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Original Article

Cathepsin activities and thermal properties of Nile tilapia (*Oreochromis niloticus*) meat during ambient storage

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ABSTRACT

Understanding the postmortem changes at ambient aquatic temperature can be useful for estimating the time of death in environmental forensic studies when little information is available. Muscle degradation was investigated in Nile tilapia (*Oreochromis niloticus*) in terms of the specific activities of cathepsins (B, H and L) and the scavenging activities and thermal transition properties of myosin and actin, to assess postmortem changes with time (0, 1, 2, 4, 8, 12, 24 and 48 h after death). The study results are relevant to ambient temperatures in Thailand, (about 30 °C). The specific activities of the three cathepsin enzymes increased significantly with postmortem time ($p < 0.05$) and had a highly significant positive relationship ($r = 0.987–0.997$, $p < 0.01$, $n = 32$). Cathepsin H had the lowest specific activity and exhibited a different type of time profile. Its lowest specific activity was observed at 8 h, which indicated a significant role at this point in time after death. The radical scavenging activities substantially decreased with the time since death, especially within the first 1 h, while no changes occurred from 2 to 8 h, or from 12 to 24 h. The thermal properties of myosin and actin were observed up to a 24 h delay. The degradation of each protein fluctuated with the delay time; actin was more sensitive to postmortem delay than myosin. Overall, the findings from the current study might be used as primary data to estimate the time of death of an aquatic animal. A potential application is for environmental forensics in relation to fish kill events associated with pollution crimes or the mass death of exported fish under transportation insurance, as well as in animal cruelty investigations.

Introduction

Postmortem changes of animals are influenced by various environmental factors including temperature, humidity, cooling rate and the growth of microorganisms (Campobasso et al., 2001). Most studies of postmortem changes have investigated terrestrial animal models, especially mammals such as rats (Kočárek, 2003), dogs (Lasseter et al., 2003), pigs (Dekeirsschieter et al., 2009) and cattle (Rhee and Kim, 2001). For aquatic animals, preservation of the products is the main purpose for the cold temperatures used in transport or storage by food industries. However, understanding the postmortem changes at the ambient temperature, can be useful for estimating the time of death when little information is available.

After death, a crude assessment of muscles and joint stiffness allows assigning the postmortem interval to one of three categories, namely pre-rigor mortis, rigor mortis, and post-rigor mortis; these three intervals relate to muscle degradation by enzymes (cathepsins), causing rapid softening of muscle texture due to the degradation of myofibrillar proteins (Jiang, 2000). Cathepsins B and L exhibited superior activity relative to cathepsin H in the white muscle of sea bass (Chéret et al., 2007), while Aoki et al. (2000) reported high activity for cathepsins B and L in the white muscles of some red, fresh fish. These findings suggest important roles of the cathepsins B, H and L in the postmortem degradation of white fish muscle.

Actin and myosin are the main actors that make muscles contract or relax (Tyska and Warshaw, 2002), while sarcoplasmic proteins are another component in muscles, suspended in the cytoplasm (Matos et al., 2011). These proteins can be degraded after death by the cathepsin enzymes (Ho et al., 2000; Ladrat et al., 2003)

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and can be monitored by differential scanning calorimetry (Schubring, 1999; Kuo et al., 2005; Beyrer and Klaas, 2007; Matos et al., 2011; Thongprajukaew et al., 2015b). The aim of this present study was to evaluate postmortem changes in the muscles of an aquatic animal. The economic freshwater fish Nile tilapia (*Oreochromis niloticus*) was chosen as a model, as it is widely cultured around the world (De Silva et al., 2004). The well-known fundamental biology of this species also supported its selection for a laboratory study. The main muscle degradation enzymes (cathepsins B, H and L) were studied concurrently with the thermal properties of actin and myosin. The degradation products were also monitored using a sensitive method, namely by diphenylpicrylhydrazyl (DPPH) radical scavenging activity. The findings from this study might be used as primary data to identify (estimate) the postmortem delay from an aquatic animal carcass in ambient tropical waters.

