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The Effects of Chicken Leg Bone Extract on Antioxidative Properties under Different Heating Condition

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ABSTRACT : The aim of this study was to extract chicken leg bone, which is a by-product of industrial poultry processing, using different heating temperatures (80, 90 and 100°C) and durations (5, 10 and 15 min). The pH value, soluble protein content, peptide content and antioxidative properties, including superoxide anion scavenging ability, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability, reducing capacity and inhibitory activity of linoleic acid peroxidation, were measured. The results showed no significant differences ($p>0.05$) in pH value among all treatments. Decreased soluble protein content and peptide content were observed in chicken leg bone extract obtained under higher heating temperatures (90 or 100°C) and longer heating durations (10 or 15 min). In antioxidative properties, the extracts which were heated at 90 or 100°C for 15 min exhibited significantly higher superoxide anion scavenging ability, DPPH free radical scavenging ability, reducing capacity and inhibitory activity of linoleic acid peroxidation ($p<0.05$).
(**Key Words** : By-product, Antioxidative Activity, Chicken Leg Bone, Heating Extract)

INTRODUCTION

Chicken soup is seen as an excellent nutritional supplement according to traditional Chinese culture. It is rich in nitrogenous compounds such as free amino acids, peptides and low molecular weight proteins that are assumed to be absorbed easily and to have regular physiological activity in a human body (Geissler et al., 1989; Man et al., 2005). Meanwhile, chicken essence is a commercial chicken soup and is reported to exhibit several functional properties (Ikeda et al., 2001; Tsi et al., 2003). Matsumura et al. (2002) revealed its preventive effect on the development of hypertension and kidney disease after feeding stroke-prone spontaneously hypertensive rats with a chicken extract. Furthermore, Geissler et al. (1996) demonstrated that a daily supplement of chicken extract stimulated haemoglobin restoration in iron deficient rats. Meanwhile, chicken extract's contribution to recovery from fatigue caused by mental workload has been reported by Nagai et al. (1996).

Reactive oxygen species (ROS) and free radicals are known to be involved in several health disorders such as hypertension, cancer, atherosclerosis and aging (Helliwell and Gutteridge, 1990; Morrissey and O'Brien, 1998). It is assumed that functional foods with antioxidants help to prevent these diseases. Thus, the development of antioxidant has gained more interest, especially with respect to those developed from natural sources based on aspects of safety and nature. Certain heating extracts obtained from beef, pork and chicken meat were reported to exhibit inhibitory activity in lipid peroxidation according to recent reports (Chan et al., 1993; Gopalakrishnan et al., 1999; Maikhunthod and Intarapichet, 2005). Also, the study of Wu et al. (2005) indicated that high contents of free amino acids, low molecular weight peptides and dipeptides such as carnosine, anserine and taurine were found in chicken essence, which contributed to its antioxidative activity.

Given greater recent concerns with environmental pollution, utilizing bioresources effectively draws significant attention, and in particular the utilization of by-products from industrial meat processing, which usually contain large amounts of proteins. With proper treatment, they could be developed as high value products. For

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instance, whey protein, blood meal, and collagen can be obtained form animal by-products. Chicken leg bone is an industrial by-product from deboning processing, which contains high protein sources (about 23% in crude protein) such as meat (4-7% meat loss which occurs during processing deboned chicken legs), cartilage and bone marrow (Cheng et al., 2008). The present study tries to utilize them through heating treatment which develops them as an antioxidant. As such, chicken leg bone was extracted using different heating conditions and determined their pH value, soluble protein content, peptide content, antioxidative properties.

