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Myofibrillar protein profile of *Pectoralis major* muscle in broiler chickens subjected to different freezing and thawing methods

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Abstract

The study examined the protein profile of *Pectoralis major* muscle in broiler chickens subjected to different freezing and thawing methods. *Pectoralis major* muscle was excised from the carcasses of twenty broiler chickens and split into left and right halves. The left half was subjected to slow freezing (-20°C) while the right half was rapidly frozen (-80°C). The samples were stored at their respective temperature for 2 weeks and assigned to either of tap water (27°C, 30 min), room temperature (26°C, 60 min), microwave (750W, 10 min) or chiller (4°C, 6 h) thawing. Changes in myofibrillar proteins following the thawing methods were monitored through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic profile indicated differences ($p < 0.05$) in intensities of the components of myofibrillar proteins among the thawing methods in both slow and rapidly frozen samples. Chiller thawing had significantly higher ($p < 0.05$) protein concentration than other methods in rapidly frozen samples. However, in slow freezing, there were no significant differences in protein concentration among the thawing methods. In rapidly frozen samples, the protein optical densities at molecular weight of 21, 27, 55 and 151kDa in tap water, chiller and room temperature thawing did not differ ($p < 0.05$). Similarly, in slowly frozen samples, protein optical densities at molecular weight of 21, 27, 85 and 151 kDa were not significantly different among chill, tap water and room temperature thawing. Microwave thawing consistently caused higher protein degradation resulting in significantly lower ($p < 0.05$) protein quality and quantity in both freezing methods.

Introduction

Poultry meat attracts considerable attention as an important source of protein and other valuable nutrients in many parts of the world. Due to the nutritionally dense nature of poultry and other meats, they are readily susceptible to chemical and microbial deteriorations, which affect the overall quality of the products. Protein degradation is not exempted in the course of spoilage. The severity of protein degradation is influenced by pH, temperature, storage time, microbial protease and endogenous factors (Ang and Hultin, 1989). The autolysis of nitrogenous compounds becomes more intense after a prolonged storage especially under inappropriate conditions (Smith, 2001). The formation of biogenic amines and oxidative rancidity often accompanied spoilage (Jadhav *et al*., 1995). In order to prevent such deterioration in meat, freezing technology is employed. However, except for microbial spoilage that is prevented, quality deteriorations in colour, flavour and texture still occur during frozen storage (Farouk and Swan, 1998). In addition, freeze-thaw process can promote lipid oxidation and protein degradation both of which can affect the quality attributes of the muscle (Sriniversan *et al*., 1997). There are variations in the degree of protein denaturation among species as influenced by postmortem storage and freeze thawing conditions. It is common for frozen meat to be thawed prior further processing. Thawing processes could further damage the muscle fibres resulting in changes in water holding capacity of muscle proteins. Although, various studies have been conducted on the effect of freezing on meat quality (Wagner and Anon, 1986; Badii and Hawell, 2011), there are limited studies on effect of subsequent thawing on changes in myofibrillar proteins in broiler meat subjected to different freezing methods. Myofibrillar proteins such as myosin, actin, troponin, and tropomyosin serve as the buiding blocks for muscle fibre and are related with the water holding capacity and the other functional properties of muscle such as gelation. The objective of this study was to determine the myofibrillar protein profile of *Pectoralis major* muscle in broiler carcass subjected to different freezing and thawing methods.

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Materials and Methods

Experimental animals, slaughtering and muscle sampling

Twenty male Cobb broiler chickens (6 weeks of age and mean live weight of 1.8 ± 0.2 kg) were obtained from a local commercial farm, transported to the slaughterhouse at the Department of Animal Science, Universiti Putra Malaysia and lairaged without feed but given ad libitum amount of drinking water for 6 h. The entire handling, slaughtering and processing management of birds were carried out according to the halal slaughtering guidelines as outlined in the MS1500:2009 (Department of Standards Malaysia, 2009). After bleeding, evisceration, and de-feathering, *Pectoralis major* muscles were excised from the left and right side of the carcasses. Excess fat and visible connective tissue were trimmed off. Samples from each half were divided into four equal portions. After cut, muscle samples were placed in labelled plastic bags and vacuum packed. The left sides of muscle were subjected to slow freezing (-20°C), while the right sides were assigned to fast freezing (-80°C) method and both were stored for a period of two weeks. Frozen samples from each freezing method were thawed by either of tap water (27°C, 30 min), room temperature (26°C, 60 min), chill temperature (4°C, 6 h) or microwave oven (100 W, 10 min) thawing. The methods of thawing employed in this study were selected to mimic the commonly applied techniques in processing plants and kitchens. All samples were assessed for their protein profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein extraction

