EXTRACTION AND ANTIOXIDANT ACTIVITY OF CARNOSINE FROM NATIVE, HYBRID NATIVE AND BROILER CHICKEN MEATS

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for the Degree of Master of Science in Food Technology

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การสกัดคาร์โนซีนจากเนื้อไก่พื้นเมือง ไก่ลูกผสมพื้นเมืองและไก่กระทง และความสามารถในการเป็นแอนตีออกซิแดนท์

นางสาวบุษยารัตน์ ไมขุนทด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree

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วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาปริมาณคาร์โนซีนในเนื้อไก่ต่างสายพันธุ์ คือ ไก่พื้นเมือง ไก่ลูกผสมพื้นเมืองและไก่กระทงจากทั้งเนื้ออกและสะโพก และสกัดสารคาร์โนซีนจากเนื้อไก่เหล่านี้เพื่อใช้เป็นสารแอนตีออกซิแดนท์ พบว่าเนื้ออกมีปริมาณคาร์โนซีนมากกว่าเนื้อสะโพกและปริมาณที่พบแตกต่างกันไปตามสายพันธุ์และเพศ ($p < 0.01$) ในการสกัดคาร์โนซีนพบว่า หากสกัดโดยการใช้ความร้อน (60, 80 และ 100 องศาเซลเซียส) สามารถเพิ่มปริมาณคาร์โนซีนในสารสกัดและลดปริมาณโปรตีนอื่นลงได้มากขึ้นเมื่อให้ความร้อนที่อุณหภูมิสูงขึ้นและปริมาณเหล็กโดยรวมยังเพิ่มขึ้นด้วย ปริมาณของแต่ละองค์ประกอบและความสามารถในการเป็นแอนตีออกซิแดนท์โดยการยับยั้งการเกิดออกซิเดชันได้ 50% ของสารสกัดที่ได้จากการให้ความร้อนที่ 80 และ 100 องศาเซลเซียสไม่แตกต่างกัน ($p > 0.05$) และเมื่อใช้สารสกัดที่ได้จากการให้ความร้อนที่ 80 องศาเซลเซียสแล้วทำให้บริสุทธิ์มากขึ้นโดยวิธีอัลตราฟิลเตรชัน พบว่าปริมาณคาร์โนซีนในสารสกัดเพิ่มขึ้น 20% ส่วนปริมาณเหล็กโดยรวมและปริมาณโปรตีนลดลงจากเดิมเมื่อสกัดด้วยความร้อนเพียงอย่างเดียว ปริมาณคาร์โนซีนในสารสกัดคาร์โนซีนจากเนื้ออกและสะโพกของไก่พื้นเมืองและลูกผสมพื้นเมืองเท่ากับ 18,498.32 - 32,874.00 และ 6,387.66 - 10,766.02 ppm ตามลำดับ ความสามารถในการเป็นแอนตีออกซิแดนท์ของทุกสารสกัดเพิ่มขึ้นเมื่อถูกใช้ในระบบออกซิเดชันในปริมาณที่มากขึ้นโดยที่สารสกัดจากเนื้อสะโพกมีประสิทธิภาพมากกว่าสารสกัดจากเนื้ออกและมากกว่าคาร์โนซีนบริสุทธิ์

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....

BUSSAYARAT MAIKHUNTHOD : EXTRACTION AND ANTIOXIDANT ACTIVITY OF CARNOSINE FROM NATIVE, HYBRID NATIVE AND BROILER CHICKEN MEATS
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The objectives of this study were to investigate carnosine content in fresh breast and thigh meat from native, hybrid native chickens and broiler, and to produce carnosine-containing antioxidant extracts from those meats. Carnosine contents in breast meats were higher than that of thigh meat, and the contents were different among breeds and between sex ($p<0.01$). In the extraction process by increasing heating temperature (60, 80 and 100°C), carnosine and total iron increased but protein contents decreased. There was not different ($p>0.05$) in each component content and ability to reduce 50% oxidation between 80°C-heated extract and 100°C-heated extract. The ultrafiltration of 80°C-heated extract was effective to decreased iron content, and increased carnosine content 20% higher than heating at 80°C. Carnosine contents of breast and thigh extracts of native and hybrid native meats were 18,498.32 - 32,874.00 and 6,387.66 - 10,766.02 ppm, respectively. Antioxidant activities of all extracts increased when amount of extract used increased. Thigh extracts exhibited greater inhibition than breast extracts and pure carnosine.

School of Food Technology
Academic Year 2003

Student's Signature...B. Maikhunthod.....
Advisor's Signature...K. Intarapichet.....

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Bussayarat Maikhunthod

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LIST OF ABBREVIATIONS

ATP	=	Adenosine triphosphates
° C	=	degree Celsius
Da	=	dalton
Fe	=	Iron
$FeCl_3$	=	Ferric chloride
g	=	gram
HCl	=	Hydrochloric acid
hr	=	hour
kg	=	kilogram
M	=	molar
mg	=	milligram
min	=	minute
mL	=	milliliter
mm	=	millimeter
mM	=	millimolar
MW	=	molecular weight
N	=	normal
nm	=	nanometer
ppm	=	part per million
µg	=	microgram
µL	=	microliter

CHAPTER I

INTRODUCTION

Lipid oxidation is not only a major cause of food deterioration, that effect alteration of foods, but also the cause of adverse effect on biological system such as human health (Zhou and Decker, 1999). It is known to be accompanied by the formation of a complex mixture of secondary products, lipid peroxides, and the secondary breakdown products including aldehydes and oxidized cholesterol derivatives. These compounds make the alterations in flavor, color, functional properties, and nutritive value of foods. In addition, they cause a health risk because of their potentially toxic. They can react with biological molecules and then, cause adverse effects including mutagenicity, toxicity and carcinogenicity to mammalian cells, and development of atherosclerosis in human (Chan et al., 1993; Chan and Decker, 1994; Zhou and Decker, 1999).

Various antioxidants play important role in biological and food systems by interfere or delay the production of lipid oxidation products (Zhou and Decker, 1999). For instance, using of antioxidants such as butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) can help control lipid oxidation in foods. However, the consumers are concerned about the use of synthetic food additives, resulting in increased demand on natural antioxidants (Chan et al., 1993). Carnosine is one of interesting natural antioxidants. It is a dipeptide presented in large amount in skeletal muscle and found to be able to inhibit lipid oxidation both in living tissue

and muscle foods (Chan and Decker, 1994). Because of the high concentrations of carnosine in white fibers it leads many researchers to study its functions. They posed its antioxidant properties that may be resulted from its ability of being a metal chelator, and free radical scavenging. It is capable of inhibiting iron-catalyzed lipid oxidation greater than individual amino acids, which are ineffective. It is suggested that carnosine has the excellent potential function when use as a natural antioxidant in biological and food systems. However, utilization of synthetic source of carnosine is limited by both economic and regulating hurdles. Carnosine-containing extracts from beef (Chan et al., 1993) and mechanically separated pork (Gopalakrishnan et al., 1999) have been shown to be capable of inhibiting lipid oxidation in vitro and salted ground pork, respectively. Nevertheless, these carnosine-containing extracts contain high amount of pro-oxidants such as iron and iron-containing compounds. The iron, presented in the extracts, might be released from heme-containing molecules such as myoglobin which found higher amount in darker meat than in whiter meat. Therefore, in order to obtain carnosine extract low in pro-oxidants, meat source contains low amount of pro-oxidants and extraction procedures should be able to reduce more pro-oxidants while remaining high carnosine. Chicken meat contains lower myoglobin content than beef and pork, thus it should be a good natural alternate source of carnosine.

Hence, the purposes of this study were to investigate carnosine content in fresh breast and thigh meats from broiler, native and hybrid native chickens, and to study the extraction of carnosine from broiler meat by heat treatment alone and heat treatment combined with ultrafiltration. In addition, these carnosine-containing extracts were evaluated their antioxidant activities *in vitro* oxidation system.

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CHAPTER II

LITERATURE REVIEWS

General Information of Carnosine

There are many naturally dipeptides found in muscle including carnosine (β-alanyl-l-histidine), anserine (β-alanyl-l-1-methylhisidine), and ophidine [β-alanyl-l-3-methlyhistidine (also known as balenine)] which were discovered by Gulevitch and Amiradgibi and Tolkatschevskaya and Ackermann in the early 1990s (Chan and Decker, 1994). Carnosine is a combination of two amino acids which are β-alanine and l-histidine, and it is a water-soluble dipeptide (Fig. 2.1).

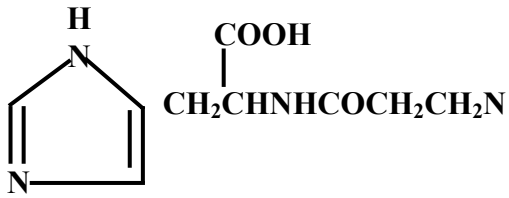

Figure 2.1 Structure of carnosine (β-alanyl-L-histidine)

Source; Decker, 1995.

It is formed by activity of carnosine synthetase (CS) as shown the reactions below.

$$\beta\text{-alanine} + ATP + CS \xrightarrow{Mg^{2+}} CS\text{-}\beta\text{-alanine-AMP} + pyrophosphate$$

$$CS\text{-}\beta\text{-alanine-AMP} + L\text{-histidine} \longrightarrow \textbf{Carnosine} + AMP + CS$$

The synthesis process of carnosine occurs mainly in muscle and brain (Chan and Decker, 1994). Carnosine is found as high as 20 mM in skeletal muscle and 5 mM in brain. It has high level due to its resistance to the proteolytic cleavage, and being equilibrium by carnosinases that are inactivate carnosine in blood or tissues (Kyriazis, 1999; Rathjen, 1999). Intact carnosine is absorbed in the small intestine (jejunum) by a specific active transport mechanism. It can be dispersed in the blood, and then transported to kidney, liver, and muscle where is found to contain the highest concentrations. Carnosine is either utilized by these tissues or hydrolyzed by carnosinase (which found in the blood, kidney, and liver) into alanine and histidine (Decker, 1995; Supplementwatch, 1999).

Although, no definite metabolic role has been ascribed to carnosine, it has been implicated in a variety of physical processes. Perhaps the best-described function of carnosine is as a "broad-spectrum" antioxidant, where it has been shown to interact with several free radical species including singlet oxygen, hydrogen peroxide, and both peroxyl and hydroxyl radicals. In addition, carnosine is able to inhibit radical-induce cellular damage induce by iron, copper and zinc. Therefore, carnosine is claimed as one of the good potential antioxidant. Furthermore, it enhances wound healing, reduces lactic acid accumulation, promotes muscle recovery and enhances muscle contraction. Its functions are suggested due to its imidazole ring and being water-soluble antioxidant. Carnosine as dipeptide, it has been shown to have greater antioxidant effect than individual amino acid constituents. This indicates the involvement of peptide linkage between alanine and histidine in antioxidant activity (Supplementwatch, 1999).

Carnosine concentrations in endogenous skeletal muscle depend on species, and are effected by diet, muscle type and animal age. For example, white muscle of chicken has higher carnosine concentration than dark muscle (Table 2.1) (Plowman and Close, 1988; Decker, 1995). Carnosine content rapidly increases within the first growth year and remains constant (Carnegie et al., 1982). But for the horse, the amount decreases with age (Chan and Decker, 1994). For the effect of diet on carnosine concentration, according to Decker (1995) reported that when the rats were fed with 5% histidine supplemented diet, carnosine concentration in rats could be 2.8 folds increased. In addition, dietary carnosine supplementation influences the concentration of carnosine in the skeletal muscle, low carnosine supplementation (0.9%) do not significantly increase carnosine concentration. While increased dietary carnosine concentration (5%) make the carnosine concentration to be 2 folds in rats. Also concentration of carnosine in rats were reduced if their diet had histidine-deficiency. The concentration of carnosine in various skeletal muscles is illustrated in Table 2.1.

Antioxidant Activity of Carnosine

Many investigators found that carnosine had capability of inhibiting lipid oxidation catalyzed by iron, hydrogenperoxide-activated hemoglobin, singlet oxygen (Decker and Faraji, 1990), and hydroxyl radicals (Chan et al., 1994). There were many oxidation systems investigated, including phosphatidylcholine liposomes, cooked ground pork and salted ground pork. The activity of carnosine as antioxidant has been studied in various systems. The antioxidant activity of carnosine has been Postulated to 2 interactions, metal chelation or free radical scavenging (Chan and

Table 2.1 Carnosine concentrations in various skeletal muscles

Source	Muscle	Carnosine mg/100 g tissue (mM)
Chicken	Leg	50 (2.9)
Gallus gallus	Pectoral	278 (16.4)
Rabbit	Leg	70 (4.1)
Oryctolagus cuniculus		
Atlantic salmon		0
Salmon salar		
Rat	Mixed	70 (4.1)
Rattus ratus		
Beef	Leg	150 (8.8)
Bos taurus		
Swine	Shoulder	276 (9.2)
Sus sp.		
Human	Quadriceps	362 (21.3)

Source; Decker, 1995.

Decker, 1994). Antioxidant activity was measured using thiobarbituric acid reactive substances (TBARS) value in most investigations (Decker and Faraji, 1990; Decker and Crum, 1993; Decker et al., 1995).