Materials and methods

Fish preparation and observation of postmortem changes

Four-month-old, sex-reversed Nile tilapia samples were collected from a private farm in Trang province, Thailand. The fish were acclimatized indoors for 15 d and were fed to satiation with a commercial diet containing 30% protein, twice daily (0700 h and 1600 h) under a 12-hr light/12-hr dark cycle. The water quality parameters during acclimatization were 29.60 ± 0.15 °C, pH 6.95 ± 0.02, dissolved oxygen 5.05 ± 0.01 mg/L and ammonia 0.94 ± 0.15 mg/L. Prior to sampling, the fish were starved for 48 h under the conditions described above. Subsequently, all the fish were killed by chilling in ice. Fish with similar body weights (105.83 ± 1.66 g) and total lengths (18.55 ± 0.14 cm) were randomly distributed into rectangular containers (32 cm width × 43 cm length × 11 cm height with 7 cm water level) at a density of 14 fish per container. The fish were floated in their rectangular containers under ambient temperature (29.60 ± 0.15 °C) and a 12-hr light/12-hr dark cycle. Four fish ($n = 4$) at each postmortem time (0, 1, 2, 4, 8, 12, 24 and 48 h) were dissected on ice and white muscle samples were collected, packed in polyethylene bags and kept at −20 °C until determinations. All analyses of white muscle were performed within 1 month after dissection.

Extraction of muscle crude extract

Crude extract of white muscle was prepared according to Chéret et al. (2007), with slight modifications. Briefly, the muscle was homogenized in buffer (1:3 wt per volume) containing 50 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA) using a micro-homogenizer (THP-220; Omni International; Kennesaw, GA, USA). The homogenate was centrifuged at 10,000 × *g*, at 4 °C for 40 min. The supernatant was collected after removing the lipid layer and kept in small portions at −20 °C.

Determination of muscle degradation enzyme activity

Protein concentration in crude extract

Determination of protein was carried out based on the method of Lowry et al. (1951). Bovine serum albumin was used as the protein standard. The protein concentrations in the crude extracts were used for quantifying muscle degradation enzyme specific activities.

Cathepsin B, H and L assays

Activities of the cathepsins B (EC 3.4.22.1), H (EC 3.4.22.16) and L (EC 3.4.22.15) were determined based on Aranishi et al. (1997), with slight modifications. Cathepsin B was assayed by mixing 200 μL of 0.4 M phosphate buffer (pH 6.0, containing 5 mM EDTA, 490 μL of 0.1% Brij35, 100 μL of 10 mM cysteine and 200 μL of 25 μM *Z*-Arg-Arg-7-amido-4-methylcoumarin) and 10 μL of crude enzyme extracts. The enzymatic reaction mixture was incubated at 40 °C for 30 min, and the reaction was stopped by adding 1.5 mL of 0.1 M sodium acetate buffer containing 0.1 M sodium monochloroacetate (pH 4.3). The cathepsins H and L were assayed as described above, except with the 0.4 M phosphate buffer (pH 6.0) replaced by 0.4 M phosphate buffer (pH 6.8, containing 5 mM EDTA, 0.1% Brij35, 10 mM cysteine and 25 μM *Z*-Arg-7-amido-4-methylcoumarin) or by 0.4 M sodium acetate buffer (pH 6.5, containing 5 mM EDTA, 0.1% Brij35, 10 mM cysteine, 25 μM *Z*-Phe-Arg-7-amido-4-methylcoumarin), respectively. Reaction activating incubation for these enzymes was performed at 45 °C or at 50 °C, in the same order. The fluorescence of liberated 7-amido-4-methylcoumarin was measured using a spectrofluorimeter (FP-8200; Jasco; Tokyo, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm.

Scavenging activity by 2,2-diphenylpicrylhydrazyl radical assay

The crude muscle extracts were obtained as described above. The 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity was determined according to the method of Thongprajukaew et al. (2015b). Radical scavenging activity (% inhibition) was calculated from $[(A_o - A_i)/A_o] \times 100$, where A_o and A_i are the absorbance of the control sample (extraction buffer in equal volume to the actual sample) and of the extract, respectively.