All frozen samples were initially placed in liquid nitrogen and ground thoroughly to a powder form using a mortar and pestle. Approximately 1 g of crushed muscle tissue was homogenized (Wiggen Hauser D-500) in 3 ml of ice-cold extraction buffer (2 mM β-mercaptoethanol, 10 mM ethylenediamine tetra-acetic acid (EDTA), 20 mM Tris Base, 6 N Hydrochloric Acid (HCl), and 50 µl/ml protease inhibitor cocktail (Calbiochem, San Diego, CA) at pH 8.3 for 15 sec. Then, the samples were centrifuged at 13,000 rpm for 20 min at 4°C after which, two aliquots of 1.5 ml of the resulted supernatant were transferred into micro-centrifuge tube. The resulted supernatant was diluted 100 fold in extraction buffer for total protein concentration determination (Bradford Assay). The remaining supernatant was stored at -80°C until subsequent analysis.

Determination of protein concentration

Total protein concentration in samples supernatant was determined by Bradford Protein Assay Kit (Bio-Rad, USA). The Bradford protein assay is a spectroscopic analytical procedure used to measure concentration of protein in a solution. For constructing the standard curve, 1.5 µg/µl of stock Bovine Serum Albumen (BSA) that was provided with the kit were diluted into six concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 µg/µl in the same extraction buffer used for protein extraction. Protein samples were diluted 100 fold in extraction buffer. A volume of 10µl of each of the standards and samples were suspended in 96-well plate with duplicate wells followed by the addition of 200 µl of Bradford dye reagent and mixed thoroughly. The plates were agitated followed by 5 min incubation at room temperature. Subsequently, the absorbance was measured using spectrophotometer (GENESYSTM, Spectronic® 20, USA) at 595 nm. Standard curve of each plate was constructed and used for the determination of total protein concentration of each sample supernatant.

Protein electrophoresis by SDS-PAGE

Gel preparation (Laemmli Buffer System)

Prior to plates assembling, the glass plates were wiped with 70% ethanol for cleansing. The plates were assembled on the casting stand, and deionized water was flush in to the surface level to check for leaking. The 12% resolving gel solution (30% acrylamide/bis, 1.5 M Tris-HCl (pH 8.8) and 10% (w/v) sodium dedocyl sulphate (SDS) was prepared and degassed in the fumehood for 15 minutes. Next, 10% (w/v) ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added into the mixtures prior to use. The mixtures were then filled into an assembled glass up to 5.5 cm and deionized water was added until it reached the upper cassette. The gel was allowed for polymerization for 45 min at room temperature. In the meantime, 4% stacking gel solution (30% acrylamide/bis, 0.5 M Tris-HCl (pH 6.8), 10% w/v SDS) were prepared. Then, 10% (w/v) ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added into the mixtures prior to use. After that, a 10 wells comb was inserted gently between the spacer. Resolving gel polymerization was allowed to take place for 45 min at room temperature.

Sample loading and protein separation

Protein samples were diluted in sample buffer (as 1:2 ratios) containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, 0.02% (w/v) bromophenol

blue and heated at 95°C for 4 minutes. A volume of 3 µl of protein ladder (PageRuler™ Prestained Protein Ladder Plus; Cat No: SM1811 from Fermentas) was loaded into the first well, while equal amount of 30 µg of each sample were loaded into the remaining wells. Proteins were separated in running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS) at pH 8.3 using a Mini-Protean Tetra Cell system (Bio-Rad; USA) under constant voltage of 120V. The electrophoresis was run until the tracking dye reached the bottom of the gel.

Coomassie blue protein bands staining

The gels from the electrophoresis run were stained with Coomassie blue staining solution (0.05% Coomassie blue, 5.0% acetic acid, 15% methanol) overnight and destained with destaining solution (10% acetic acid and 30% methanol) until excessive background disappeared.

Protein bands visualization by Densitometer

A densitometer (GS-800 Calibrated Imaging Densitometer, Bio-Rad) was used to visualize and quantify the protein band. Data were analysed by Quantify One® software for molecular weight and quantify optical densities of the proteins band visualized.

Statistical analysis

The data were analyzed using the GLM procedures of SAS Version 9.2 software (Statistical Analysis System, SAS Institute Inc, Cary, NC, USA) in a completely randomized design. Mean differences were considered significant at $p < 0.05$ and separated by Duncan multiple range test.