A. Antioxidant activity of carnosine compared to individual amino acids

Due to the hydrophilic nature of carnosine, it has ability to inhibit water-soluble catalyzed oxidation, such as hydroxyl radical catalyzed oxidation (Chan et al., 1993), transition metals and hemoglobin (Decker and Faraji, 1990). Chan and colleagues (1994) studied an antioxidant activity of carnosine (25 mm), individual amino acids (25 mm histidine or β-alanine) and mixture of amino acids in hydroxyl radical catalyzed oxidation, using an Fe^{2+} and H_2O_2 system, and phosphatidylcholine liposome and measured by electron paramagnetic resonance (EPR). Carnosine activity was determined by calculation of hydroxyl radical ($OH^{\bullet}$) concentration from hydroxyl radical spin peak height in EPR spectra, and measuring TBARS for oxidation reactions. The results showed that the ability of individual amino acids to inhibited oxidation are less than carnosine (Table 2.2). β-alanine increased $OH^{\bullet}$ production unlike histidine which quenched 49.1% of $OH^{\bullet}$ produced. The hydroxyl radical quenching ability of histidine + β-alanine mixture was similar to histidine alone, which are 54.4% and 50.9% of control, respectively, and oxidation inhibition activities were 77.5% and 78.1% of control, respectively. The similar antioxidant abilities of both mixtures were suggested due to direct formation of radical on its imidazole ring, but β-alanine did not involve in antioxidant ability. Carnosine was the most effective antioxidant by inhibiting hydroxyl radical production and oxidation about 74.8% and 34.7% of control, respectively. It is indicated that linkage between

amino acids is involved in the ability of peptide to scavenge hydroxyl radicals and scavenging activity of histidine moiety. Thus, antioxidant activity of carnosine was more effective than histidine and histine + β-alanine mixture (Chan et al., 1994; Wade and Tucker, 1998).

B. Chelation of metal ions

The chelating activity of carnosine depends on the type of metal ions. According to Decker and colleagues (1992), the antioxidant activity of carnosine in combination with either iron or copper was determined by ^{1}H nuclear magnetic resonance spectra. Carnosine (10 mM) was dissolved in deuterium oxide containing (trimethlysily) propionic acid as the internal standard, and $FeCl_2$ and CuCl (10 μM) were added immediately before analysis. It was reported that carnosine acted differently in oxidation systems (Fig. 2.2). Spectra for carnosine alone (Fig. 2.2a) and Fe^{2+}-carnosine (Fig. 2.2c) were similar. But the spectrum of Cu^{1+}-carnosine (Fig. 2.2b) was different from other in the absence of peaks at 6.9 and 7.7 ppm. These peaks represented C-4 and C-2 of imidazole ring of carnosine, respectively. This was suggested that carnosine formed complex with copper but not with iron. The researchers concluded the result of this study by following Brown in previous study, 4 molecules of carnosine chelate 1 molecule of copper through the N-3 of imidazole ring in tetramer form causing the absence of C-4 and C-2 peaks. However, this study was found that carnosine could inhibit Fe-catalyzed and Cu-catalyzed microsomal lipid oxidations by inhibiting the production of TBARS (data not shown). This means carnosine acted as antioxidant in Cu-catalyzed oxidation by metal chelating, unlikely in Fe-catalyzed oxidation system.

Table 2.2 Hydroxyl radical ($OH^{\bullet}$) quenching and antioxidant activity of amino acids and carnosine.

	$OH^{\bullet}$conc as % of control[a]	oxidation as % of control[b]
carnosine (β-Ala-His)	25.2 ± 0.9	34.7 ± 1.4
β-Ala-Ala	108.4 ± 2.9	96.4 ± 4.1
His	50.9 ± 6.3	78.1 ± 6.1
β-Ala	244.6 ± 10.1	98.4 ± 2.5
His+β-Ala	54.4 ± 2.2	77.5 ± 1.9

[a] Hydroxyl radical generation system included 5 mMH_2O_2, 5 mM $FeSO_4$, and 100 DMPO in 0.1 M phosphate buffer (pH 7.4). Amino acid or dipeptide concentrations were 25 mM. [b]Antioxidant activity of dipeptide or amino acids (25 mM) was determined in a model system containing $FeSO_4$ (1 mM0, H_2O_2 (0.5 mM), and phophatidyl-choline liposomes in 0.1 M phosphate buffer (pH 7.4). Extent of oxidation was determined by measuring TBARS.

Values represent mean ± standard deviation (*n* =3).

Source; Chan et al., 1994.

Takenaka and colleagues (1997) also reported antioxidant activity of carnosine in metal chelating. The effect of carnosine on the destruction of vitamin B_{12} (B_{12}) with vitamin C (C) in the presence of copper on multivitamin-mineral supplemented food was studied. The B_{12} destruction was assumed to be concerned with radical generated by C in the presence of copper. In this system, B_{12} was converted to B_{12} analogues which could block B_{12} metabolism and B_{12} would loss of biological activity. Carnosine concentrations of 1-10 mM were used in their experiment. The reaction mixture contained 0.5 mM B_{12}, 10 mM C, and various concentration of

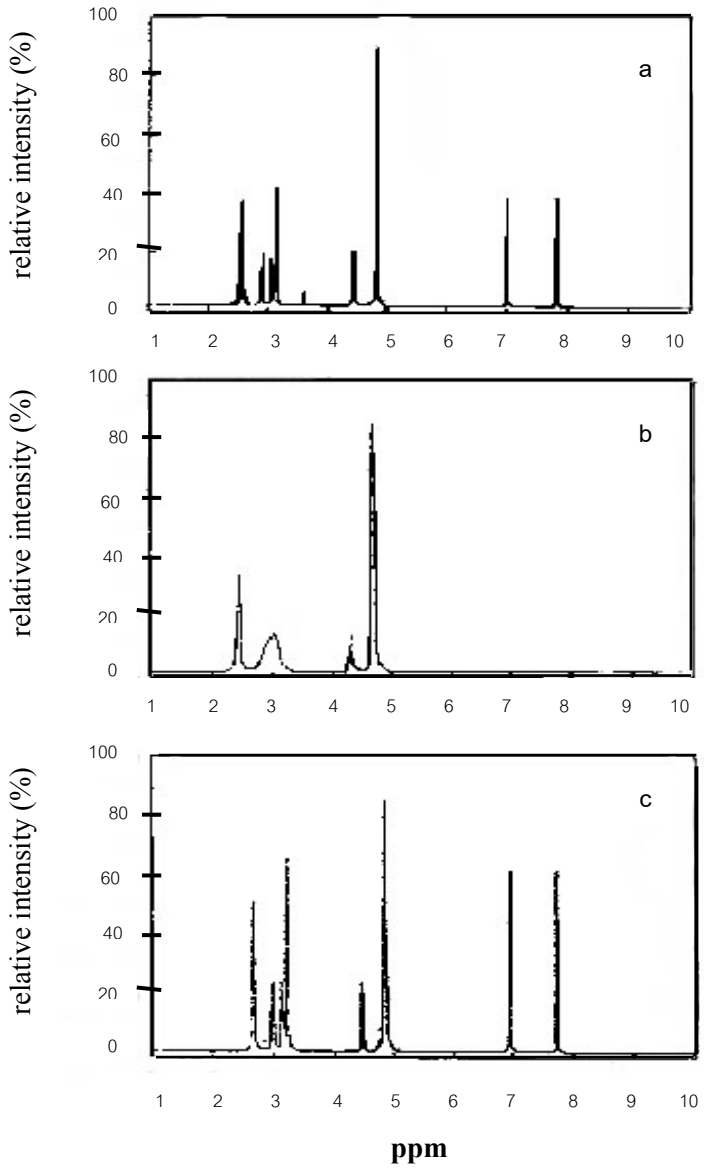

Figure 2.2 ^{1}H NMR spectra for carnosine (a), carnosine plus Cu^{1+}(b), and carnosine plus Fe^{2+}(c). Carnosine concentration was 10 mM and copper and iron concentrations were 10 μM. Peaks at 2.6, 3.0, 3.2, 4.7, 7.7, and 6.9 ppm represent the protons of β-CH_2 of β-Ala, β-CH_2 of His, α-CH_2 of β-Ala, α-CH of His, and C-2 and C-4 of the imidazole ring of carnosine, respectively. Source; Decker et al., 1992.

carnosine. It was found that carnosine. It was found that 70% of control B_{12} was destroyed in system that consisted of B_{12}-C-copper. The destruction of B_{12} was significantly reduced by 10% of control sample by adding of 10 mM carnosine (Fig. 2.3). This observation suggested that carnosine acted as copper chelator, resulting in protecting B_{12} from the destruction.

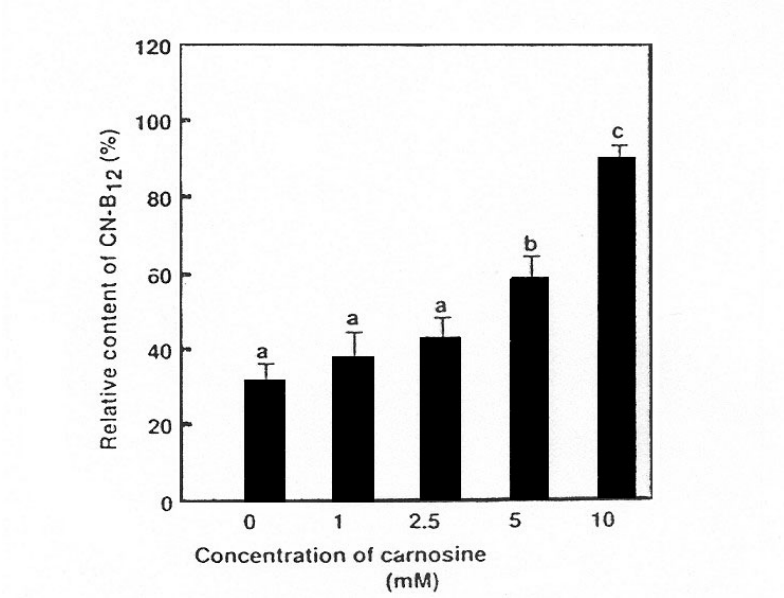

Figure 2.3 Effect of carnosine on the destruction of vitamin B_{12} by vitamin C in the presence of copper. Source; Takenaka et al., 1997.

Lee and Hendricks (1997) also reported the metal chelation of carnosine in copper-catalyzed oxidation in liposome. From their investigation, even low concentration of carnosine showed strongly inhibited peroxidation resulting in smaller TBARS values in copper-catalyzed peroxidation system. The concentration about 1-10 mM carnosine, inhibited lipid peroxidation at 89.1-95.0% (Table 2.3). They

suggested that the formation carnosine-copper complex (metal chelation) might contribute to the strong inhibition of lipid peroxidation in this system.

Table 2.3 Effect of carnosine on metal and ascorbic acid-dependent lipid peroxidation in phosphatidlycholine liposomes[a]

Carnosine (mM)	TBARS (nmole/mL liposome)
	Cu (II)
0 (control)	4.41 ± 0.14
1	0.48 ± 0.01
5	0.44 ± 0.02
10	0.31 ± 0.03
15	0.28 ± 0.04
20	0.22 ± 0.02

[a]Data represent the mean ± SD of two determinations.

Source; Lee and Hendricks, 1997.

C. Free-radical scavenging

The chelating agent such as ethylenediamine tetraacetic acid (EDTA) has no effect on the ability of hydrogenperoxide-activated myoglobin unless there are hydrogen donors or free radical scavengers present in system. In addition, chelating agent is not inhibiting singlet oxygen or lipoxidase to promote the lipid peroxides formation without involvement of free metal (Decker and Faraji, 1990).

Decker and Faraji (1990) tested carnosine ability to inhibit several different lipid oxidation catalysts in oxidation of liposome. The catalysts were iron, hemoglobin and singlet oxygen. The result showed that carnosine (25 mM) inhibited activity of catalysts 65-96% of activity (Table 2.4). The most effective inhibition was

on hydrogenperoxide-activated hemoglobin, by measuring TBARS. The result also showed that carnosine was heat stable because of the antioxidant activity of heated carnosine (at 100°C, 15 min) was similar to unheated carnosine.

Table 2.4 Inhibition of different lipid oxidation catalysts by carnosine.

Catalysts	Percent inhibition[a]	
	Carnosine (25 mM)	Heated carnosine (25 mM)[b,c]
Fe + ascorbate	77	76
H_2O_2 + hemoglobin	96	96
Singlet oxygen	61	65
Lipoxidase	35	39

[a]Percent of inhibition was calculated as {1 - (TBARS produced in the presence of carnosine ÷ TBARS produced in the absence of carnosine)} × 100. [b]Carnosine was heated for 15 min in a boiling water bath and immediately cooled on ice. [c]Heated samples were not significantly different than unheated sample ($P< 0.05$).
Source; Decker and Faraji, 1990.

The results above provide better understanding that carnosine has antioxidant activity in metal chelation and also in free radical scavenging or donate hydrogen ions. From the study of Chan et al. (1994) mentioned above, the free radical was bound to the imidazole ring of carnosine. Results of investigations above, it can be confirmed that carnosine has potential antioxidant activities. Thus, carnosine was used in the many studies of its ability to inhibit the oxidation in food systems. Carnosine was studied its activity in combination with tocopherol to inhibit oxidation in ground turkey. The mixture provided greater keeping quality of ground turkey than individual substance (Calvert and Decker, 1992). Moreover, carnosine showed the

ability to control discoloration of meat and lipid oxidation of the steak by chelating free radical form the oxidation of oxyhemoglobin, resulting in extend its shelf life (Lee et al., 1999). Carnosine also showed the efficiency in medicinal application. It could be utilized in medicinal practices against lipid oxidation, which cause adverse effect of health (Chan and Decker, 1994). It is known that there is variety of diseases can be treated by the use of carnosine. There was a study reported carnosine could prevent cataract in animal. Animal study showed that carnosine could preserve nerve cells from damage and death. This suggests that carnosine might be employed to treat patients with stroke. Carnosine can be employed to treat the diabetic patient for preventing the complications, such as cataract, kidney failure. In addition, some studies found that carnosine is effective in treating human in corneal erosion and other corneal disease. For instance, it can heal ulcer infected by virus and eyes infected by bacteria (Kyriazis, 1999; Jordan, 1999).

Extraction of Carnosine

In muscle, there are many kind of water-soluble substances, including carnosine, iron-containing compound, and heme-containing compound. These compounds also exist in a water-soluble muscle extract. Nonprotein-bound ion, as low molecular weight (LMW) iron, has capability of producing superoxide anion, hydrogen peroxide and hydroxyl radical which can promote lipid oxidation. Heme-containing compounds, such as hemin, methemoglobin and metmyoglobin can be the active catalysts of lipid oxidation both *in vitro* and biological membranes. Therefore, to produce an effective antioxidant extracted from muscle, techniques used should be able to reduce level of prooxidants and maintain high levels of carnosine (Chan et al., 1993).