MATERIALS AND METHODS

Preparation of chicken leg bone extracts

The method referred to Gopalakrishnan et al. (1999) with slight modification. Chicken leg bones (broiler) were obtained from a poultry processing factory in Tai-Chung, Taiwan and cut into small pieces. 100 g of chicken leg bone were ground with 200 ml of water using a blender (Waring commercial, USA) and then heated under different temperatures (80, 90 and 100°C) and durations (5, 10 and 15 min). After cooling to ambient temperature, the chicken leg bone extracts were centrifuged at 10,000×g for 10 min, filtered through a filter paper (Advantec No.1) and stored at -80°C.

pH value measurement

The pH value of the heating extract was measured using a FET (field effect transistor) pH electrode (Model PY-P30; Sartorius, Goettingen, Germany) attached to a pH meter (Model PB-20; Sartorius).

Soluble protein content

The extracts were diluted properly in distilled water. The soluble protein content was determined according the method of Lowry (Peterson, 1979). Absorbance was measured at 750 nm using a spectrophotometer (U2000; Hitachi, Tokyo, Japan). Bovine serum albumin (BSA) was used as a standard.

Peptide content

Peptide content was measured according to Church et al. (1983). The OPA (*o*-phthaldialdehyde) reagent was prepared daily by dissolving 40 mg OPA in 1 ml of methanol, and mixed with 25 ml of 100 mM sodium tetraborate (borax) buffer, 2.5 ml of 20% SDS and 100 μl of β-mercaptoethanol. The volume was adjusted to 50 ml. Twenty micro liters of heating extract was added into 1.5 ml of OPA reagent, and incubated for 2 min at room temperature. The absorbance was read at 340 nm by spectrophotometer. Gly-Leu was used as the standard.

Measurement of antioxidant activity

1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability : The analysis of DPPH scavenging activity was performed according to the method of Shimada et al. (1992). Briefly, chicken leg bone extracts (0.8 ml) was added into 0.2 ml of 1 mM DPPH which was prepared with ethanol. After incubated at room temperature in a dark room for 30 min, the absorbance of the mixtures was spectrophotometrically measured at 517 nm. BHT (1.0 mg/ml) was measured as the positive treatment, and distilled water was used as a control. The percentage of DPPH radical-scavenging activity was expressed as: (1-(absorbance of samples at 517 nm)/(absorbance of the control group at 517 nm))×100%.

Superoxide anion scavenging ability : The assay refers to the method of Robak and Gryglewski (1988), where 120 μM of phenazine methosulfate (PMS), 936 μM of β-nicotinamide adenine dinucleotide (NADH) and 300 μM of nitro blue tetrazolium (NBT) were prepared with 0.1 M phosphate buffer (pH 7.4), respectively. 0.3 ml of PMS, NADH, sample, and NBT were added in a tube in turn and mixed well. After being incubated at 25°C for 5 min, the absorbance was read at 560 nm. The superoxidase dismutase (SOD) (S2515, Sigma) was presented as a positive control, while distilled water was used as a control. The scavenging percentage of superoxide anion was calculated as previous description.

Determination of reducing capacity : The reducing capacity was modified according to the method of Yen and Chen (1995). Sample solutions (1 ml) were added to 0.5 ml of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixed solutions were incubated in a water bath at 50°C for 20 min. 0.5 ml of 10% trichloroacetic acid (TCA) was mixed with the sample solutions to stop the reaction. The mixtures were then centrifuged at 3,000×*g* for 10 min. The supernatants were added into deionized water and 0.1% iron (III) chloride hexahydrate at a ratio 1:1:1. The reaction was allowed to proceed for 10 min. The absorbance was measured at 700 nm by a spectrophotometer. The higher absorbance was an indication of a greater reducing capacity (Yen and Chen, 1995).

Inhibition of linoleic acid peroxidation : The method achieved refers to Shimoni et al. (1998) with minor modifications. Samples (100 μl) were added to mixtures containing 2 ml linoleic acid emulsion: 0.28% Tween-20 and 0.28% linoleic acid was mixed in 0.2 M phosphate buffer (pH 6.6). The mixtures were then incubated in the dark at 37°C. The samples (100 μl) were withdrawn into 7 ml of 80% ethanol at 15 h. The linoleic acid oxidation was assayed by absorption of conjugated dienes at 234 nm. Butyl hydroxytoluene (BHT) was used as a positive group (1.0 mg/ml), and distilled water was used as a control.