Thermal transition properties

The thermal transition properties of muscle were analyzed using a differential scanning calorimeter (DSC7; PerkinElmer; Waltham, MA, USA). A 20 mg muscle sample was placed in an aluminum sample pan, sealed, allowed to equilibrate at room temperature and then heated with comparison against an empty reference pan. Thermal changes were recorded from 25 °C to 120 °C at a rate of 5 °C/min. Myosin and actin were identified using the denaturation temperatures as described by Skipnes et al. (2008) and Matos et al. (2011). Thermal parameters—onset (T_o), peak (T_p) and conclusion (T_c) temperatures of protein denaturation, temperature range ($T_c - T_o$) and the transition enthalpy (ΔH)—were recorded automatically.

Statistical analysis

Data were analyzed using the SPSS version 17 software package (SPSS Inc.; Chicago, IL, USA), and all data were expressed as the mean ± standard error of mean (SEM, $n = 4$). Significant differences between means were determined using Duncan's multiple range test, with significance tested at $p < 0.05$. Pearson's correlation coefficients (r) for each pair of measured parameters were reported.

Results

Specific activities of cathepsins B, H and L

The specific activities of cathepsins B (Fig. 1A), H (Fig. 1B) and L (Fig. 1C) increased with the time delay after death. Generally, a slight increase in specific activity was observed within the first

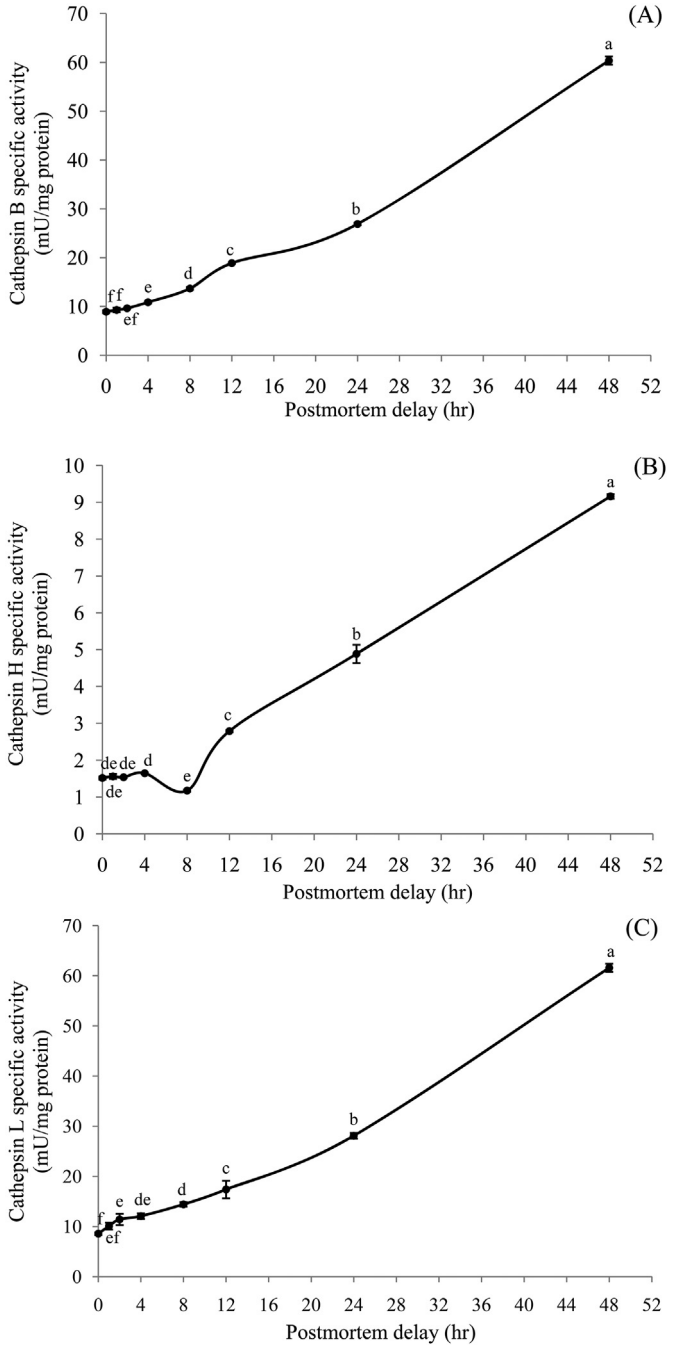

Fig. 1. Postmortem changes in specific activity of cathepsin B (A), H (B), and L (C), in white muscles of Nile tilapia carcass within 48 h after death. Data are expressed as mean ± SEM (n = 4). Different lowercase letters indicate significant differences between observation time points ($p < 0.05$).