Chan and colleagues (1993) studied extraction of carnosine from beef muscle. In addition, Gopalakrishnan and colleagues (1999) also studied the extraction of carnosine from mechanically separated pork. In both studies, carnosine was extracted by water extraction and heat treatment or ultrafiltration (molecular weight cut off 10,000) separately in attempt to remove any prooxidant compounds as much as possible. They found that heat treatment was effective to remove other compounds but it increased the releasing of irons from their bound proteins. Ultrafiltration was more effective to remove both hemin and iron-containing compound than heat treatment. However, there was more amount of low-molecular-weight iron containing compound in permeate than heated extracted. In addition, loss of carnosine could occur during extraction steps in each treatment.

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CHAPTER III

CARNOSINE - CONTAINING ANTIOXIDANT EXTRACTS FROM BROILER MEATS: HEAT EXTRACTION, ULTRAFILTRATION AND ANTIOXIDANT ACTIVITIES

ABSTRACT

Carnosine contents in broiler fresh meats were investigated. Breast meat had 7-folds carnosine higher than thigh meat. To obtain high carnosine in extracts from chicken meats, carnosine was extracted from breast and thigh meat by water extraction and then, partially purified by (1) heat treatment at 60, 80 and 100°C, and (2) isolation of low-molecular-weight iron containing compounds by ultrafiltration(UF) 5,000 molecular weight cut off. Carnosine, protein and total iron contents of all the extracts were determined, and antioxidant activities by TBARS method were investigated. Increasing temperature affected on decreasing of protein contents, whereas carnosine and total iron content and antioxidant activity increased. The results showed no significant difference in each component content and ability to reduce oxidation by 50% between 80°C- and 100°C-heated extract, but they were different from 60°C-heated extract. The UF permeate from the 80°C-heated extract had approximately 20% carnosine higher, but 40% protein and 10-30% total iron contents lower than that of 80°C-heated extract. However, meat extracts gave the greater activity than pure carnosine at the same concentration levels of carnosine in oxidation system.

INTRODUCTION

Oxidative reactions have been implicated in the development of food deterioration affecting color, flavor, texture and nutritional value. Furthermore, the toxic substances formed by these reactions cause the development of numerous diseases including atherosclerosis and cancer (Decker, 1995; Kansci et al., 1997; Zhou and Decker, 1999). In recent years, there has been some interests in the antioxidant potential of carnosine in meats (Calvert and Decker, 1992; Decker and Hultin, 1992; Lee et al. 1999; O'neill et al., 1999). Carnosine is a naturally occurring skeletal muscle dipeptide consisting of β-alanine and histidine. Carnosine content can be affected by diet and fiber types of animals. Carnosine shows the antioxidant activity by playing role as metal chelator and free-radical scavenger (Chan and Decker, 1994). In copper-catalyzed oxidation system, carnosine inhibits the oxidation by chelating copper, as shown by inhibiting the destruction of vitamin B_{12} in the presence of copper, and also by strongly inhibiting the peroxidation of copper-catalyzed oxidation in liposome (Lee and Hendricks, 1997; Takenaka et al., 1997). But it only works as free radical scavenger in the iron-catalyzed oxidation system (Decker and Faraji, 1990; Decker et al., 1992). In biological system, carnosine is used in medical practices to prevent the oxidation which cause eye-cataract and aging. In addition, it has been shown to suppress the tumor growth in rat (Boissonneault et al., 1998; Jordan, 1999; Kyriazis, 1999). In previous experiments, carnosine was extracted from many skeletal muscle sources including beef and pork by using either heat treatment or ultrafiltration (Chan et al., 1993; Gopalakrishnan et al., 1999). The carnosine-containing extracts obtained were capable of inhibiting *in vitro* lipid

oxidation and in meat products. However, the extracts contained high amount of pro-oxidants, such as free iron and iron-containing proteins. Therefore, in order to obtain carnosine extract low in pro-oxidants, muscle used should contain low amount of pro-oxidants and extraction procedures should be able to reduce more pro-oxidants while remaining high carnosine. Chicken meat contains lower myoglobin content than beef and pork (Hazell, 1982), thus it should be a good muscle alternate source of carnosine. The purposes of this study were to extract carnosine from breast and thigh of broiler meats by water and heat extraction combined with ultrafiltration for further purification. Carnosine extracts were determined for carnosine, protein and total contents and their antioxidant activities by TBARS.

MATERIALS AND METHODS

Chemicals

Carnosine, o-phthaldehyde (OPA), soybean phosphatidylcholine, β-mercaptoethanol (BME), bovine serum albumin (BSA), and Folin Ciocalteu's phenol reagent were purchased from Sigma Chemical (St. Louis, MO, USA). Standard Fe (1000 mg/mL) was obtained from Merck KgaA (Darmstadt, Germany). Methanol and acetonitrile were HPLC grade, and all other chemicals were reagent grade or purer.

Raw Materials

Broiler meats were purchased from Lotus Supercenter Store (Nakhon Ratchasima, Thailand). Breast and thigh meats were used for the experiment. The

meats were ground using a domestic mincer, then, vacuum packed and stored at –20°C until used.

Chemical Composition Analysis of Fresh Meats

Fresh meat samples from each part of chicken meat were determined for moisture content according to AOAC (1997), protein content by Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard, carnosine content by high performance liquid chromtography (Cornet and Bousset, 1999; Gopalakrishnan et al., 1999), and total iron content by atomic absorption spectrophotometry (James, 1995).

Preparation of Meat Extracts for Determining Total Carnosine Content in Fresh Meats

The meat extracts were prepared according to Aristoy and Toldra′ (1991) with slight modification. The frozen minced meat was thawed under running tap water. One part of this minced meat was homogenized in 4 parts of precooled (4°C) 0.1 N HCl with an AM-8 homogenizer (Nihonseiki, Kashi Ltd., Tokyo, Japan) for 8 min on the ice chamber. The homogenate was centrifuged at 10,000×g by Sorvall RC-5C plus (Dupont, Delaware, USA) at 4°C for 20 min. The supernatant was filtered through Whatman #4 filter paper, and then, the filtrate was subjected to freeze-drying. Frozen (-45°C) filtrate was freeze-dried for 72 hr using HETO FD3 (Heto-Holten A/S, Allerod, Denmark). Subsequently, the dried filtrates were determined for total carnosine content present in broiler meats by the procedure described in determination of carnosine content.

Extraction of Carnosine by Heat Treatment

Heat extraction of carnosine was performed following the procedure of Chan et al. (1993) and Gopalakrishnan et al. (1999) with slight modification. The frozen minced meat was thawed under running tap water. One part of minced meat was homogenized with 2 parts of precooled (4°C) distilled deionized water in an AM-8 homogenizer in the ice chamber for 8 min. The homogenate was centrifuged at 20,000×g for 30 min at 4°C using Sorval RC-5C plus, and then, the supernatant was filtered through Whatman #4 filter paper. The water-extracted filtrate was subjected to heat treatment at 60, 80 and 100°C for 10 min in water bath and cooled in ice bath. Heated extract was centrifuged to remove precipitated proteins at 5,000×g for 20 min. Supernatant was filtered through Whatman #4 filter paper. The filtrate was freeze-dried using a HETO-FD3 for 72 hr, and analyzed for carnosine, protein and total iron content and antioxidant activity. Temperature at which maximum carnosine content and low total iron content obtained was used to prepare the heated liquid extract prior to subsequently treated by ultrafiltration for further purification.

Ultrafiltration

The heated extract, prepared at selected temperature, was subjected to ultrafiltration. The separation between low and high molecular weight fractions of the extract was obtained by passing liquid extracts through the 5,000 molecular weight cut off ultrafiltration membrane (PES) (Vivaflow 50, Vivascience, Sartorius AG, Goettingen, Germany). Permeate containing low molecular weight fraction was collected, freeze-dried and kept for further analyses.

Analysis of Extract Compositions

Protein content. Protein contents of undried and dried extracts were determined by Lowry method (Lowry et al., 1951) using BSA as a standard. The dried extracts (2.0000 – 3.0000 mg) were reconstituted with 1 mL phosphate buffer (5 mM, pH 7.0) prior to determination.

Total iron content. The total iron contents in fresh meats and dried extracts were measured at 248.3 nm by means of atomic absorption spectrophotometry using AAnalyst 100 (Perkin Elmer, Norwalk, CT, USA). Accurate weight of sample (3.0000 g for fresh meat and 0.2500 g for dried extract) was ashed in a muffle furnace (Carbolite CSF1200, Cheffield, England) at 500°C until a white or light gray ash obtained. Five mL of concentrated hydrochloric acid was added to the crusible containing the ash, boiled for 5 min, transferred to a beaker, adjusted volume to about 40 mL and then, boiled for 10 min. The mixture was cooled, filtered through Whatman #1 filter paper into a 50-mL volumetric flask, made up to volume with deionized water, and measured at 248.3 nm. The iron concentrations were calculated from a standard curve generated using known concentration of iron (0-10 μg/mL) (James, 1995).

Carnosine content. Carnosine contents in fresh meats, undried and dried extracts were determined using high performance liquid chromatography (HPLC), derivatizing the extracts by OPA working solution. The OPA working solution was prepared 24 hr before use by dissolving 27 mg of OPA in 500 μL of absolute alcohol. Five mL of 0.1 M sodium tetraborate ($Na_2B_4O_7.10H_2O$) (pH 9.5) was added, followed

by adding 50 μL of BME. The mixture was throughly mixed, stored in a tightly closed container, and kept in the dark. The reagent can be kept for several days (Antoine et al., 1999).

Sample preparations for HPLC analysis were based on the methods of Gopalakrishnan and colleagues (1999) and Cornet and Bousset (1999) with slight modification. For liquid extract, 2 mL of the extracts or 100 mg dried extract reconstituted in 2 mL of 5 mM phosphate buffer, pH 7.0 was mixed with 2 mL of 0.4 M perchloric acid and boiled for 5 min to precipitate proteins, and centrifuged at 2,000×g for 20 min by Labofuge 400R #8179 (Heraeus Instruments, Germany). The supernatant was filtered through 0.45 μm membrane filter. The filtrate (10 μL) was derivatized with 10 μL of OPA working solution just prior to injection. The derivatized samples were analyzed on Agilent 1100 series HPLC with an autosampler (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). OPA derivatized carnosine was separated using a mobile phase of 0.3 M sodium acetate (pH 5.5), methanol and acetonitrile (75:15:10) at flow rate at 0.5 mL/min with Eclipse XDB-C18 (4.6×150 mm, 5 micron) column. Fluorescence detector was set at an excitation wavelength 310 nm and emission wavelength 375 nm. A standard curve was prepared using pure carnosine solution with 10-1000 ppm. Carnosine concentration was calculated by chemstation Rev.A.09.03 (1417) (Agilent Technologies 1990-2002).

Evaluation of Antioxidant Activity

Antioxidant activity of extract was determined by its ability to inhibit iron/ascorbate catalyzed oxidation of phosphatidylcholine liposomes, which was modified from Decker and Faraji (1990), Kansci and colleagues (1997), and Gopalakrishnan and colleagues (1999). Lipid oxidation was performed in a 2.0 mL model system containing 1 mg phosphatidylcholine liposomes/mL of 5 mM phosphate buffer (pH 7.0) plus lipid oxidation catalyst and also various concentration of extract.

Preparation of reagents. Sodium ascorbate and $FeCl_3$ solution, acted as the catalysts, were separately prepared in degassed ultra-pure water freshly before use (Kansci et al., 1997). The MDA standard was prepared according to Botsoglou and colleagues (1994). 1,1,3,3-tetraethoxypropane (TEP) was weighed (100 mg) in a screwed-capped test tube, diluted with 10 mL of 0.1 N HCl, immersed into a boiling water bath for 5 min, and cooled immediately under tap water. Stock solution of MDA (100 μg/mL) was prepared by transferring the hydrolyzed TEP solution into a 100-mL volumetric flask and diluting to volume with water.

Analysis of thiobarbituric acid reactive substances (TBARS). Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS). The pure carnosine or extract was dissolved in phosphatidlycholine liposomes suspension to give a various concentration of 0-12.5 mg/mL. After 10 min, phospholipid oxidation was induced by adding sodium ascorbate and $FeCl_3$ solutions to a final concentration 45 μM of each. The oxidation mixture was incubated in a

37°C water bath for 30 min, followed by adding 2 mL of thiobarbituric acid (TBA) reagent (0.02 M in water). The tightly closed tube was heated in boiling water bath for 15 min, cooled and centrifuged at 4,000×g for 15 min. The absorbance of supernatant was measured at 532 nm using UV/VIS 916 Spectrophotometer (GBC Scientific Equipment, Ltd., Victoria, Australia). Concentration of MDA in oxidation system was calculated from the MDA standard curve (0-7 μg/mL). The ability of inhibition was calculated as follows:

$$\%\text{inhibition} = \frac{\text{(MDA conc. in absence of extract)-(MDA conc. in presence of extract)}}{\text{MDA conc. in presence of extract}} \times 100$$

The oxidation activity of extract was expressed as amount of FD-extract or the concentration of carnosine used in system that could inhibit 50% of oxidation (EC50). The EC50 of extract was also compared with EC50 of pure carnosine.

Statistical Analysis

All experiments were performed in duplicates. Each replicate was chemically analyzed in triplicate samples. Statistical analysis was evaluated in Completely Randomized Design (CRD) with Statistical Analysis System (SAS Institute, Inc., 1995). Analysis of Variance (ANOVA) and means comparison by Duncan's Multiple Range Test (DMRT) were analyzed.