Table 1. The pH value, soluble protein content and peptide content of heating extracts from chicken leg bone using various heating conditions

	80°C			90°C			100°C			T	D	T×D
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min			
pH	6.7±0.25	6.65±0.27	6.75±0.11	6.77±0.34	6.75±0.12	6.78±0.22	6.64±0.11	6.68±0.15	6.75±0.17	ns	ns	ns
Soluble protein content (mg/ml)	7.30±1.07^{a}	1.86±0.34^{b}	2.74±1.6^{b}	6.15±1.68^{a}	2.33±0.58^{b}	2.27±0.15^{b}	6.22±0.58^{a}	2.03±0.12^{b}	2.11±0.28^{b}	***	ns	ns
Peptide content (mg/ml)	12.65±1.0^{bc}	13.2±1.8^{ab}	10.8±0.8^{cd}	13.48±1.4^{ab}	10.36±0.2^{d}	10.87±0.8^{cd}	14.67±0.8^{a}	12.68±1.0^{bc}	12.25±1.4^{bcd}	*	**	ns

Data are expressed as mean±standard deviation.

$^{a-d}$ Data within the same row with different superscripts are significantly different ($p<0.05$). (n = 3).

T = Heating temperature effect; D = Heating duration effect; T×D = Heating temperature×duration interaction.

ns: $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Antioxidant activity (%) = (1-(absorbance of samples at 234 nm)/(absorbance of the control group at 234 nm))×100%.

Statistical analysis

The statistical analyses were performed using the SAS System for Windows V8 (SAS, 2000). Differences among mean values were compared using the one-way analysis of variance (ANOVA) and Tukey's test. Effect of heating time (three levels: 5, 10 and 15 min), temperature (three levels: 80, 90 and 100°C) and interaction between these two factors were analyzed by a two-way ANOVA. Significance was reported at the $p<0.05$ level.

RESULTS AND DISCUSSION

Chicken leg bone extract

There were no significant differences ($p>0.05$) in the pH values of heating extracts obtained form various heating conditions (Table 1). The pH values were not influenced by different heating temperatures and durations. Significantly higher soluble protein contents were found in an extract when it was heated at 80°C for 5 min ($p<0.05$) (Table 1). Under the same heating temperature (80°C), the soluble protein content of extract decreased form 7 mg/ml to 2 mg/ml when the duration was increased from 5 to 10 min. However, no other decrease in soluble protein content was observed when longer duration was carried out (15 min). Similar results were found in extracts under 90 and 100°C heating temperatures. Generally, protein denaturation was resulted form protein unfolding and aggregation during heating procedure. In particular, folding global protein usually denatured when heated, which led to a decrease of soluble protein content (Belitz and Grosch, 1999). Gopalakrishnan et al. (1999) had discussed the antioxidant activity of mechanically separated pork extracts and found that the soluble protein content decreased during heating procedure. Moreover, lower soluble protein content was obtained in extract under higher heating temperatures. The report of Osborn et al. (2003) indicated that myoglobin would be denatured and deposited after heating, and dramatic denaturation was discovered in the initial stage of heating, followed by little changes in protein denaturation, which were similar to current observations. The peptide contents of chicken leg bone extract are shown in Table 3. The OPA reagent tended to react with the amino group of small peptides (below 6 kDa in molecular weight) or free amino acids. The extract with the condition of heating at 100°C for 5 min had the highest peptide content (14.7 mg/ml), while lower peptide content was observed when the heating time was extended. Also, other heating temperatures (80 and 90°C) showed the same trends. It is known that peptides are usually released from protein during food processing or enzymatic hydrolysis (Korhonen and Pihlanto, 2003). It was also reported that heating treatment contributed to an increase of peptide in beef (Spanier et al., 1990). Bauchart et al. (2006) who compared the changes of small peptide contents (<5 kDa) in beef between aged and cooked beef, which revealed that about double the peptide contents were increased after aging period, whereas several times of the peptide content were obtained in aging meat after cooked. Specifically, a significant increase was found in di-peptide such as carnosine and anserine, which suggested that heating treatment influenced the increase of peptide content in meat more than aging.