4 h, followed by a dramatic increase. The cathepsins B and L exhibited similar characteristics within the 48 h of observation. Both these enzymes had their highest specific activities at the end of the sampling, and the values increased approximately 6.26 fold (60.98 ± 0.59 mU/mg protein on average) compared to the initial activity at 0 h delay (9.74 ± 0.24 mU/mg protein on average). Cathepsin H exhibited a different type of time profile and had lower specific activities than cathepsins B and L. Its lowest specific activity was observed at 8 h after death, and this did not significantly differ from its activities at 0 h–2 h delay times. The two highest specific activities of this enzyme were 6.03 fold and 7.83 fold relative increases at 0 h and 8 h sampling time, respectively.

Scavenging activity of white muscle

The radical scavenging activity of white muscle is illustrated in Fig. 2. The values substantially decreased with the time elapsed since death, especially within the first 1 h. No differences in the values were observed in the time delay interval 2–8 h, or between 12 h and 24 h. The lowest scavenging activity was observed at 48 h after death, with about a 40 fold decrease from the initial rate at 0 h.

Thermal changes of myosin and actin

The thermograms of white muscle showed two endothermic characteristics (Fig. 3). In the control sample, denaturation of myosin occurred at comparatively low temperatures for the first peak (T_o = 44.45 ± 0.22 °C, T_p = 46.88 ± 0.03 °C, T_c = 48.62 ± 0.23 °C), while the following peak corresponded to the denaturation of actin (T_o = 68.31 ± 0.50 °C, T_p = 71.04 ± 0.50 °C, T_c = 73.42 ± 0.37 °C). Fluctuations in results for both proteins were observed within the first 24 h postmortem (Table 1), while the protein peaks became undetectable by the end of observations. Actin was more sensitive to the postmortem changes than myosin, as indicated by the significantly decreased T_o at 12 h after death. The main changes in T_o, T_p and T_c for both proteins were observed at 24 h and decreased with postmortem delay. The values of T_c–T_o, fluctuated and increased with postmortem delay. No significant changes occurred in ΔH or ΣΔH for either protein within the first 24 h after death.

Discussion

Muscle protein degradation was reported to have increased significantly with postmortem delay in fish muscle, both when ice-stored and when super-chilled (Gaarder et al., 2012). Available information is scarce regarding protein degradation in a fish carcass that floats in the ambient aquatic conditions of the tropics. The cathepsins B, D, H and L are cysteine proteases, mainly present in lysosomes. Cathepsin D was not observed in the current study because its role in fish protein hydrolysis should not be significant since it hydrolyzes myofibrils at an optimal pH range from 3 to 5 (Makinodan et al., 1982; Jiang, 2000). These enzymes are released into both cytoplasm and intracellular spaces as a consequence of lysosomal disruption and induce the postmortem pH to fall and the intramuscular ionic strength to increase (Yates et al., 1983). Aoki et al., 2000 and Chéret et al. (2007) reported that the main postmortem degradation of white muscle could be attributed to cathepsins B and L. These enzymes hydrolyze the major muscle structural proteins: B hydrolyzes connectin, nebulin and myosin; and L hydrolyzes connectin, nebulin, myosin, collagen, α-actinin and troponins T and I (Yamashita and Konagaya, 1991). The observed pH in muscle tissue after death is near optimal for both these enzymes (Sainclivier, 1983). The rapid activity increases observed in the current study indicated that the rate of protein breakdown by cathepsins B and L was faster than that by cathepsin H. This finding was in agreement with data for sea bass, as well as for beef (Wang and Xiong, 1999), suggesting that cathepsin H plays a secondary role in the postmortem proteolysis of fish (Chéret et al., 2007). A novel not previously reported observation was the significant decrease of cathepsin H activity at 8 h after death. This may have been due to the postmortem pH drop from 7.0 to 6.5 during the period of rigor mortis in fish, while it later rises to values close to 7 (Sainclivier, 1983). This pH point (pH 6.5) may negatively affect