RESULTS AND DISCUSSIONS

Chemical Composition of Fresh Meats

Carnosine and total iron content of fresh breast and thigh meats are shown in Table 3.1. The total iron content of thigh (17.19 μg Fe/g muscle) was 1.3 folds of breast muscle (12.73 μg Fe/g muscle) which agreed with Ratchanee (1999). Thigh meat has higher total iron content because it contains higher amount of myoglobin and darker (red) color than the breast. The degree of redness is closely related to the amount of myoglobin and/or blood supply in those muscles. Breast meat has lower myoglobin content than thigh meat. Thus, the darker muscle, the higher total iron content (Hazell, 1982; Pearson and Young, 1989). Carnosine content in breast meat is approximately 5-7 times more than in thigh meat which agreed with previous studies by Decker (1995), and Cornet and Bousset (1999). Chicken breast (white fiber) can be categorized into fast-twitch glycolytic fiber (type IIB), chicken thigh (red fiber) is the slow-twitch oxidative fiber (type I) (Meyer, 2003). Type IIB fibers generate ATP by anaerobic fermentation of glucose to lactic acid. Because there are limited capillary supply to deliver oxygen. Hence, it has a fast contraction and is easily fatigued resulting from accumulation of lactic acid. Type I fibers have a good capillary supply for efficient gas exchange, and also high myoglobin. Thus, they are built for aerobic metabolism. They have a slow contraction and are highly resistant to fatigue (Illingworth, 2004). One of the biological roles of carnosine is buffering. Carnosine acts as a buffer to provide pH-buffering capacity, by regulating pH which decrease due to lactic acid accumulation (Chan and Decker, 1994). This role of carnosine can explain why higher concentration was found in breast muscle where

high levels of anaerobic metabolisms are commonly occurrence. This is in good agreement with the study of Davey (1960) who reported that the aerobic capacity of muscle was inversely related to the dipeptide concentration when carnosine was measured in muscle of several. Furthermore, Sewell and colleagues (1992) found the strong positive correlation between carnosine content and type IIB fiber in the equine middle gluteal muscle. This experiments showed that carnosine contributed as much as 46% to the buffering of H^+ produced by type IIB fibers, and interpreted the important of this dipeptide in stabilizing the pH in anaerobic contraction.

Table 3.1 Chemical compositions of fresh broiler meats

sample	%Protein (wet basis)	%Moisture(wet basis)	Fe (μg /g meat)		carnosine (μg /g meat)	
			Wet Basis	Dry basis	Wet basis	Dry basis
Breast	22.13	75.22	12.73	51.39	718.65	2900.12
Thigh	18.65	75.15	17.19	69.17	104.23	419.42

Heat Treatment

The composition of heated extracts

Considering in undried (UD)-heated extracts by comparing in the same meat group (Table 3.2 for breast extract and Table 3.3 for thigh extract), it was found the increasing of carnosine content with increasing temperature even they were not significantly different ($p>0.05$) when compared with untreated extract. Protein content of the extract decreased with increasing temperature. UD-80C and UD-100C

(both breast and thigh groups) had a similar protein contents which were less than UD-60C ($p<0.05$). Heating of extracts at 60, 80 and 100°C could reduce protein approximately 58-60, 87-90 and 88-92%, respectively, compared to the untreated extract. Freeze dried-heated extract (FD) had similar trend to its UD extract, i.e. carnosine content increased with increasing temperature, but protein content decreased. Carnosine contents and protein contents of FD-80C and FD-100C were similar. This result conformed to the previous study by Gopalakrishnan and colleagues (1999) who extracted carnosine from mechanically separated pork by varying temperature at 60, 70 and 80^{o}C. They found that protein content in UD-extracts were significantly decreased ($p<0.05$) with increasing temperature. Heat treatment for removing proteins from untreated extracts may cause protein denaturation by aggregation. An aggregation caused by folding of globular proteins lead to solubility reduction and then, can be precipitated by centrifugation (Belitz and Grosch, 1999). Although, the protein concentration of UD-extracts significantly decreased ($p<0.05$), there was no significant difference ($p>0.05$) among carnosine contents of extracts. This could be due to loss of carnosine during extraction steps. It was agreed with Chan and colleagues (1993) in extraction of carnosine from beef muscle. They suggested that lack of complete recovery of carnosine might be due to the destruction of carnosine or association of carnosine with precipitated proteins during processing.

The total iron contents of FD-heated extracts of broiler meat are shown in Table 3.2 and 3.3. It was found that the total iron content increased with increasing temperature there was no significant difference between FD-80C and FD-100C ($p>$

Table 3.2 Composition of undried (UD), freeze-dried (FD) broiler breast extracts which had been subjected to heat treatment.

Treatments	Carnosine		Protein		Total iron
	UD (μg/ml)	FD (μg/g)	UD (mg/ml)	FD (mg/g)	FD (μg/g)
Br-untreated	845.09 [a]	ND	21.94 [a]	ND	ND
Br-60C	857.65 [a]	33,200.85 [b]	8.63 [b]	415.30 [a]	98.01 [b]
Br-80C	874.46 [a]	38,434.04 [a]	2.04 [c]	140.63 [b]	109.75 [a]
Br-100C	884.76 [a]	40,380.13 [a]	1.81 [c]	121.79 c	113.75 [a]

Br = breast; 60C, 80C, and 100C represent the extracts which have been extracted by heating to 60°C, 80°C or 100°C for 10 min, respectively. Numbers with different letters within the same column are significantly different ($p \leq 0.05$);

ND = not determined

Table 3.3 Composition of undried (UD), freeze-dried (FD) broiler thigh extracts which had been subjected to heat treatment.

Treatments	Carnosine		Protein		Total iron
	UD (μg/ml)	FD (μg/g)	UD (mg/ml)	FD (mg/g)	FD (μg/g)
Th-untreated	261.94 [b]	ND	18.87 [a]	ND	ND
Th-60C	268.31[b]	12,918.65 [a]	7.87 [b]	437.50 [a]	200.83 [b]
Th-80C	280.72 ab	13,899.73 [a]	2.34 [c]	159.55 [b]	215.73 [a]
Th-100C	297.61 [a]	14,104.50 [a]	2.22 [c]	147.16 [b]	220.95 a

Th=thigh; 60C , 80C, and 100C represent the extracts which have been extracted by heating to 60°C, 80°C or 100°C for 10 min, respectively. Numbers with different letters within the same column are significantly different ($p \leq 0.05$);

ND = not determined.

0.05) but significant difference from FD-60C ($p>0.05$). The total iron content of FD-Br-60C, FD-Br-80C, and FD-Br-100C were 98.01, 109.75, and 113.75 μg/g FD extract, respectively. The total iron content of FD-Th-60C, FD-Th-80C, and FD-Th-100C were 200.83, 215.25, and 220.95 μg/g FD extract, respectively. If the total iron content was calculated in term of iron content in FD extract/g of fresh meat, the data would show the decrease of total iron content when heating temperature was increased. The total iron contents of FD-Br-60C, FD-Br-80C, and FD-Br-100C were 3.29, 2.72 and 2.94 μg/g meat, respectively. The total iron content of FD-Th-60C, FD-Th-80C and FD-Th-100C was 4.51, 3.44, and 3.75 μg/g meat, respectively (data not shown). The total iron content in term of μg Fe/g meat, would agree with Chan and colleagues (1993). They found that heating untreated extract of beef muscle could remove iron-containing protein. That means after heating the extract, the iron-containing proteins were denatured, aggregated and removed by centrifugation. Thus, the higher temperature, the less total iron content would be observed.

On the other hand, if total iron contents were compared in term of μg Fe/g of FD-extract, the result would not agree with the above suggestion. It was found that total iron in dried extract increased with decreasing temperature of heat treatment. Chan and colleagues (1993) reported this finding in dry-heated extract. Although, the total iron content decreased with increasing temperature of extraction, the low-molecular-weight (LMW) iron would increase 2.6-2.9 folds compared with untreated extract. This was concluded from the previous reports by Igene and colleagues (1979), and Chen and colleagues (1984) that heating beef muscle extract at 63-70^{o}C released protein-bound iron from the samples. Therefore, in this study, it can be

concluded that the LMW iron should be the main component of total iron content in chicken extracts. Unlike in the beef extracts which hemin is the main source of iron (Chan et al, 1993). Beef muscle consists about 89% myoglobin in total iron but chicken muscle consists only 12% myoglobin. The myoglobin is categorized into high-MW iron compound, with MW at least 17,000 Da. In chicken meat has higher LMW-iron (<12,000 Da) content than beef (Hazell, 1982). Hence, in this study, it can be suggested that most of iron in heated extract should be the LMW iron compound since increasing heating temperature increased the releasing of LMW-iron, as the total iron content (μg/g FD extract) were in the order of Br-100C > Br-80C > Br-60C and Th-100C > Th-80C > Th-60C, respectively, for breast extracts and thigh extracts.

According to the results of carnosine, total iron and protein contents obtained above, heating at 80°C for 10 min was taken in account for preparation of the extract for further purification by ultrafiltration.

Antioxidant activity of heated extracts

From TBARS determination, antioxidant activity of the extracts was expressed in percentage of inhibition calculated from the reduction of MDA in oxidation system. The result showed that antioxidant activity increased with increasing amount of FD-heated extract in the oxidation system for both breast and thigh extract groups. This agreed with the previous studies of Decker and colleagues (1992), Chan and colleagues (1993), Decker and colleagues (1995) and Gopalakrishnan and colleagues (1999). The antioxidant activities of broiler meat extracts were remarkably lower than pure carnosine. As shown in Table 3.4, amount of pure carnosine between 7.5-12.5

mg/mL in oxidation system produced constant antioxidative activity at approximately 92% inhibition, whereas the activities of all heated extracts were still increasing. The inhibition ability of heated extracts remained gradually increasing even reaching the maximum amount of 12.5 mg/mL of extract used. The final percentages of inhibition (at 12.5 mg/mL) of the heated extracts were approximately 51-78% inhibition. Comparison of antioxidant activities among heated extracts was demonstrated in Fig.3.1. Fig. 3.1B and 3.1D illustrated antioxidant activities of heated breast and thigh extracts in mg/mL. The FD-80C and FD-100C showed the similar antioxidant activities which were greater than that of FD-60C. In addition, Fig. 3.1A and 3.1B showed the antioxidant activities of breast and thigh extracts in term of carnosine concentrations in the extracts used in oxidation system, respectively. Similar trend of %inhibitions was obtained as shown in term of amount of extract used in the system.

Table 3.4 The ability of inhibiting oxidation by heated extracts from broiler meat compared with pure carnosine

Amount of extract (mg/mL)	% Inhibition						
	Pure cns	Br-60C	Br-80C	Br-100C	Th-60C	Th-80C	Th-100C
2.5	28.05	21.91	7.87	10.10	19.37	12.66	14.21
5	83.14	29.54	27.68	28.91	28.17	34.46	35.37
7.5	90.75	39.14	43.03	47.62	39.83	54.41	52.51
10	92.04	45.79	59.18	58.27	47.73	69.01	65.42
12.5	91.69	51.41	66.63	67.15	58.67	77.75	74.92

cns = carnosine; Br = breast and Th = thigh; 60, 80, and 100C represented the heated treatment at 60, 80 and 100°C for 10 min, respectively.

In addition, the antioxidant activity was also interpreted as the concentration of carnosine or amount of extract in oxidation system that could reduce 50% oxidation (EC50) compared with pure carnosine (Fig. 3.2). Fig.3.2A and 3.2B illustrated the EC50 of heated extracts from breast meat in term of amount of extract and to in term of carnosine concentration in oxidation system, respectively. Similar pattern of comparison among heated extracts from thigh meat were shown in Fig. 3.2C and 3.2D, respectively. The result showed that there were not significantly different in EC50 between 80C and 100C extracts ($p>0.05$). Considering the EC50 in of term of amount (mg/mL) extract used in oxidation system (Fig. 3.2A and 3.2C), pure carnosine had the greatest antioxidant activity which EC50 was 3.5 mg/mL. There was no significant difference in EC50 between FD-80C and FD-100C extracts ($p>0.05$), which were significantly lower than that of FD-60C extract ($p<0.05$). When EC50s were compared in term of carnosine concentration (mM) in present in the extracts used oxidation system (Fig. 3.2B and 3.2D) EC50s of the extracts were much lower than that of pure carnosine. The EC50 of pure carnosine was 15.45 mM , whereas Br-heated extracts were 1.4-1.75 mM and Th-heated extracts were 0.4-0.6 mM of oxidation system. It is obvious that pure carnosine had very low efficiency to reduce 50% oxidation when compared with heated extracts ($p<0.01$). The antioxidant activities of heated extracts increased with increasing temperatures of extraction, in the order of 100C ≥ 80C > 60C.

Controversy was found in correlation of the total iron content and antioxidant activity since the iron containing compounds act as oxidation catalysts. Higher extraction temperature, higher amount of iron was extracted from the meat (Table 3.2

and 3.3). However, heating at higher temperature could be more effective to remove other pro-oxidants as well, such as phospholipid and heme protein, lower protein contents with higher temperature applied (Table 3.2 and 3.3). In addition, there might be due to the presence of other LMW antioxidants such as phosphates and amino acids which might be released more with increasing temperature of extraction (Chan et al., 1993). Besides there is anserine (β-alanyl-l-1-methyl histidine) in skeletal muscle of vertebrates to play biological role similar to carnosine (Boldyrev et al., 1988; Chan and Decker, 1994; Cornet and Booussset, 1999). Anserine shows the antioxidant activities in muscle and brain (Kohen et al., 1988) and *in vitro* oxidation systems (Wu et al., 2003). This dipeptide would be released by heat treatment then, it would take parts in antioxidant activity of carnosine in this study. In addition, the carnosine contents in 80C and 100C extracts were higher than in 60C extracts. Thus, these compounds could act as synergist in antioxidant activity of carnosine in the extracts. This suggestion might also support the EC50 of pure carnosine which was higher value (lower antioxidant activity) than those of 80C and 100C extracts.

Heated-Ultrafiltration Extraction

The UD-80C extract was subjected to ultrafiltration (UF) in attempt to remove LMW iron containing compounds, and the carnosine was in UF permeate part.

