma Plot 12, 2012). An independent-samples *t*-test was employed to determine the level of significant differences between burnt and normal meat.

3. Results

pH values of both burnt and normal portions in the same specimens from 15 individuals are shown in Figure 1 and Table 1. The burnt meat gave lower pH in comparison with normal meat with a significant difference ($p < 0.001$).

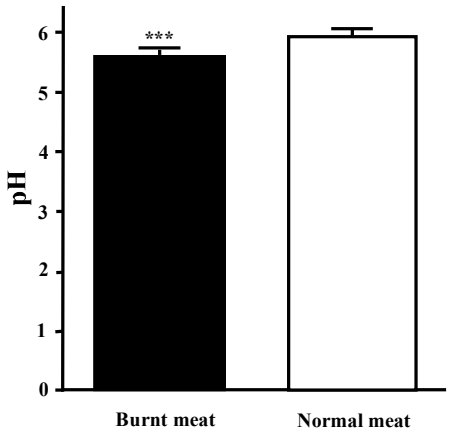

Figure 1. *pH of burnt (n=15) and normal meat (n=15). Significant difference is shown at the level of p < 0.001 (***) by t-test.*

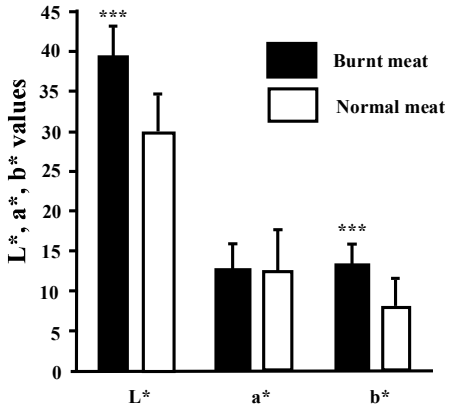

Figure 2. *The color parameters of burnt (n=15) and normal meat (n=15). Significant difference is shown at the level of p < 0.001 (***) by t-test.*

Comparison in tuna meat color between burnt and normal meat is shown in Figure 2 and Table 1. The L* values of both meats were significantly different ($p < 0.001$). Namely, higher

L* values were observed in burnt meat. There was no significant difference in the a* value between burnt and normal meat ($p > 0.05$). However, the b* value of burnt meat was significantly higher than that of normal meat ($p < 0.001$).

Lactic acid contents in tuna meat are shown in Figure 3 and Table 1. The content in burnt meat was clearly higher than that in normal meat, giving a significant difference ($p < 0.001$).

The SOD activity in burnt and normal portion is shown in Figure 4 (A) and Table 1. The activity was higher in normal than burnt meat. They were significantly different ($p < 0.01$).

The activity of an antioxidant enzyme, GR, in normal and burnt meat is shown in Figure 4 (B) and Table 1. The activity was higher in normal than burnt meat, with a significant level of difference ($p < 0.05$).

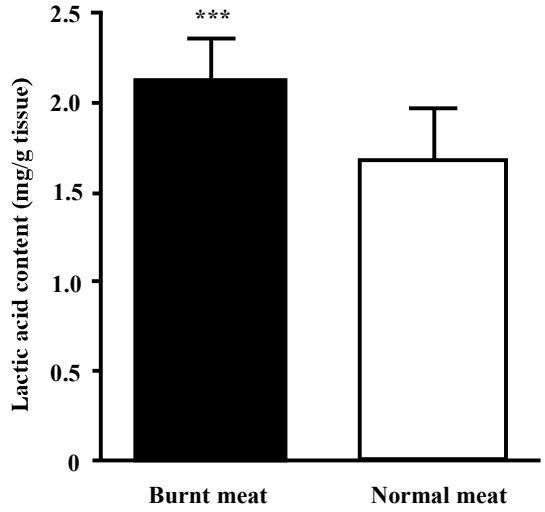

Figure 3. *The lactic acid content of burnt (n=15) and normal meat (n=15). Significant difference is shown at the level of $p < 0.001$ (***) by t-test.*