Results and Discussion

Total protein concentration

The total protein concentrations in the slowly and rapidly frozen of *Pectoralis major* muscles subjected to different thawing methods are presented in Table 1. In the slowly frozen samples, no difference in total protein concentration was observed among the thawing methods (Table 1) while in rapidly frozen samples, there were differences in total protein concentration among the thawing methods ($p < 0.05$). The highest total protein concentration was indicated by samples subjected to CT thawing method (0.676 ± 0.019), compared to other thawing methods. However, the total protein concentration in RT, TW and MW samples were not significantly different.

The observations in fast frozen samples could be due to differences in protein denaturation and

Table 1. Total protein concentration in slowly and rapidly frozen *Pectoralis major* muscles subjected to different thawing methods

Freezing methods	Thawing methods				
	RT	TW	CT	MW	P value
Slow freezing (-20°C)	0.628±0.017	0.627±0.014	0.670±0.019	0.660±0.021	0.240
Fast freezing (-80°C)	0.618±0.011[b]	0.590±0.013[b]	0.676±0.019[a]	0.589±0.029[b]	0.007

[a,b] means in a row with different superscripts are significantly different at $p < 0.05$.
RT - room temperature; TW - tap water; CT - chille temperature;
MW - microwave.

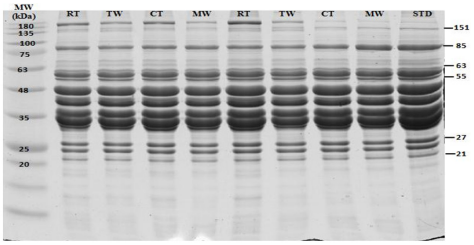

Figure 1. SDS-PAGE patterns of *Pectoralis major* muscle proteins subjected to room temperature (RT), tap water (TW), chiller (CT), and microwave (MW) thawing on 12% acrylamide gel stained with Commassie blue staining solution. Each lane was loaded with equal amount (30 µg) of protein.

protein solubility during the thawing process. Xiong and Decker (1995) reported that freeze–thaw process induced protein denaturation due to loss in salt-soluble protein. The formation of disulfide bonds might be responsible for decrease in solubility. The accelerated denaturation of protein might have resulted in increased disulfide bond formation (Thanonkaew *et al.*, 2006). Vojdani (1996) attributed reduction in protein solubility to a change in balance of interaction between protein and water, and protein intermolecular interaction, leading to a scenario where interaction between protein and water is waned while protein intermolecular interaction is reinforced. Furthermore, Decker *et al.* (1993) reported that cross-linkages are formed among proteins due to loss of ordered tertiary structure as proven by the decrease in solubility. From the present results, MW tends to show decline in solubility related to upsurge in protein denaturation during freeze-thaw process. This could results from liberation of pro oxidant compounds and oxidative enzymes from cellular organelles that were ruptured (Xia *et al*., 2012). Therefore, there could be a greater precipitation of soluble protein in the supernatant due to decreased sulphyhydryl group content with greater disulfide bond content (Xiong *et al*., 2000).

Electrophoretic banding patterns and protein band intensity

The electrophoretic profiles of myofibrillar proteins on 12% acrylamide gels are shown in Figure 1. Protein bands appeared to be of different pattern among the thawing treatments were considered and

Table 2. Effect of different thawing methods on optical density of protein bands in slowly and rapidly frozen *Pectoralis major* muscle

Freezing methods	Molecular weight (kDa)	Thawing methods				
		RT	TW	CT	MV	P value
Slow freezing (-20°C)	21 kDa	0.207±0.011^{a}	0.191±0.010^{a}	0.195±0.010^{a}	0.134±0.005^{b}	0.003
	27 kDa	0.425±0.024^{a}	0.388±0.023	0.405±0.021^{a}	0.329±0.017^{b}	0.053
	55 kDa	0.419±0.024	0.399±0.018	0.408±0.018	0.373±0.015	0.254
	63 kDa	0.187±0.006	0.176±0.005	0.183±0.008	0.169±0.005	0.296
	85 kDa	0.336±0.010^{ab}	0.362±0.020^{ab}	0.385±0.020^{a}	0.299±0.010^{b}	0.016
	151 kDa	0.136±0.005^{ab}	0.139±0.006^{ab}	0.148±0.008^{a}	0.120±0.005^{b}	<0.001
Fast freezing (-80°C)	21 kDa	0.213±0.012^{a}	0.188±0.010^{a}	0.205±0.012^{a}	0.128±0.006^{b}	0.002
	27 kDa	0.440±0.024^{a}	0.396±0.019^{a}	0.419±0.027^{a}	0.285±0.018^{b}	<0.001
	55 kDa	0.429±0.017^{a}	0.413±0.015^{a}	0.421±0.019^{a}	0.324±0.017^{b}	<0.001
	63 kDa	0.188±0.006	0.210±0.035	0.178±0.005	0.159±0.006	0.278
	85 kDa	0.317±0.010^{b}	0.398±0.020^{a}	0.397±0.020^{a}	0.248±0.010^{c}	<0.001
	151 kDa	0.140±0.005^{a}	0.145±0.006^{a}	0.153±0.009^{a}	0.112±0.003^{a}	<0.001