Composition of ultrafiltration permeate (UFP)

Carnosine contents in UFP of both breast and thigh were obviously higher than in heated extracts and was significantly different ($p<0.05$) (Table 3.5) with 47816.91 and 18206.34 μg/g FD extracts for Br-UFP and Th-UFP, respectively**. The** carnosine contents increased about 20%, and protein contents decreased about 40%

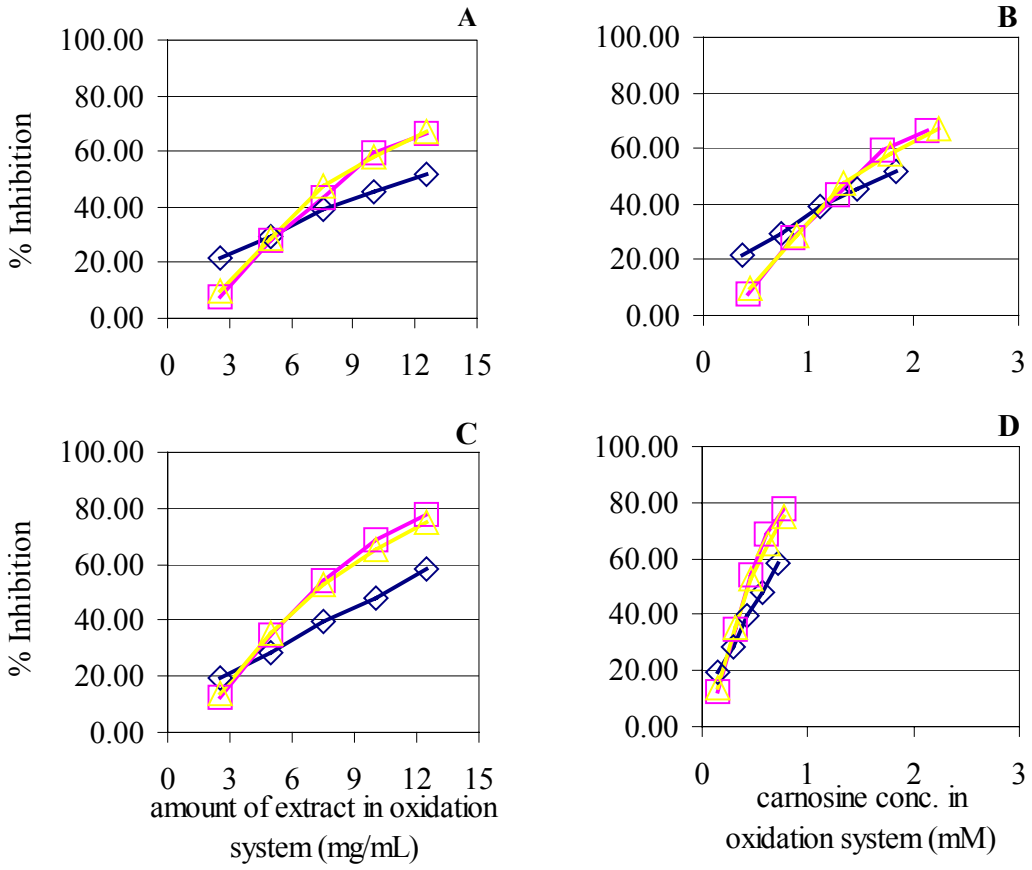

Figure 3.1 Effects of extract concentration on antioxidant activity as determined by TBARS, expressed in term of % inhibition. A and B = systems contained breast extracts; C and D = system contained thigh extracts; A and C = comparison of % inhibiting oxidation in term of amount of extract used in oxidation system; B and D = comparison of % inhibiting oxidation in term of carnosine concentration in oxidation system.

(◇ = 60C, □ = 80C, △ = 100C)

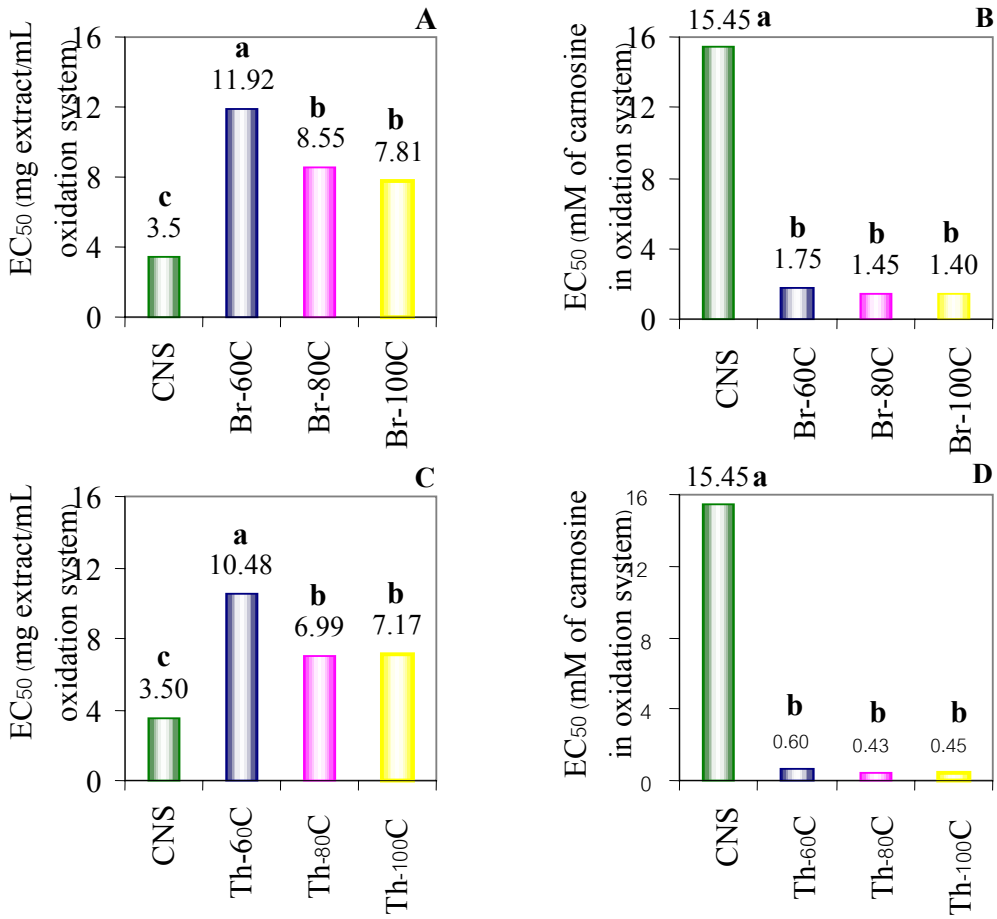

Figure 3.2 Comparison among heated extracts and pure carnosine (CNS) used in oxidation system that necessary to reduce by 50% of oxidation as determined by TBARS (EC50). A and B = systems contained breast extracts; C and D = systems contained thigh extracts; A and C = comparison of EC50s in term of amount of extract used in the system (mg/mL); B and D = comparison of EC50s in term of carnosine concentration used in the system (mM).

EC50s with different letters are significantly different ($p<0.05$).

for both UD- and FD-UFP compared with the 80C extract. The total iron contents of Br-UFP and Th-UFP decreased 10% and 40%, respectively, compared with the 80C extracts. These results showed an agreement with previous study of Chan and colleagues (1993) and Gopalakrishnan and colleagues (1999) in beef and pork. Their studies showed higher removal of LMW iron compounds and other proteins by UF than did in this experiment. This may due to higher content of LMW iron compounds (less than 5,000 Da) in UFP of broiler meat than in beef and pork.

Antioxidant activity of ultrafiltration permeate (UFP)

As similar trend to the heated extracts, antioxidant activity of UFP increased with increasing concentration of carnosine or amount of extract in oxidation system (Fig. 3.3). The UFP showed less ability to inhibit oxidation than that of 80C extract for both breast and thigh group. The EC50 of UFP was higher than that of the 80C extract ($p<0.05$) in both breast and in thigh meat extracts (Fig 3.4). When comparing in term of carnosine concentration used (mM) in oxidation system, the EC50s of Br-UFP and Th-UFP were 2.25 and 0.67 mM of oxidation system, respectively. Whereas, the EC50s of Br-80C and Th-80C extracts was 1.45 and 0.45 mM, respectively (Fig. 3.4B and 3.4D). It means that UFP has less antioxidant activity than that of 80C extract. Even though, the UFP had higher carnosine content and lower total iron content than did the 80C extract. However, UFP had greater activity than pure carnosine. On the contrary, when comparing in term of the amount of extract used (Fig. 3.4A and 3.4C), pure carnosine had the greatest antioxidant activity with the lowest EC50. This evidence was similar to the comparison of EC50 among the heated extracts (Fig. 3.2). The lower activity of UFP could be due to loosing of

Table 3.5 Composition of undried (UD), freeze-dried (FD) of 80C-heated extracts and permeate of ultrafiltration (UFP) from breast and thigh meats

Treatments	Carnosine		Protein		Total iron
	UD (μg/ml)	FD (μg/g)	UD (mg/ml)	FD (mg/g)	FD (μg/g)
BREAST EXTRACTS					
Br-80C	874.46 [a]	38,434.04 [b]	2.04 [a]	140.63 [a]	109.75 [a]
Br-UFP	1105.88 [ab]	47,816.91 [a]	1.28 [b]	73.63 [b]	98.30 [b]
THIGH EXTRACTS					
Th-80C	280.72 [b]	13,899.73 [b]	2.34 [a]	159.55 [a]	215.73 [a]
Th-UFP	367.63 [a]	18,206.34 [a]	1.41 [b]	82.70 [b]	114.24 [b]

Br=breast: Th=thigh extracted; Numbers with different letters within the same column of each muscle type are significantly different ($p \leq 0.05$).

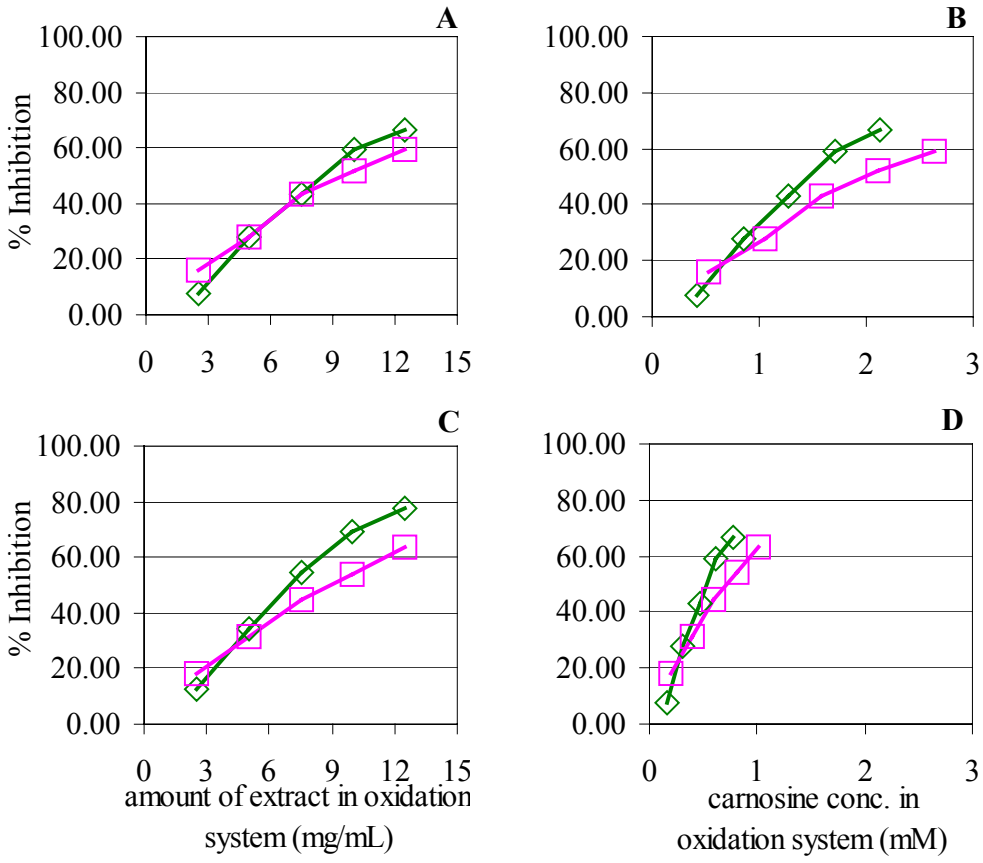

Figure 3.3 Effects of 80C-heated extract and UFP concentrations on antioxidant activity as determined by TBARS, expressed in term of % inhibition. A and B = systems contained breast extracts; C and D = system contained thigh extracts; A and C = comparison of % inhibiting oxidation in term of amount of extract used in oxidation system; B and D = comparison of % inhibiting oxidation in term of carnosine concentration in oxidation system.(◇ = 80C-heated extract; □ = UFP (ultrafiltration permeate))