Free radical residue scavenging ability

The resulting superoxide scavenging ability was found in all treatments (Table 2). A lower scavenging ability of only about 25% was observed in extracts obtained from heating at 80°C for 5 to 15 min, whereas the extracts treated with heating at 90 or 100°C for 10 min had a significantly higher scavenging ability (48%, 52%)($p<0.05$). However, longer heating duration (>10 min) was not beneficial to an increase in superoxide anion scavenging ability in extracts. In other words, about 100% of the scavenging activity was increased when the heating temperature was raised from 80 to 90 and 100°C for more than 10 min. In DPPH scavenging ability (Table 3), the extracts heated at 80, 90 and 100°C for 5 min showed poor scavenging ability. Significant increases of activity were obtained in extracts when the heating duration was extended to 10 min ($p<0.05$) at different heating temperatures. Moreover, at the same

Table 2. Free radical residue scavenging ability of heat extracts from chicken leg bone using various heating conditions

	80°C			90°C			100°C			T	D	T×D
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min			
Scavenging effect on superoxide anion (%)	22.75±2.6^{bc}	21.94±9.0^{bc}	25.90±5.5^{b}	14.57±4.5^{c}	48.20±3.5^{a}	52.40±3.6^{a}	26.84±4.31^{b}	54.60±3.4^{a}	54.38±4.5^{a}	***	***	***
Scavenging effect on DPPH (%)	ND	45.38±0.8^{b}	53.64±1.9^{a}	ND	34.65±2.6^{c}	46.41±2.7^{b}	12.14±3.2^{d}	34.39±1.3^{c}	54.78±5.0^{a}	***	**	***

Data are expressed as mean±standard deviation. ND = Not determined.

The scavenging effect on superoxide anion of SOD (5,000 U/ml) was 82.86%, presented as positive control.

The scavenging effect on DPPH of BHT (1 mg/ml) was 82.02%, presented as positive control.

$^{a-d}$ Data within the same row with different superscripts are significantly different ($p<0.05$). (n = 3).

T = heating temperature effect; D = heating duration effect; T×D = heating temperature×duration interaction.

** $p<0.01$; *** $p<0.001$.

heating temperature, the extracts showed the significantly higher activity at a longer heating duration (15 min) ($p<0.05$). It has been stated that some antioxidants with free radical scavenging ability could be derived from food protein after heating (Taylor and Richardson, 1980; Hofmann et al., 1999). Tong et al. (2000) indicated that heating treatment led to protein unfolding, which contributed to provide protons for scavenging free radical residues as a consequence of exposing the internal function group of protein. In addition, heating treatment benefited to the release of small peptides from food protein. Chen et al. (2003) revealed that milk possessed antioxidative activity following heating treatment, which was related to low molecular protein fragments generated from milk protein during heating treatment. Wu et al. (2003) stated that chicken essence contained large amounts of free amino acids, di-peptides and low molecular peptides which contributed to provide protons for scavenging free radical residues. The observations of this study showed that higher free radical scavenging ability in extracts obtained from higher heating temperature (90 and 100°C) for longer heating duration (>10 min) were assumed to relate to small peptides resulted from heating treatment.