^{a,b} means in a row with different superscripts are significantly different at $p < 0.05$.
RT- room temperature; TW- tap water; CT-chill temperature; MW-microwave

selected as proteins of interest. The differences in intensity among the bands might have been influenced by denaturation. This observation supported the result of previous study where higher concentrations of the smaller molecular weight proteins were found in electrically stimulated muscles (Uytterhaegen *et al.*, 1992). In this study, the proteins of interest vary from 21, 27, 55, 63, 85, and 151 kDa in their molecular weights. The range of molecular weight detected in this study was in agreement with the previous profile presented by Penny (1980).

The differences in optical density of the proteins of interest among the different thawing methods subjected to slowly and rapidly frozen meat samples are as shown in Table 2. In both slowly and rapidly frozen samples, at molecular weight of 21 kDa, there were significant differences in the optical density of protein bands among the thawing methods. Samples subjected to MW had the lowest value that was significantly different ($p < 0.05$) from other thawing methods. However, there were no significant differences among RT, TW and CT thawing methods. Similar trends were observed at molecular weight of 27 kDa in both rapidly and slowly frozen samples. This observation was in line with the findings of Martinez *et al.* (2001) who reported an association between lower molecular weight protein bands (below 35 kDa) and myofibrillar protein degradation as seen in the consistently lower values of MW thawing.

In slowly frozen samples, there were no significant differences in the optical density of protein bands among the thawing methods at molecular weight of 55 kDa. Conversely, in rapidly frozen samples, there were significant differences among the thawing methods at molecular weight of 55 kDa. These results are similar to those of Jung *et al*. (2000) who reported that the density of α-actin with molecular weight around 50 kDa was greater in the supernatant than in control after treatment at room temperature and decreased in precipitates with increasing temperature.

In both slowly and rapidly frozen samples, the optical density of protein bands at 63 kDa were not affected by thawing methods. Previous studies have classified some proteins as stable myofibrillar components. Dutson (1982) reported that degradation of stable proteins occurred only under specific or controlled condition, such as at a very high temperature. Thus, the absence of differences in optical density of 63 kDa proteins among the thawing methods reflects stability of these proteins against various thawing conditions.

In proteins at 85 kDa, both slowly and rapidly frozen samples showed significant differences in intensity among the thawing treatments (Table 3). In slowly frozen samples, the CT method gave the highest value compared to MW, RT and TW methods. Meanwhile, in rapidly frozen sample, TW and CT showed highest values, compared to other thawing methods. Significance difference was also noted in samples subjected to RT and MW thawing method. This observation was in agreement with the report of Yamamoto *et al.* (1979) who found that the band of proteins at 90 kDa did not appear in samples subjected to heat treatment.

In both freezing methods, the optical density of protein bands at 151 kDa were different among the thawing methods ($p < 0.05$). Highest optical density was presented by samples subjected to CT thawing compared to RT, TW, and MW thawing. However, the RT and TW samples did not differ. In the slowly frozen samples, RT, TW and CT differ significantly from MW. However, there was no difference in optical density among the RT, TW, and CT thawing methods. This observation was in line with the findings of Locker and Wild (1984) who reported higher instability in the high molecular weight proteins. Furthermore, it was opined by Wang (1982) that high molecular weight proteins can be solubilized when heated in a water bath at 50°C for 20 min.

Conclusion

Thawing influenced protein concentration and protein band intensity in both slowly and rapidly frozen *Pectoralis major* muscle in broiler chickens. In rapid freezing, samples subjected to chill thawing have higher protein concentration than other thawing methods. The profile of protein band intensity showed that in rapidly frozen samples, protein optical density were similar among RT, TW and CT thawing at molecular weight of 21, 27, 55 and 151 kDa while MW thawing had consistently lower intensity. The same trend was observed in slowly frozen samples at molecular weight of 21, 27, 85 and 151 kDa. Microwave thawing resulted in lower protein quantity and quality in both rapid and slow freezing. Further

study to characterize proteins in *Pectoralis major* in broiler chickens subjected to different freezing and thawing methods is suggested.

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