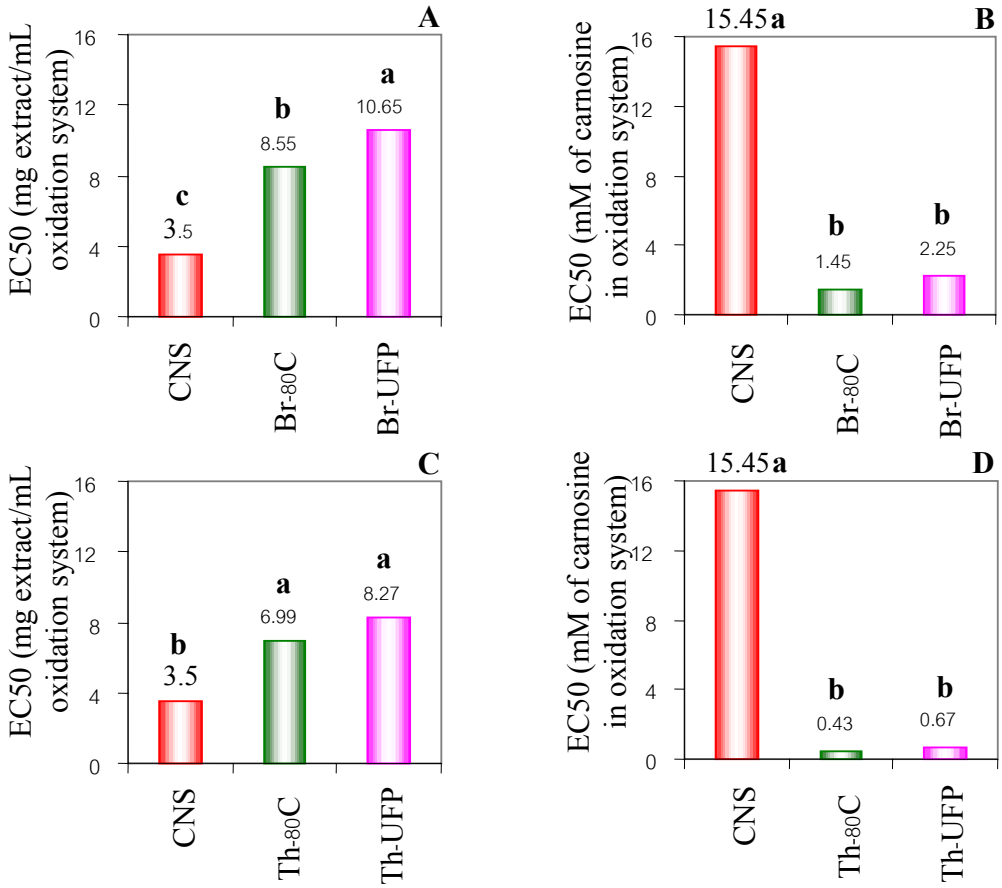

Figure 3.4 Comparison among 80C-heated extract, UFP and pure carnosine (CNS) used in oxidation system that necessary to reduce by 50% of oxidation as determined by TBARS (EC50). A and B = systems contained breast extracts; C and D = systems contained thigh extracts; A and C = comparison of EC50s in term of amount of extract used in the system (mg/mL); B and D= comparison of EC50s in term of carnosine concentration used in the system (mM). EC50s with different letters are significantly different ($p<0.05$).

other synergistic antioxidant compounds in the remaining retentate of the UF membrane. Consequently, the ratio of antioxidant compounds (including carnosine) to other LMW pro-oxidants (including LMW iron compounds) could be decreased. Thus, there could be only carnosine or carnosine with small amount of other antioxidant compound left in UFP resulting in lower antioxidant activity of the UFP than that of the 85C extract. However, the presence of other low molecular weight antioxidant molecules in UFP caused greater antioxidant activity (Fig. 3.4B and 3.4D) than pure carnosine when compared in term of carnosine concentration presented in oxidation system.

CONCLUSIONS

Chicken breast meat had 7-folds carnosine content higher than thigh muscle. In the heat treatment, increasing temperature resulted in increasing of carnosine concentration and total iron content but decreasing in protein content of the heated extracts. The results showed no significant differences in the amount of each component and EC50 values between 80C and 100C extracts, but their contents were different from that of 60C extract. After 80C extract was passed through ultrafiltration, carnosine content in UFP increased. In addition, ultrafiltration was effective to remove low-molecular weight iron compounds. The antioxidant activities of all the extracts increased when extract concentrations in the oxidation system were increased. The antioxidant activity of pure carnosine was obviously lower than those of heated extracts and UFP at the same level of carnosine used in oxidation system due to synergistic effects of other antioxidant compounds in the chicken meat extracts.

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CHAPTER IV

EXTRACTION AND ANTIOXIDANT ACTIVITY OF CARNOSINE FROM CHICKEN MEATS: BETWEEN DIFFERENCES BREEDS OF CHICKENS

ABSTRACT

Carnosine contents in fresh meat from native, hybrid native chickens were different among breeds ($p<0.01$) and between sex ($p<0.01$) of chickens. Breast meat contained 2-4 fold higher carnosine content than thigh meats. It was found higher carnosine content in female meat than in male meat. After extracted by heating at 80 °C and passed through ultrafiltration, carnosine contents in extracts were different among breeds ($p<0.01$) and between sex ($p<0.01$) of chickens, also. Furthermore, the carnosine contents in breast extracts still were higher than in thigh extracts by 2-4 folds with found higher content in female extract than in male extract. The different carnosine contents in extracts were influenced from feed quality, also. There was no relation between total iron content and carnosine content on the antioxidant activity of extract. However, compared in term of carnosine concentration in extract used in oxidation system, they showed remarkably greater antioxidant activity than pure carnosine. In addition, thigh extract had more effective inhibiting ability than breast extract.

INTRODUCTION

There are many functional compounds found in skeletal muscle of vertebrate animal, such as free amino acids, dipeptides, and non-protein nitrogenous compounds. Some of these compound have been implicated and purposed as the active compounds for biological activities in vertebrates, such as dipeptides including carnosine (Wu and Shiau, 2002). Carnosine (β-alanylhistidine) plays important role in physiological functions, such as a potent intracellular pH-buffer, inhibiting of oxidation, and neurotransmitter function (Chan and Decker, 1994; Wu and Shiau, 2002). From the broad-spectrum antioxidant activities, carnosine was studied the ability of inhibiting oxidation in various food systems and in medical practices (Calvert and Decker, 1992; Decker and Crum, 1993; Jordan, 1999; Kyriaziz, 1999; Lee et al., 1999). Carnosine content can be affected by fiber types of animal which are influenced from breed, sex, age and breeding of animals (Abe and Okuma, 1995). Muscle fiber types can be divided into three main groups: white glycolytic muscles rich in type IIB fibers, red oxidative muscles rich in type I and IIA fibers and intermediate muscles (Cornet and Bousset, 1999). Plowman and Close (1988) reported that white meat, such as chicken meat, had higher carnosine content than dark meats**.**

Nagai and colleagues (1996) studied effects of chicken essence on the recovery from fatigued. They found that the students, who consumed the chicken essence, felt more active and less fatigued during the workload and their performance to perform a mental arithmetic test and a short term memory test were also improved. They concluded that the chicken essence has a potential to metabolize stress-related

substance in blood oxidants and to promote recovery from mental fatigue. Among the ingredients in this essence used, there was carnosine in several hundred milligrams of 140 mL essence. Carnosine are regarded as candidates for the cortisol metabolism accelerator. In addition, Wu and Shiau (2002) investigated carnosine content in six types of commercial chicken essences. There were carnosine contents ranged from 8-162 mg/100 g essence. The lowest carnosine content was found in the essence made from chicken bones. As the essential compound in chicken extracts, carnosine content depending on breed, sex and age of animal, the better knowledge of its content and extraction from different breeds of chicken meats would lead to gain more understanding of carnosine in various breeds chickens. Hence, the purposes of this study were to investigate carnosine contents in fresh breast and thigh of native, 4-lines cross breeds and 5-lines cross breeds chickens meats, and produce a natural carnosine-containing antioxidant extract from those meats using heat treatment combined with ultrafiltration. These extracts were also evaluated for their antioxidant activities. In addition, protein and total iron contents were determined due to their important involvement in antioxidant activity of the extracts.

MATERIALS AND METHODS

Chemicals

Carnosine, o-phthaldehyde (OPA), soybean phosphatidylcholine, β-mercaptoethanol (BME), bovine serum albumin (BSA), and Folin Ciocalteu's phenol reagent were purchased from Sigma Chemical (St. Louis, MO, USA). Standard Fe (1000 mg/mL) was obtained from Merck KgaA (Darmstadt, Germany). Methanol

and acetonitrile were HPLC grade, and all other chemicals were reagent grade or purer.

Raw Materials

Three types of chickens were investigated; native, 4-lines cross breeds, and 5-lines cross breeds chickens. Native chicken was raised *ad libitum* by conventional feeding, 4-lines and 5-lines cross breeds chickens obtained from commercial poultry farms were fed *ad libitum* by commercial formula feed. The experiment was performed when the chicken live weight reached 1.8 kg. This study was taken by comparison among breeds and sexes of chickens within the breast and thigh meats group, separately. All chicken meats samples were ground using a domestic mincer, then vacuum packed and stored at –20°C until used.

Chemical Composition Analysis of Fresh Meat

Fresh meat samples were determined for moisture and ash contents according to AOAC (1997), protein content by Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard, carnosine content by high performance liquid chromatography (Cornet and Bousset, 1999; Gopalakrishnan et al., 1999), and total iron content by Atomic Absorption Spectrophotometry (James, 1995).

Preparation of Meat Extracts for Determining Total Carnosine Content in Fresh Meats

The meat extracts were prepared according to Aristoy and Toldra′ (1991) with slight modification. The frozen minced meat was thawed under running tap water.

One part of this minced meat was homogenized in 4 parts of precooled (4°C) 0.1 N HCl in an AM-8 homogenizer (Nihonseiki, Kashi Ltd., Tokyo, Japan) for 8 min on the ice chamber. The homogenate was centrifuged at 10,000×g by Sorvall RC-5C Plus (Dupont, Delaware, USA) at 4°C for 20 min. The supernatant was filtered through Whatman #4 filter paper, and then, the filtrate was subjected to freeze-drying. Frozen (-45°C) filtrate was freeze-dried for 72 hr using HETO FD3 (Heto-Holten A/S, Allerod, Denmark). Subsequently, the dried filtrates were determined for total carnosine content present in chicken meats by the procedure described in determination of carnosine content.

Extraction of Carnosine by Heat Treatment Combined with Ultrafiltration

The procedure was modified from Chan et al. (1993) and Gopalakrishnan et al. (1999). The minced meat was thawed under running tap water. The minced was homogenized in 2 volumes of precooled (4°C) distilled deionized water in an AM-8 homogenizer in the ice chamber for 8 min. The homogenate was centrifuged at 20,000×g for 30 min at 4°C using Sorval RC-5C Plus, and then, the supernatant was filtered through Whatman #4 filter paper. The filtrate was heated in water bath to 80°C for 10 min and cooled in ice bath. Heated extract was centrifuged to remove precipitated proteins at 5,000×g for 20 min. Supernatant was passed through the 5,000 molecular weight cut off ultrafiltration membrane (PES) (Vivaflow50, Vivascience, Sartorius AG, Goettingen, Germany). The ultrafiltration permeate was freeze-dried using a HETO-FD3. The freeze-dried extract (FD-Ext) was determined for carnosine, protein, and total iron content, and evaluated for antioxidant activity.

Analysis of Extract (Ext) Compositions

Protein content. Protein content in FD-Ext was determined by Lowry method (Lowry et al., 1951) using BSA as a standard. FD-Ext (2.0000-3.0000 mg) was reconstituted with 1 mL phosphate buffer (5 mM, pH 7.0) prior to determination.

Total iron content. The total iron content in fresh meats and FD-Exts were measured at 248.3 nm by means of atomic absorption spectrpphotometry using AAnalyst 100 (Perkin Elmer, Norwalk, CT, USA). Accurate weight of sample (3.0000 g for fresh meat and 0.2500 g for FD-Ext) was ashed in a muffle furnace (Carbolite CSF1200, Cheffield, England) at 500°C until a white or light gray ash obtained. Five mL of concentrated hydrochloric acid was added to the crusible containing the ash, boiled for 5 min, transferred to a beaker, adjusted volume to about 40 mL and then, boiled for 10 min. The mixture was cooled, filtered through Whatman #1 filter paper into a 50-mL volumetric flask, made up to volume with deionized water, and measured at 248.3 nm. The iron concentrations were calculated from a standard curve generated using known concentration of iron (0-10 μg/mL) (James, 1995).

Carnosine content. Carnosine contents in fresh meats and FD-Exts were determined using high performance liquid chromatography (HPLC), derivatizing the extracts by OPA working solution. The OPA working solution was prepared 24 hr before use by dissolving 27 mg of OPA in 500 μL of absolute alcohol. Five mL of 0.1 M sodium tetraborate ($Na_2B_4O_7.10H_2O$) (pH 9.5) was added, followed by adding 50 μL of BME. The mixture was throughly mixed, stored in a tightly closed

container, and kept in the dark. The reagent can be kept for several days (Antoine et al., 1999).

Sample preparations for HPLC analysis were performed based on the methods of Gopalakrishnan et al. (1999) and Cornet and Bousset (1999) with slight modification. The 100 mg FD-Ext reconstituted in 2 mL of 5 mM phosphate buffer, pH 7.0 was mixed with 2 mL of 0.4 M perchloric acid and boiled for 5 min to precipitate proteins, and centrifuged at 2,000×g for 20 min by Labofuge 400R #8179 (Heraeus Instruments, Germany). The supernatant was filtered through 0.45 μm membrane filter. The filtrate (10 μL) was derivatized with 10 μL of OPA working solution just prior to injection. The derivatized samples were analyzed on Agilent 1100 series HPLC with an autosampler (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). OPA derivatized carnosine was separated using a mobile phase of 0.3 M sodium acetate (pH 5.5), methanol and acetonitrile (75:15:10) at flow rate at 0.5 mL/min with Eclipse XDB-C18 (4.6×150 mm, 5 micron) column. Fluorescence detector was set at an excitation wavelength 310 nm and emission wavelength 375 nm. A standard curve was prepared using pure carnosine solution with concentration of 10-1000 ppm. Carnosine concentration was calculated by Chemstation Rev.A.09.03 (1417) (Agilent Technologies 1990-2002).

Evaluation of Antioxidant Activity

Antioxidant activity of FD-Ext was determined by its ability to inhibit iron/ascorbate catalyzed oxidation of phosphatidylcholine liposomes, which was modified from Decker and Faraji (1990), Kansci and colleagues (1997), and

Gopalakrishnan and colleagues (1999). Lipid oxidation was performed in a 2.0 mL model system containing 1 mg phosphatidylcholine liposomes/mL of 5 mM phosphate buffer (pH 7.0) plus lipid oxidation catalyst and also various concentration of FD-Ext.