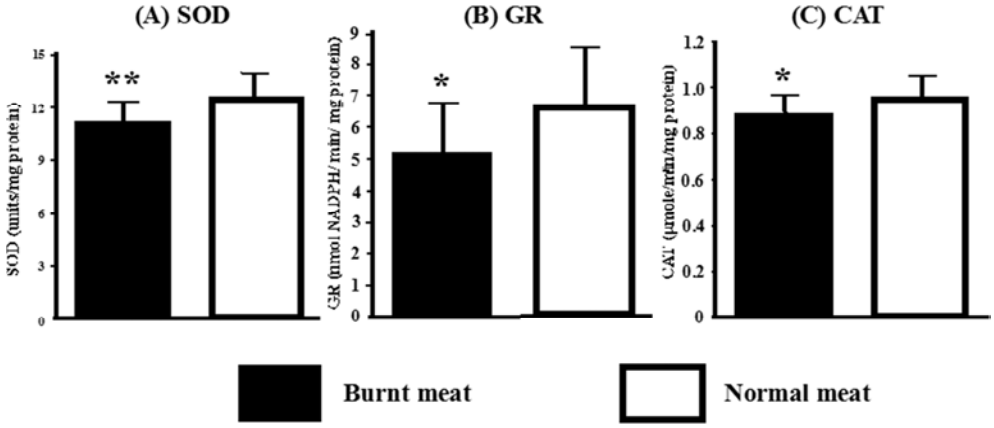

Figure 4. *The antioxidant enzymes activities of burnt (n=15) and normal meat (n=15). Significant difference is shown at the level of $p < 0.001$ (***) by t-test. (A) superoxide dismutase (SOD), (B) glutathione reductase (GR) and (C) catalase (CAT) activities.*

The CAT activities in burnt and normal meat are shown in Figure 4 (C) and Table 1. The activity in burnt meat was lower than that in normal meat with a significant difference ($p < 0.05$).

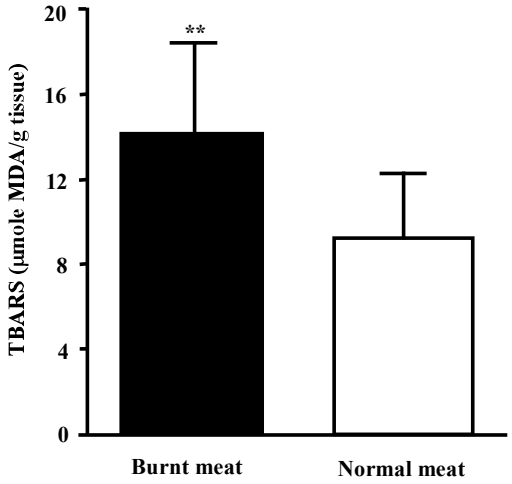

Figure 5. *Thiobarbituric acid reactive substances (TBARS) of burnt (n=15) and normal meat (n=15). Significant difference is shown at the level of $p < 0.001$ (***) by t-test.*

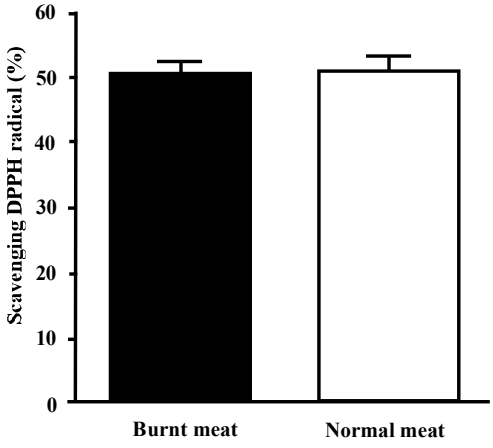

Figure 6. *The level of scavenging 2,2-diphenyl-1- picrylhydrazyl (DPPH) radicals of burnt (n=15) and normal meat (n=15).*

TBARS values in burnt and normal meat are shown in Figure 5 and Table 1. The lipid peroxidation in burnt meat was much higher ($p < 0.01$) than that in normal meat. The TBARS value was 14.08±4.32 μmol MDA/g tissue in burnt meat, and 9.32±3.04 μmol MDA/g tissue in normal meat.