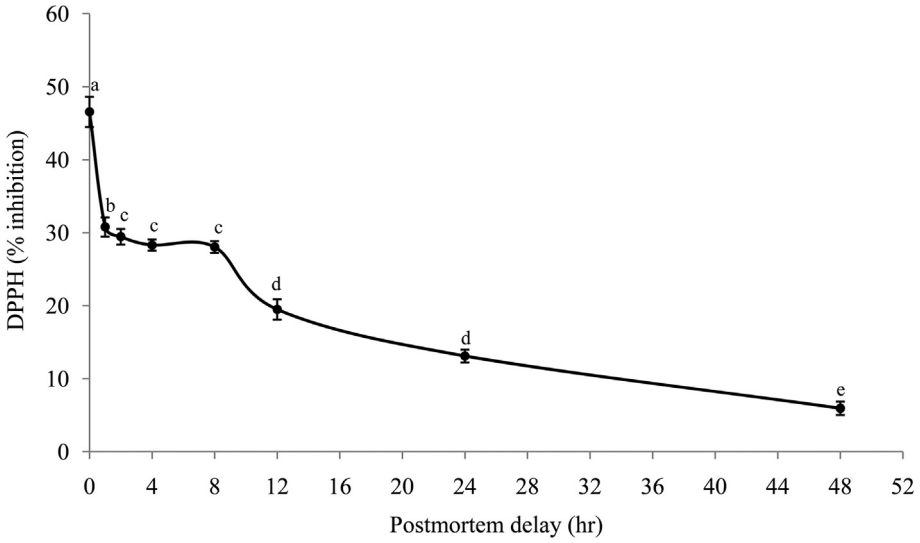

Fig. 2. Postmortem changes in the 2,2-diphenyl-2-picrylhydrazyl scavenging activity of the white muscles of Nile tilapia carcass within 48 h after death. Data are expressed as mean ± SEM ($n = 4$). Different lowercase letters indicate significant differences between observation time points ($p < 0.05$).

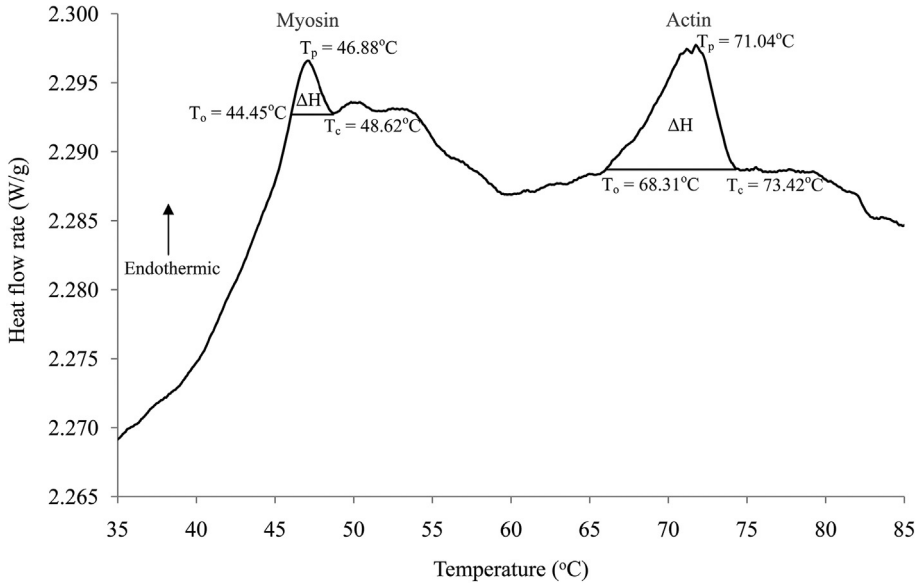

Fig. 3. Thermal transition properties of muscle from control Nile tilapia (0 h after death). T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, ΔH = enthalpy.

the amphoterism of amino acid side chains at the active site of the enzyme, affecting activity.