Preparation of reagents. Sodium ascorbate and $FeCl_3$ solution, acted as the catalysts, were separately prepared in degassed ultra-pure water freshly before use (Kansci et al., 1997). The MDA standard was prepared according to Botsoglou and colleagues (1994). 1,1,3,3-tetraethoxypropane (TEP) was weighed (100 mg) in a screwed-capped test tube, diluted with 10 mL of 0.1 N HCl, immersed into a boiling water bath for 5 min, and cooled immediately under tap water. Stock solution of MDA (100 μg/mL) was prepared by transferring the hydrolyzed TEP solution into a 100-mL volumetric flask and diluting to volume with water.

Analysis of thiobarbituric acid reactive substances (TBARS). Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS). The pure carnosine or extract was dissolved in phosphatidlycholine liposomes suspension to give a various concentration of 0-12.5 mg/mL. After 10 min, phospholipid oxidation was induced by adding sodium ascorbate and $FeCl_3$ solutions to a final concentration 45 μM of each. The oxidation mixture was incubated in a 37°C water bath for 30 min, followed by adding 2 mL of thiobarbituric acid (TBA) reagent (0.02 M in water). The tightly closed tube was heated in boiling water bath for 15 min, cooled and centrifuged at 4,000×g for 15 min. The absorbance of supernatant was measured at 532 nm using UV/VIS 916 Spectrophotometer (GBC

Scientific Equipment, Ltd., Victoria, Australia). Concentration of MDA in oxidation system was calculated from the MDA standard curve (0-7 μg/mL). The ability of inhibition was calculated as follows:

$$\%\text{inhibition} = \frac{\text{(MDA conc. in absence of extract)-(MDA conc. in presence of extract)}}{\text{MDA conc. in presence of extract}} \times 100$$

The oxidation activity of FD-Ext was expressed as amount of FD-Ext or the concentration of carnosine used in system that could inhibit 50% of oxidation (EC50). The EC50 of FD-Ext was also compared with EC50 of pure carnosine.

Statistical Analysis

All experiments were performed in duplicates. Each replicate was chemically analyzed in triplicate samples. Statistical analysis was evaluated by Factorial in Completely Randomized Design (CRD) with Statistical Analysis System (SAS Institute, Inc., 1995). Analysis of Variance (ANOVA) was analyzed and means comparison was done by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSIONS

Chemical Composition of Fresh Chicken Meats

Content of individual composition in fresh meat was presented in Table 4.1 and 4.2 for breast and thigh meats, respectively. There were no significant differences among breeds and between sex of chicken in protein content ($p>0.05$). Protein contents of breasts and thighs were ranged of 21-24% and 19-21%, respectively.

Table 4.1 Chemical composition of fresh breast meats from different breeds (Bd) and sex (S) of chickens

Composition	N		K		T		SEM	Effect		
	F	M	F	M	F	M		Bd	S	Bd *S
Moisture (%)	73.82^{bc}	73.23^{c}	73.24^{c}	74.89^{a}	74.53^{ab}	74.67^{a}	0.21	0.01	ns	0.01
Protein (%)	23.96^{a}	23.75^{a}	23.83^{a}	23.43^{a}	21.69^{a}	22.46^{a}	0.36	ns	ns	ns
Carnosine (μg/g wet meat)	993.23^{b}	818.55^{c}	1,200.05^{a}	684.82^{c}	929.03^{b}	621.23^{d}	35.18	0.01	0.01	0.01
Total Iron (μg/g wet meat)	11.94^{ab}	10.87^{bc}	12.91^{a}	9.78^{c}	9.71^{c}	11.89^{ab}	0.39	ns	ns	0.01

N= native, K= 4-lines cross breeds, and T= 5-lines cross breeds chickens; F =Female and M= Male

Effect = level of statistical differences by breed, sex and interaction between breeds and sex: 0.01 = highly significant, 0.05 = significant, and ns = not significant differences. SEM = Standard Error of Means

Means with different letter are significantly different ($p<0.05$).

Table 4.2 Chemical composition of fresh thigh meats from different breeds (Bd) and sex (S) of chickens

Composition	N		K		T		SEM	Effect		
	F	M	F	M	F	M		Bd	S	Bd *S
Moisture (%)	76.24^{bc}	76.79^{ab}	75.59^{c}	77.58^{a}	76.34^{bc}	76.96^{ab}	0.21	ns	0.01	ns
Protein (%)	21.17^{a}	19.50^{a}	19.67^{a}	19.98^{a}	20.16^{a}	19.92^{a}	0.22	ns	ns	ns
Carnosine (μg/g wet meat)	311.93^{bc}	271.01^{c}	304.88^{c}	279.57^{c}	429.76^{a}	363.06^{b}	11.62	0.01	0.01	ns
Total Iron (μg/g wet meat)	14.54^{b}	16.22^{a}	12.76^{c}	15.72^{ab}	15.84^{a}	15.90^{a}	0.38	0.05	0.01	0.05

N= native, K= 4-lines cross breeds, and T= 5-lines cross breeds chickens; F =Female and M= Male

Effect = level of statistical differences by breed, sex and interaction between breeds and sex: 0.01 = highly significant, 0.05 = significant, and ns = not significant differences. SEM = Standard Error of Means

Means with different letter are significantly different ($p<0.05$).

Total iron content of breast was lower than that of thigh meat. This result was consistent with other studies (Hazell, 1982; Pearson and Young, 1989; McCormick, 1994; Kongkachuichai et al., 2002) that red meat is more supplied with blood vessels and contains more myoglobin and hemoglobin than light meat. Therefore, it would be expected to have higher contents of iron. In breast meats group (Table 4.1) total iron content was not significantly different compared among breeds, and between sex ($p>0.05$), whereas it was significantly different among breeds ($p<0.05$) and between sex ($p<0.01$) in thigh meats group (Table 4.2). Most studies reported that 99.5-100% of chicken breast meat was white (fast glycolytic) fibers which contained limited less amount of myoglobin, resulting in non-significant differences in total iron content between breast meats from different breeds (Smith and Fletcher, 1988; Lengerken et al., 2002). In addition, Meyer (2003) and Illingworth (2004) reported that chicken thigh meats composed of red and intermediate fibers. The ratio of these fibers may be different among chicken breeds depending on their activities, resulting in different myoglobin and total iron contents.

Carnosine contents were significantly different when compared among breeds ($p<0.01$) and between sex ($p<0.01$) for both breast (Table 3.1) and thigh (Table 3.2) groups. Breast had 2-4 folds carnosine content higher than thigh. However, carnosine contents were not significantly different among thigh meats ($p>0.05$) comparing between the interaction of breeds and sex, but there was differences among breast meats ($p<0.01$). This result agreed with many previous studies (Davey, 1960; Decker, 1995; Cornet and Bousset, 1999) that white muscle had higher carnosine content than dark muscle. Davey (1960) reported the high carnosine content

associated with white muscles which maintain their energy-rich phosphate ester supplies by anaerobic means. Therefore, in view of the power of carnosine, it becomes a factor of great importance to act as buffer in physiological range of pH effected from lactic acid accumulation. Comparing between sex, it was found that carnosine content of female meat was higher than that of male meat ($p<0.01$). At the same live weight (LW), female chicken is older than male chicken (Kotula, 1991). In this study regardless of age, the LW of chicken used was the same at 1.8 kg, that might be meaning that female was older than male. Thus, results showed higher carnosine content from female. It agreed with Chan and Decker (1994) who reported carnosine content of poultry skeletal muscle increased with age. At the same LW, native chicken had growth time about twice as longer as that of cross breeds chicken, even 4-lines and 5-lines cross breeds chickens were improved on the purposes of obtaining meat qualities that similar to native chicken. The faster growth depended on feed quality. Four-lines cross and five-lines cross breeds were fed with commercial feed *ad libitum*, whereas native chicken was fed with conventional feed by allowing scavenge during the day time in the simple sheds or farm house (Haitook et al., 2003). Thus, according to the results, feed quality might have more effect on carnosine content than age of the birds since, the older native chickens had no remarkably higher carnosine content than cross breeds chickens in the same sex. This result was supported by Wattanachant and colleagues (2004) who reported that different feeding affected on the composition of chicken meat, such as amino acids and fatty acids. Hence, the commercial feed should be more enrichment than conventional feed, resulting in high concentration of amino acids, including histidine which are the precursors of carnosine systhesis.

Chemical Compositions of Carnosine-Containing Extracts (Ext) from Different Chicken Breeds.

The chemical composition of Ext from breast and thigh meat was shown in Table 4.3 and 4.4, respectively. Differences within the contents of each Ext component were observed among breeds ($p<0.01$) and between sex ($p<0.01$) for both types of meats. Similar to fresh meats, that extracts from breast (Br-Ext) had 2-4 folds carnosine contents higher than that of extracts from thigh (Th-Ext). Carnosine contents in Br-Ext and Th-Ext were 18,498 - 32,874.11 and 6,387.66 - 10,766.02 ppm in Ext, respectively. Carnosine contents were different among breeds and between sex of which female-Exts were greater than in male-Exts. These results agreed with the previous studies of Davey (1960), Decker (1995), and Cornet and Bousset (1999) that there were higher carnosine content in white fibers than in red fibers. Consequently, similar results would be observed in the meat extracts. Furthermore, not only fiber type affected on carnosine content, Chan and Decker (1994) reported that it could be affected by species and age of animal. Br-Ext and Th-Ext had approximately 3-6 folds and 6-10 folds total iron content higher than their fresh meats, respectively. Total iron contents in Br-Exts were ranged 41.02 - 83.95 ppm (Table 4.3) while in Th-Exts were ranged 82.11 - 150.86 ppm (Table 4.4). Protein contents in Br-Exts and Th-Exts were between 72.40 - 79.79 and 33.01 - 50.77 mg/g, respectively. Total iron contents of Th-Exts were greater than that of Br-Exts, thus conformed to the total iron content of fresh meats shown in Table 4.1 and 4.2. Regarding to the remarkably high total iron content in Th-Exts, it would be suggested that iron was released from myoglobin or hemoglobin molecules into the extract during heating at 80°C. According to Schricker and Miller (1983), and Chen et al.

Table 4.3 Chemical composition of carnosine extracts (Ext) of breast meats from different breeds (Bd) and sex (S) of chickens

Component	N		K		T		SEM	Effect		
	F	M	F	M	F	M		Bd	S	Bd *S
Carnosine (μg/g Ext)	32,874.11^{a}	30,332.11^{b}	31,297.39^{ab}	22,978.72^{c}	23,355.16^{c}	18,498.32^{d}	928.48	0.01	0.01	0.01
Protein (mg/g Ext)	79.79^{a}	72.57^{c}	76.30^{b}	79.40^{a}	74.99^{bc}	72.71^{c}	0.76	0.01	0.01	0.01
Total Iron (μg/g Ext)	54.12^{c}	48.42^{cd}	45.16^{de}	41.02^{e}	67.28^{b}	83.95^{a}	3.16	0.01	0.01	0.01

N= native, K= 4-lines cross breeds, and T= 5-lines cross breeds chickens; F =Female and M= Male

Effect = level of statistical differences by breed, sex and interaction between breeds and sex: 0.01 = highly significant,

0.05 = significant, and ns = not significant differences. SEM = Standard Error of Means

Means with different letter are significantly different ($p<0.05$).

Table 4.4. Chemical composition of carnosine extracts (Ext) of thigh meats from different breeds (Bd) and sex (S) of chickens

Component	N		K		T		SEM	Effect		
	F	M	F	M	F	M		Bd	S	Bd *S
Carnosine (μg/g Ext)	9,168.31[b]	7,910.12[c]	10,159.37[ab]	7,872.40[c]	10,766.02[a]	6,387.66[d]	227.67	0.01	0.01	ns
Protein (mg/g Ext)	36.53[d]	46.55[b]	33.09[e]	40.31[c]	50.77[a]	41.27[c]	1.46	0.01	0.01	0.01
Total Iron (μg/g Ext)	82.11[d]	96.40[c]	111.04[b]	115.20[b]	119.96[b]	150.86[a]	4.53	0.01	0.01	0.01

N= native, K= 4-lines cross breeds, and T= 5-lines cross breeds chickens; F =Female and M= Male

Effect = level of statistical differences by breed, sex and interaction between breeds and sex: 0.01 = highly significant,

0.05 = significant, and ns = not significant differences. SEM = Standard Error of Means

Means with different letter are significantly different ($p<0.05$).

(1984), these released irons would be compounds with lower molecular weight than 5,000 Da. Nonheme iron content in heated samples were found ranged from less than 10% to more than 100% depending on the length and severity of heat treatment. Heat treatment caused the oxidation cleavage of porphyrin ring followed by releasing of the iron.