Table 1. *Comparison of the parameters between burnt and normal meat from bluefin tuna.*

	Burnt meat	Normal	Unit	Significance
pH	5.6±0.1	6.0 ± 0.1		$p < 0.001$***
Lactic acid	2.12±0.24	1.69±0.28	mg/g tissue	$p < 0.001$***
L*	39.4±3.7	30.1±4.6		$p < 0.001$***
a*	12.6±3.4	12.5±5.2		$p > 0.05$
b*	13.1±2.8	8.2±3.4		$p < 0.001$***
SOD	11.1±1.2	12.5±1.3	units/mg protein	$p < 0.01$**
GR	5.1±1.7	6.6±1.9	nmol NADPH/min/mg protein	$p < 0.05$*
CAT	0.876±0.095	0.953±0.113	μmol/min/mg protein	$p < 0.05$*
TBARS	14.08±4.32	9.32±3.04	μmol MDA/g tissue	$p < 0.01$**
DPPH	50.42±1.99	50.90±2.15	%	$p > 0.05$

The levels of scavenging DPPH radicals were 50.42±1.99% and 50.90±2.15% for burnt meat and normal meat, respectively, and did not differ significantly between burnt and normal meat ($p > 0.05$) (Figure 6, Table 1).

4. Discussion

In this study, burnt meat phenomenon of Pacific bluefin tuna (*T. orientalis*) landed on Taiwan was investigated. The characteristics of burnt meat varied as other researchers have already reported [3, 4], namely, the pale or brown color of meat, exudative water, peculiar smell, softness and so on. Similar phenomena are also observed in other livestocks such as poultry, beef and pork, which are called pale, soft, and exudative (PSE) meat [26, 27, 28]. Our results showed that pH of normal meat was generally higher than that of burnt meat and the lactic acid content was generally higher in burnt than normal meat. These results suggest that the taste of burnt meat became sour. After death, anaerobic glycolysis proceeds in muscle where lactic acid is subsequently generated and accumulated [29]. Following the accumulation of lactic acid, the pH decrease affects the quality of meat [30]. Struggling of bluefin tuna during capture might cause quick lactic acid accumulation, resulting in a rapid drop of muscle pH.

Block [31] reported that burnt meat is associated with the hardest stress which tuna experience during rapid capture. Arroyo Mora et al. [32] reported that the stressful slaughter method which would favor faster glycolytic process also influences the occurrence of burnt meat in yellowtail (*S. quinqueradiata*). In pigs, high post-mortem temperature and low muscle pH are the possible factors for PSE [33, 34]. Similarly, when pigs are stressed, the glycogen content in muscle is reduced by its conversion to lactic acid, lowering pH of meat [35]. The lower pH and higher lactic acid content were also observed in PSE [33, 36, 37, 38].

From a morphological point of view, large and thick body as well as thick skin and scales of tunas probably lead to difficulty in heat radiation and cause high post-mortem temperature after struggling. Besides, due to the existence of the endothermic system in tunas, counter-current heat exchangers function to keep their body temperature above that of seawater when they are alive [39]. Such special physiology of counter-current heat exchangers could maintain elevated temperatures in deep red-muscle fibers densely surrounding the spine where burnt meat most likely distributes [40, 41, 42]. Konno and Konno suggested that insufficient cooling of deeper muscle close to the spine is probably the reason why burnt meat is produced [6]. Therefore, the muscle around the spine is considered to be subjected to burnt meat more easily than the other parts.

Watson et al. reported high habitat temperatures cause burnt meat [43]. In Taiwan, fishermen catch bluefin tuna from April to July. After the middle of June, the water temperature elevation possibly increases the frequency of burnt meat. The results in laboratory experiments in the fish tank at higher temperature also suggest that insufficient cooling triggers the production of burnt meat [32]. Accordingly, the ambient warm air temperature may also promote the occurrence of burnt meat.

Furthermore, the burnt meat showed degradation of the protein of around 50 kDa in SDS-PAGE [4], and the major degraded component corresponded to creatine kinase. This enzyme may be very susceptible to be degraded by higher temperature and lower pH. Itoi et al. reported the levels of serum creatine kinase were increased in rainbow trout (*Oncorhynchus mykiss*) after heat treatment [44]. It suggests that creatine kinase may be leaked from muscle to serum after heat event in burnt meat. It also indicates that burn meat has deep relationship with heat damages. Hence, it is very likely that the generation of burnt meat might be caused by the combination of several factors.