Reducing capacity

Reducing capacity of chicken leg bone heating extract is shown in Table 3. Poor reducing capacity was observed in extracts treated with heating at 80°C from 5 to 15 min. The extract showed a significant increase in activity ($p<0.05$) when the higher heating temperature (90 and 100°C) was executed from 5 to 15 min. The highest reducing capacity was obtained in extracts treated with heat at 90 and 100°C for 15 min. The research of Wu et al. (2005) exhibited that the free radical residue scavenging ability and reducing capacity were both raised at the same time when the concentration of chicken essence was increased, which seemed to positively relate free radical residue scavenging ability with reducing capacity. Similar observations were found in the current study, where high heating temperatures (90 and 100°C) and longer heating duration (15 min) treatments possessed strong free radical residue scavenging ability and reducing capacity.

Inhibition of linoleic acid peroxidation

In the antioxidant assay, inhibitory activities of linoleic acid peroxidation were discovered in all chicken leg bone hearting extracts (Table 3). Among all treatments, extracts treated with heat at 90°C for 15 min or 100°C for 10 and 15 min exhibited the highest inhibitory activity (about 33%). The results of current study suggest that there was a positive correlation among free radical residue scavenging ability, reducing capacity and inhibitory activity of linoleic acid peroxidantion. Yu et al. (2002) stated that the antioxidant usually possessed more than one kind of

Table 3. Reducing capacity and inhibitory activity of linoleic acid peroxidation of heat extracts from chicken leg bone using various heating conditions

	80°C			90°C			100°C			T	D	T×D
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min			
Reducing capacity (Absorbance at 700 nm)	0.30±0.04^{cd}	0.35±0.03^{cd}	0.26±0.05^{d}	0.47±0.04^{a}	0.48±0.11^{a}	0.56±0.02^{a}	0.36±0.06^{bc}	0.46±0.04^{ab}	0.56±0.06^{a}	***	***	***
Inhibitory activity of linoleic acid peroxidation (%)	22.17±0.9^{c}	24.11±1.1^{c}	28.74±4.9^{b}	21.21±1.4^{c}	23.00±0.3^{c}	32.51±1.9^{b}	22.71±0.8^{c}	30.00±3.8^{ab}	33.14±0.9^{a}	***	***	ns

Data are expressed as mean±standard deviation.

The reducing capacity of BHT (1 mg/ml) was 1.42, presented as positive control.

The inhibitory activity of linoleic acid peroxidation of BHT (1 mg/ml) was 69.8%, presented as positive control.

$^{a-d}$ Data within the same row with different superscripts are significantly different ($p<0.05$). (n = 3).

T = Heating temperature effect; D = Heating duration effect; T×D = heating temperature×duration interaction effect.

ns: $p>0.05$; *** $p<0.001$.

antioxidative properties such as free radical residue scavenging activity, reducing capacity, and iron or copper ion chelating ability for inhibition of linoleic acid peroxidation, which agreed with current observations. As a consequence of this study, it was discovered that the extracts treated with heat at 90 and 100°C for 15 min did not have a significant higher soluble protein content and peptide content in comparison with other treatments. It was suggested that their higher activity on superoxide anion and DPPH free radical scavenging activity, reducing capacity and inhibition of lipid peroxidation seem to be related with the release of specific antioxidants such as low molecular weight protein fragment, peptide and free amino acid derived form chicken leg bone protein during heating treatment rather than the increase of peptide content. Intarapichet and Maikhunthod (2005), who reported on the comparison of antioxidative activity of heating extract obtained from different strains of chicken breast and thigh meat, indicated that the content of di-peptide, carnosine was known as a potent antioxidant in breast meat heating extract and was 5-fold higher than thigh meat heating extract; however, no significant difference on antioxidative activity was found, which suggested that the antioxidative activity of thigh meat heating extract must be contributed by specific antioxidants or synergistic antioxidants such as low molecular peptide, phosphate or free amino acid. Additionally, Chan et al. (1993) stated that low molecular antioxidant could be released from meat protein during heating treatment.

The chicken leg bone is an industrial by-product during processing, which contains high protein sources such as meat, cartilage and bone marrow. The findings of this study contribute to the utilization of industrial by-products. Nevertheless, further study is necessary to analyze and identify the related antioxidants in chicken leg bone heating extract.

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