Antioxidant Activity of Carnosine-Containing Extracts (Ext)

TBARS value was measured for antioxidant activity of Ext and expressed in percentage of inhibition, calculated from the reduction of MDA in oxidation system. The greater antioxidant activity was observed when higher amount of Ext used in oxidation system for both Br-Ext and Th-Ext (Fig. 4.1). These results consistent to previous studies of Decker and colleagues (1992), Chan and colleagues (1993), Decker and colleagues (1995), and Gopalakrishnan and colleagues (1999). Comparison of antioxidant activities among the extracts was illustrated in Fig. 4.1. Antioxidant activity in term of amount of Br-Ext and Th-Ext used (mg/mL) in oxidation system were presented in Fig. 4.1A and 4.1C, respectively. Fig. 4.1B and 4.1D presented antioxidant activities of Br-Ext and Th-Ext in term of carnosine concentration in the extracts (mM), used in oxidation system, respectively. Similar results of % inhibitions among Br-Exts and among Th-Exts, in term of amount of Ext used in the system, were observed that the higher amount of Ext used, the greater antioxidant activity obtained (Fig. 4.1A and 4.1C). On the other hand, when antioxidant activities compared in term of carnosine concentration used in the system, different activities among Br-Exts and among Th-Exts (Fig. 4.1B and 4.1D) were found. If compared between Br-Exts and Th-Exts, the activities of Th-Exts greater

than that of Br-Exts at the same levels of carnosine concentration used in the system. In addition, the antioxidant activity was interpreted in term of amount of Exts and carnosine concentration used in oxidation system that could reduce 50% oxidation (EC50) campared with pure carnosine as shown in Fig. 4.2 and 4.3. When compared in term of amount of Ext used in the system, pure carnosine showed obviously greater antioxidant activity than Br-Exts (Fig. 4.2A) and Th-Exts (Fig. 4.3A). The EC50 of pure carnosine was 3.54 mg/mL while EC50s of Br-Exts and Th-Exts were 9.62-13.30 and 8.24 - 13.20 mg/mL, respectively. In contrast, pure carnosine showed definitely lower activity than Br-Exts (Fig. 4.2B) and Th-Exts (Fig. 4.3B) when compared in term of carnosine presented used in oxidation system. The EC50 of pure carnosine was 15.63 mM whereas the EC50s of Br-Exts and Th-Exts were 0.79 - 1.81 and 0.25 - 0.54 mM, respectively. Higher efficiency in antioxidant activity of Exts than pure carnosine might be due to the presence of other antioxidants such as phosphates, and amino acids and low molecular weight peptides which released during heat treatment (Chan et al., 1993). In addition, there is anserine in chicken muscle which play role similar to carnosine (Boldyrev et al., 1988; Chan and Decker, 1994; Cornet and Bousset, 1999; Wu et al., 2003). Thus, it would take parts in synergistic antioxidant activity of carnosine in the extracts. Furthermore, maillard reaction (MR) products occurred in extract during heat treatment could add synergistic effect to the extracts as well. The MR may be occurred by the reaction of amino acids and ribose from nucleic acids or other carbonyl compounds (Pokrny′, 1981; Hultin, 1985). The maillard reaction intermediates (MRI), such as reductone like compounds, occurred while browning state not yet reached. MRI products have been shown to have high antioxidant activities (Eichner, 1981; Bailey and Um, 1992).

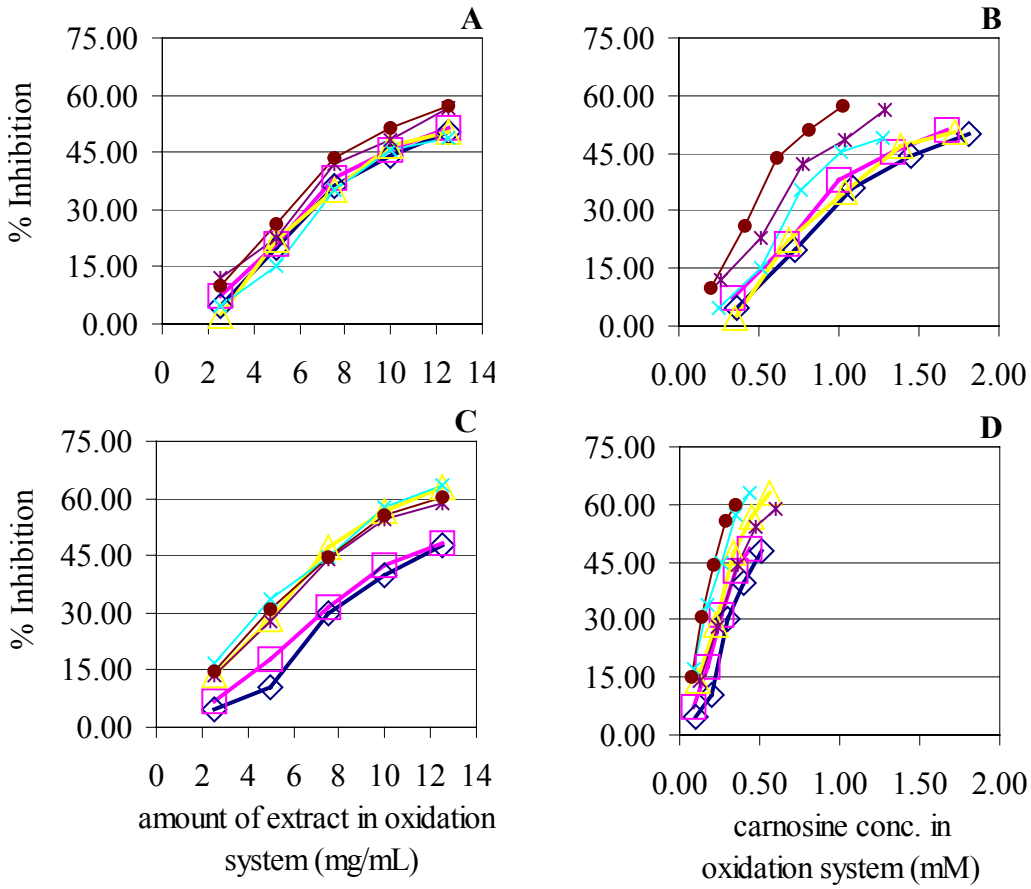

Figure 4.1 Effects of extract (Ext) concentration on antioxidant activity as determined by TBARS, expressed in term of % inhibition. A and B = systems contained breast extracts; C and D = system contained thigh extracts; A and C = comparison of % inhibiting oxidation in term of amount of extract used in oxidation system; B and D = comparison of % inhibiting oxidation in term of carnosine concentration in oxidation system.

(◇ = NF, □ = NM, △ = KF, × = KM, = TF, = TM)

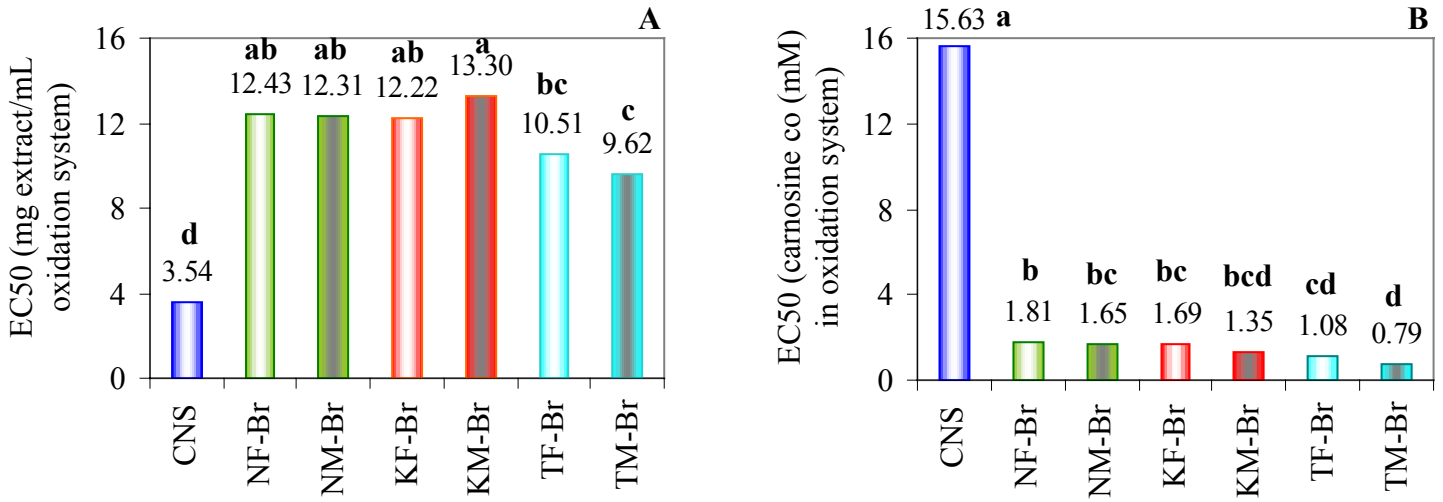

Figure 4.2 Amount of breast (Br)-extract or carnosine conc. in Br-extracts used in oxidation system that necessary to reduce by 50% of oxidation as determined by TBARS(EC_{50}) which compared with pure carnosine (CNS). A= comparison of EC50s in term of amount of extract used in the system (mg/mL); B = comparison of EC50s in term of carnosine concentration used in the system (mM). EC50s with different letters are significantly different ($p<0.05$).

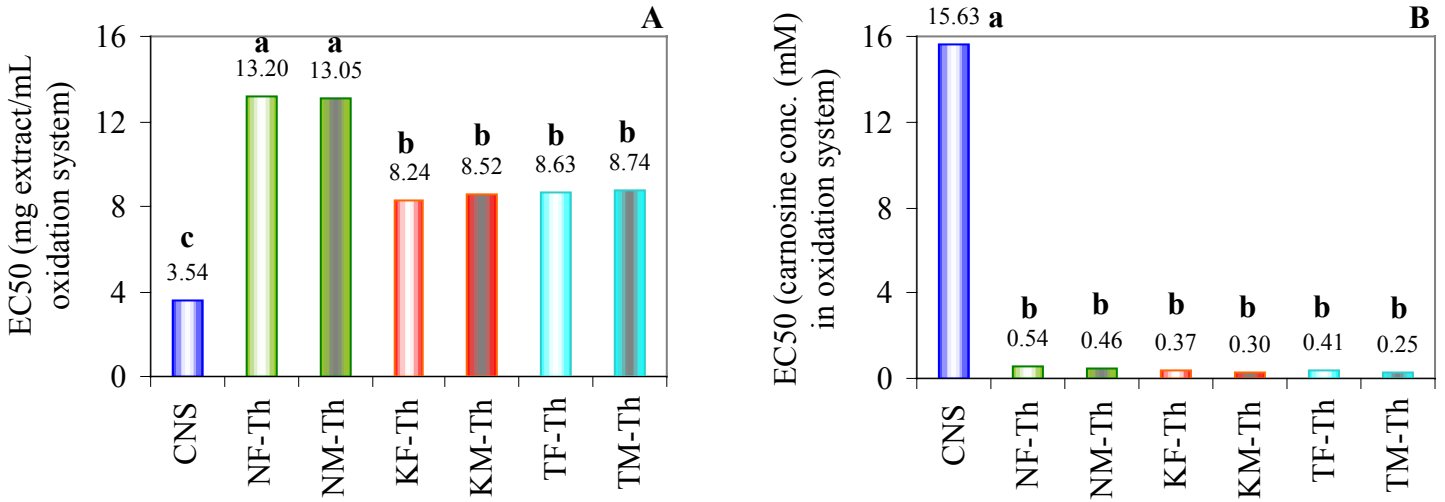

Figure 4.3 Amount of thigh (Th)-extract or carnosine conc. in Th-extracts used in oxidation system that necessary to reduce by 50% of oxidation as determined by TBARS(EC50) which compared with pure carnosine (CNS). A= comparison of EC50s in term of amount of extract used in the system (mg/mL); B = comparison of EC50s in term of carnosine concentration used in the system (mM). EC50s with different letters are significantly different ($p<0.05$).

Th-Ext is expected to contain more MRI products since pH of red meat is higher than white meat (Davey, 1960). In addition, higher amount of myoglobin and hemoglobin in thigh meat would give the Th-Ext upon heating with higher nonheme compounds and small protein fractions, such as histidine residue released from heme molecule, which provide better synergistic effects in the extracts. This evidence is agreed with the finding of Decker and Faraji (1990) who reported that inhibition of lipid oxidation of carnosine was highest in the system containing H_2O_2-hemoglobin comparing with system containing Fe-ascorbate, singlet oxygen and lipoxidase.

CONCLUSIONS

This study demonstrated that carnosine contents in fresh meat for both breast and thigh meats were significantly different among breeds and between sex of chickens. Breast meats contained higher carnosine contents than thigh meats. Carnosine content in female chicken meat was higher than that of male chicken meat. Breeds and sex were suggested to have influence on carnosine content. Similar results were obtained in their extracts. When compared with pure carnosine at the same concentration, chicken extracts exhibited higher antioxidant activities. Extracts of thigh meats gave higher inhibition efficiency than that of breast meats even though they contained lower amount of carnosine.

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CHAPTER V

OVERALL CONCLUSIONS

Chicken breast contained higher carnosine content than thigh meat in all chicken breeds. There were the highly significant differences in carnosine contents among breeds and between sex of chickens. Broiler breast meat contained 7 folds higher while native and hybrid native breast contained only 2-4 folds higher carnosine content than that of their thigh meats. After extraction by heat treatment, the carnosine and total iron contents increased whereas protein content decreased with increasing temperature. The 80°C-heated extract was obtained to further purified by ultrafiltration because it contained high carnosine content and lower total iron content which was not significantly different from 100°C-heated extract. The ultrafiltration permeate had significantly higher carnosine content, lower total iron and protein contents than that of 80°C-heated extract. Carnosine-containing extracts from native and hybrid native chicken meats were different in carnosine, total iron and protein contents, affected by breeds and sex. The antioxidant activities of these extracts were greater than that of pure carnosine in term of carnosine concentration in extract used in oxidation system. The antioxidant activities of thigh extracts were greater than that of breast extracts. Although, heat treatment combined with ultrafiltration can be an effective method resulting in high carnosine content in extracts and removing more iron compounds, total iron content is still high. The further study is need for better efficient to remove iron and other pro-oxidants.

BIBLIOGRAPHY

Miss Bussayarat Maikhunthod was born in Nakhon Ratchasima, Thailand on Saturday December 17th, 1977. She finished high school from Suranaree Wittaya School, Nakhon Ratchasima. In 1998, she received Bachelors Degree in B.Sc. (Food Technology) from Institute of Agricultural Technology, Suranaree University of Technology, Thailand. Then she continued her Master Degree in School of Food Technology at Institute of Agricultural Technology, Suranaree University of Technology. Her research topic was extraction and antioxidant activities of carnosine from native, hybrid native and broiler chicken meats. While she was studying in Master Degree, she had experiences by working as the teaching assistant in school of food technology, SUT, in Food processing laboratory, and Cereal and legume products technology, and working as a research assistant in the topic a comparative study of characteristics, chemical composition and sensory qualities of hybrid native chicken, commercial broilers and laying male chicks.