Based on Pearson's correlation analysis, the three observed cathepsins degraded muscle protein concurrently, as indicated by the highly positive pairwise correlation coefficients between enzyme specific activity and postmortem delay ($r = 0.987–0.997$, $p < 0.01$, $n = 32$), suggesting that the three cathepsins function synergistically on muscle proteins. In a comparison to a terrestrial animal, cathepsins degraded fish muscle faster than bovine muscle (Chéret et al., 2007). This may have been due to the fish having a less complex muscle structure relative to the bovine muscle studied. In the current study, floating the fish carcass in water also supported the hydrolysis capacity of the enzymes. Based on a prior investigation in the same species, however, the degradation of muscle was less than that of the gastrointestinal tract (Hahor et al., 2016). Longer observation times than those of the current study might help to understand better the roles of these enzymes.

Postmortem changes of the physical dimensions of one or more of the intercellular space or intercellular/extracellular fluids significantly decrease the electrical conductivity (Querido, 1992). This prior finding was supported by the significant decrease in scavenging activity with postmortem delay observed in the current study. The postmortem degradation of cells can have toxic effects through produced peroxides and free radicals that damage all components (Lubec et al., 1996). Although the hydrolysis by proteolytic enzymes can release a mixture of various peptides that act as electron donors in DPPH system (Lassoued et al., 2015), these constituents are not sufficient to compensate for the overproduction of free radicals after death. Moreover, at longer hydrolysis times, the formed peptides have shorter chains and are less effective in scavenging DPPH• radicals (Lassoued et al., 2015).

Table 1
Thermal transition characteristics of muscle proteins in sex-reversed Nile tilapia in relation to postmortem time delay. Data are calculated from duplicate determinations and are expressed as mean (standard error of the mean).

Postmortem delay (hr)	Myosin					Actin					ΣΔH (J/g)
	T_o (°C)	T_p (°C)	T_c (°C)	T_c–T_o (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	T_c–T_o (°C)	ΔH (J/g)	
0	44.45^{a} (0.22)	46.88^{a} (0.03)	48.62^{a} (0.23)	4.18^{b} (0.44)	0.39^{a} (0.01)	68.31^{a} (0.50)	71.04^{a} (0.50)	73.42^{a} (0.37)	5.12^{c} (0.87)	0.34^{a} (0.09)	0.73^{a} (0.10)
1	43.35^{ab} (0.38)	46.67^{a} (0.06)	48.68^{a} (0.22)	5.33^{ab} (0.16)	0.42^{a} (0.10)	66.28^{b} (0.40)	70.67^{a} (0.35)	73.22^{a} (0.20)	5.94^{abc} (0.20)	0.31^{a} (0.05)	0.76^{a} (0.13)
2	43.42^{ab}(1.17)	46.55^{a} (0.44)	48.43^{a} (0.39)	5.01^{ab} (0.78)	0.37^{a} (0.05)	67.06^{ab} (0.50)	70.42^{a} (0.12)	72.72^{a} (0.04)	5.66^{bc} (0.54)	0.33^{a} (0.07)	0.70^{a} (0.02)
4	43.65^{ab} (0.11)	46.21^{a} (0.09)	48.28^{a} (0.12)	4.63^{ab} (0.23)	0.43^{a} (0.01)	66.26^{b} (0.08)	70.29^{a} (0.03)	72.59^{a} (0.01)	6.33^{abc} (0.06)	0.42^{a} (0.03)	0.85^{a} (0.04)
8	42.87^{ab} (0.09)	46.21^{a} (0.09)	48.71^{a} (0.33)	5.84^{ab} (0.24)	0.54^{a} (0.05)	67.09^{ab} (0.02)	70.42^{a} (0.06)	72.79^{a} (0.04)	5.70^{bc} (0.06)	0.39^{a} (0.03)	0.92^{a} (0.02)
12	42.16^{ab} (1.69)	47.00^{a} (0.29)	47.90^{a} (0.34)	4.56^{ab} (0.06)	0.43^{a} (0.09)	65.99^{b} (0.06)	69.92^{a} (0.47)	73.26^{a} (0.05)	7.27^{ab} (0.11)	0.39^{a} (0.03)	0.81^{a} (0.12)
24	39.72^{b} (0.03)	43.46^{b} (0.33)	45.96^{b} (0.27)	6.24^{a} (0.29)	0.32^{a} (0.04)	62.20^{c} (0.60)	66.79^{b} (0.15)	70.12^{b} (0.27)	7.92^{a} (0.34)	0.33^{a} (0.04)	0.68^{a} (0.04)
48	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

T_o = onset temperature; T_p = peak temperature; T_c = conclusion temperature; T_c–T_o = temperature range; ΔH = enthalpy; nd = not detected.
Different lowercase superscripts in the same column indicate significant differences between observations with different postmortem delays ($p < 0.05$).