Bluefin tuna contain the high amount of polyunsaturated fatty acids, up to 400 mg eicosapentaenoic acid (EPA) and 1200 mg docosahexaenoic acid (DHA) per 100 g of muscle [45]. ROS generated through oxidative stress attacks polyunsaturated fatty acids, enhancing lipid peroxidation in meat, thus causing deterioration of meat. TBARS concentrations were found to be higher in burnt meat, suggesting that polyunsaturated fatty acids were at least partially damaged by ROS [46], resulting in higher oxidation level of lipid. Extensive struggling might enhance the generation of a large amount of ROS due to strenuous physical exercise, followed by lipid peroxidation in muscle, leading to burnt meat. The higher TBARS values were also found to be characteristic of PSE meat [47].

Antioxidant enzymes such as SOD, GR and CAT play the major roles in protecting cells from damages by free radicals [48]. In the present study, the activities of these enzymes were slightly inactivated in burnt meat, but seem to maintain the

similar level compared to those in normal meat. SOD transforms free radical O_2^- to H_2O_2 to reduce ROS. The higher activity of SOD has been demonstrated in PSE and red, firm and non-exudative (RFN) pork as reported by Chen et al. [47]. Cornforth et al. showed that low muscle pH affected the function of mitochondria, where Mn SOD is localized [49]. That may be why the activity of SOD is lower in burnt meat.

CAT seems to be the most effective enzyme to retard oxidation and deterioration, by inhibiting oxidation of oxyMb to metMb [50]. GR catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting against oxidative stress and maintaining the reducing environment inside cells [51]. Low pH and high temperature brought about exudative meats, resulting in lower water-holding capacity of denatured protein in burnt meat [52]. SOD, CAT and GR are localized in the cytosol. These antioxidant enzymes in the present study showed lower activities in burnt than normal meat. The higher activities in normal meat catalyze ROS more efficiently and thus might favor for maintaining the freshness. High antioxidative capacity in tissues would prevent lipid oxidation. However, the level of scavenging DPPH radicals did not show significant differences between burnt and normal meat. There were not only the above antioxidant enzymes but also antioxidant vitamins such as tocopherol and ascorbic acid even in burnt meat. This might be the reason why the ability of scavenging free radicals did not differ significantly between normal and burnt meat.

The L* and b* values of burnt meat were significantly higher than those of normal one. Higher L* values make meat look paler, whereas higher b* values make it more yellowish. The Mb content in meat and the proportion of Mb redox forms are the important factors affecting the color of meat, and lipid peroxidation is also related with pigment oxidation [53]. The proportion of the Mb redox forms has been reported to be fundamentally influenced by ROS [54]. Guidi et al. concluded that the increased formation of metMb resulted from the raised ROS level [55]. The high metMb content in meat might be the factor that causes the increase of b* value in burnt meat. Higher L* and b* values were also observed in PSE of chickens [36, 37]. Therefore, it is suggested that higher antioxidant enzyme activities or some antioxidant chemical material in tuna meat reduced the ROS production and led to significant reduction of the TBARS values, thus preventing conversion of oxyMb to metMb.

Although the burnt meat problem has existed in Taiwan for a long time, related studies have been quite rare. This issue must be faced in Taiwan not only from the economical point of view but also for effective utilization of limited tuna resources. In the present study, the biochemical properties of burnt meat were examined, and as far as we know, this is the first report for the burnt meat in Taiwan. However, a lot of questions are still to be addressed for the burnt meat of tuna. In the previous study [5], we interviewed some workers dealing tuna about burnt meat and collected some special information. Nine out of thirty workers claimed that solar or lunar irradiation may also be related to burnt meat. It might be associated with physiological conditions of tuna which are affected by the movement of their heavenly bodies. The protein denaturation and the proportion of the Mb redox forms in burnt tuna meat are on the way to be considered as the changes accompanied by the occurrence of burnt meat.

5. Conclusion

In the present study, strong struggle of tuna throughout the catch and high water temperature were considered to be the main causes of tuna burnt meat. In addition to the theory, the present study also explored the role of free radicals and lipid peroxidation in the occurrence of tuna burnt meat. The results showed that higher levels of free radicals together with lipid peroxidation products would promote tuna burnt meat. This conclusion could give a hint to prevent burnt meat and also favors the tuna aquaculture and livestock industry to reduce burnt meat occurrence ratio.

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