However, based on the current observations, the biochemical determination of this scavenging activity appears to be a sensitive indicator of the time delay since death.

Differential scanning calorimetry (DSC) is used to assess the protein conformation, and energy changes can explain structural changes such as protein denaturation. Denaturation of myosin and actin can be caused by a drop in pH (Tyska and Warshaw, 2002), and is followed with degradation by the proteolytic enzymes. Kuo et al. (2005) reported that the denaturation temperature of muscle protein was lowered in a postmortem specimen and the current study observed decreased values for T_o, T_p and T_c, and an increased value in T_c–T_o with postmortem delay. These changes indicated the transformation or partial degradation of proteins by proteolytic cleavage. Thongprajukaew et al. (2015a) reported that a broad T_c–T_o range was due to the heterogeneity of polymer chain lengths. Increased values in the current study might have been caused by the production of diverse polypeptide chains through enzymatic hydrolysis. This effect of the time delay after death has also been observed in the red muscles of the same species (Nonthaput et al., 2015), as well as in red snapper, red mullet and catfish (Schubring, 1999). According to the current data, actin was more sensitive to postmortem changes than myosin since significantly decreased T_o was observed at 12 h after death. This was in agreement with the stable nature of tropical fish myosin reported by Schubring (1999). However, the transition peaks of the proteins were no longer detected at 48 h after death, suggesting complete absence of native protein forms. This finding was in agreement with earlier investigation of the red muscles of Nile tilapia (Nonthaput et al., 2015) and with studies of the rigor mortis phase (within 2–24 h after death) of frozen Atlantic salmon (Roth et al., 2006) and of frozen barramundi within 3–24 h after death (Wilkinson et al., 2008). Therefore, the muscles become flexible again as the proteins are completely degraded (Tyska and Warshaw, 2002). In higher-order animals, rigor mortis in rats develops within 5–24 h after death (Krompecher, 1994), while in humans during summer (12.0–46.5 °C) or winter (−2.6–35.4 °C) this can take 8–30 h or 7–36 h, respectively (Dalal et al., 2006). These differences in the time delay between aquatic and terrestrial animals are consequences of the muscle structure and configuration of blood circulation (Gillis and Biewener, 2001). Furthermore, the current study was conducted in an ambient water environment which may have caused faster degradation rates due to both the availability of water and the comparatively high temperatures in the tropics. There were no significant differences observed within 24 h from death for ΔH or ΣΔH. Both these thermal parameters indicate the amount of proteins in native form (Matos et al., 2011). Beyrer and Klaas (2007) also reported no changes in total ΔH for single- or double-frozen cod fillets. Similarly, while the temperature shift in the DSC thermogram can be used as an indicator of meat freshness, ΔH cannot be used (Kuo et al., 2005). Therefore, prior studies support the conclusion that T_o, T_p, T_c and T_c–T_o are more sensitive to the qualitative postmortem changes in proteins than are ΔH or ΣΔH.

The specific activities of cathepsins, the radical scavenging activity and the degradation of myosin and actin were significantly affected by the postmortem delay time. Based on the current observations, the degradation rate of fish muscle in an ambient aquatic environment in the tropics was faster than that reported for terrestrial animals. In continuation of this reported research, a histopathological examination of the muscles for their postmortem changes is currently underway in this same fish species. The findings from the current study suggested that the time of death of an aquatic animal, left to decay in ambient tropical waters, can be estimated from muscle samples from the carcass. This knowledge has potential application in environmental forensics in relation to fish kill events associated with pollution crimes, as well as in animal cruelty investigations. However, fish kills can have a large variety of differing circumstances (chemicals, pH, water temperature and environmental factors among others) that can interfere with the processes examined in the current study. Therefore, each species might require work specific to it under a variety of circumstances before the approach of the current study could be used in a court of law.

Conflict of interest

The authors declare that there is no conflict of interest.

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