

Optimizing Carnosine Containing Extract Preparation from Chicken Breast for Anti-glycating agents

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Abstract

Optimization of carnosine and anserine extraction from chicken breast was performed using response surface methodology (RSM) to obtain the maximized physiological activities for anti-glycation and anti-oxidation. The optimum extraction conditions were water extraction for 1.6 h in the case of the 20-wk laying hen muscle and water extraction for 2.12 h in the case of 90-wk laying hen muscle. Higher carnosine and anserine contents were measured in the 20-wk laying hen muscle, along with higher physiological activities, which increased in direct proportion with the dipeptide contents. The extracts prepared from the 20-wk laying hen under optimum conditions showed 57% inhibition of advanced glycated end-product formation, 64% inhibition of lipid peroxidation, and 61% of DPPH radical scavenging effects. On the other hand, 52% inhibition of AGE formation, 62% inhibition of lipid peroxidation, and 53% of DPPH radical scavenging effect were demonstrated within the 90-wk laying hen. In addition, the ratio of carnosine was a key indicator for the physiological activities of the extracts.

Key words: carnosine, anserine, extraction, optimization, chicken

Introduction

Chicken has been perceived as body-warming food in traditional medicine, with carnosine and anserine being the key components for its bioactivity (Li *et al.*, 2012). Carnosine (β -Ala-His) and anserine (β -Ala-His(3-Me)) are imidazole-containing dipeptides of β -alanine and L-histidine, found in the skeletal muscle, heart, and central nervous system (Mora *et al.*, 2008; Tian *et al.*, 2007) of vertebrates (chicken, pork, cattle, etc). Carnosine and anserine play important roles in physiological functions such as potent intracellular pH buffering, anti-oxidation, and anti-glycation (Decker *et al.*, 2000; Dukic-Stefanovic *et al.*, 2001; Intarapichet and Maikhunthod, 2005; Lee *et al.*, 1998). These peptides have gained considerable attention as anti-oxidant food supplements for eye disorders (Boldyrev *et al.*, 2004; Chan and Decker, 1994; Yanai *et al.*, 2007). Since the formation of advanced glycated end-products (AGEs) and lipid peroxidation are closely related to degenerative diseases such as diabetes, aging, Alzheimer's di-

sease, and cancer (Ahmed, 2005; Gunter *et al.*, 2007, Johnson and Lund, 2007), carnosine and anserine are receiving much attention as natural antioxidants (Hipkiss and Brownson, 2000; Kohen *et al.*, 1988; Nabetani *et al.*, 2012).

White muscle is known to contain a higher amount of carnosine than does red muscle tissue (Boldyrev *et al.*, 2004; Brown, 1981; Hipkiss, 2005; Plowman and Close, 1988). Chicken is a representative animal with higher carnosine content among the white muscle tissue animals (Boldyrev and Severin, 1994; Kim *et al.*, 2012). A few efforts have been made by other researchers to identify the optimum extraction conditions of the dipeptides. For example, Maikhunthod and Intarapichet (2005) attempted heat and ultrafiltration processes with acidic extraction, and Nabetani *et al.* (2012) applied membrane separation to prepare a functional peptide extract containing carnosine and anserine from chicken. Most of the reported carnosine extract preparation methods involve acid extraction, followed by de-proteinization using ethanol treatment, which could alter the actual carnosine concentration. In addition, no systemic investigation has been attempted for optimizing carnosine and anserine extraction while preserving the maximum physiological activities. Herein, we have optimized the extraction process for peptide mixtures with the highest anti-oxidation and anti-gly-

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cation activity through response surface methodology (RSM) using multiple objective optimizations.

Materials and Methods

Chemicals and reagents

All the reagents used in this study were of the highest purity available. Acetonitrile, methanol, ethanol, bovine serum albumin (BSA), methylglyoxal, L-ascorbic acid, α, α -diphenyl- β -picrylhydrazyl (DPPH), hydrochloric acid (HCl) 35%, sodium chloride (NaCl), ferrous chloride, boric acid, *o*-phthaldialdehyde (OPA), L-carnosine, and L-anserine were purchased from Sigma Aldrich Co. (USA).

Experimental design and optimization of extracting conditions by statistical analysis

RSM, an empirical modeling technique, was used to estimate the relationships between a set of experimentally observed results. Central composite design (CCD) was used for predicting the optimum extraction conditions for carnosine and anserine from chicken breast using Design-Expert (version 8.0, State-Ease Inc., USA). The details of CCD for a given range of parameters based on coded and actual levels are shown in Table 1, with two independent variables, extraction time and ethanol concentration. Low, middle, and high levels of each variable were designated as -1, 0, and +1, respectively. Overall, thirteen combinations were chosen in random order according to the CCD configuration for four dependent variables: (i) concentrations of carnosine and anserine, (ii) anti-glycation activity, (iii) DPPH radical scavenging, and (iv) inhibition of lipid peroxidation. To obtain a reliable estimate of the optimum extracting conditions, all data were fitted to a modified Gompertz function using a non-linear regression model. The results of the thirteen combinations were fitted to a quadratic polynomial model. Statistical testing of the model was performed at $p < 0.05$ by analysis of variance (ANOVA) to test the significance and adequacy of the model. Finally, simultaneous optimization for the ex-

traction was performed using Design-Expert (version 8.0, State-Ease Inc., USA) to identify the conditions for the highest physiological activities such as anti-glycation activity, DPPH radical scavenging, and inhibition of lipid peroxidation.

Extraction of carnosine and anserine from chicken muscle

Chicken samples (20 and 90 wk laying hen) were received from Department of Animal Science and Technology, Chung-Ang University, Korea. At the end of each week, the chickens were sacrificed by cervical dislocation, a method approved by Animal Care Committee of Chung-Ang University. Carnosine and anserine were extracted according to the method proposed by Auh *et al.* (2010), with a slight modification. Muscle samples from chicken breast were isolated, frozen at -80°C , and freeze-dried for 72 h. The dried muscle samples were finely ground, and 0.5 g of the ground muscle was suspended in 12 mL of solvent (0-70% of ethanol) and incubated at 25°C with moderate shaking for 1 to 3 h. Subsequently, the mixture was centrifuged at 14,000 g for 30 min, and then, 300 mL of the supernatant was mixed with 900 mL of cold ethanol and kept for 30 min at 4°C . The supernatant obtained after centrifugation was filtered through a membrane syringe filter (MCE type, 0.45 μm , Advantec, MFS Inc., Japan) and freeze dried. Lyophilized samples were stored at -40°C until further analysis.

HPLC analysis

Carnosine and anserine concentrations were determined in accordance with the methods reported by Aristoy *et al.* (2004) and Auh *et al.* (2010). An HPLC system (Gilson Medical Electronics, USA) equipped with a fluorescence detector (Agilent, USA) and a Spherisorb SCX column (4.6 \times 250 mm, Waters Co., USA) was used for the analysis. 10 mL of the sample solution (10%, w/v) was mixed with 100 mL of OPA solution and placed for 2 min at room temperature in the dark. Subsequently, 20 mL of the derivatized sample was injected into the HPLC system. The mobile phase was composed of 80% of phase A (20% acetonitrile in 6.6 mM HCl) and 20% of phase B (phase A containing 0.8 M NaCl), and the elution was effected at 0.6 mL/min for 30 min at 40°C . Fluorescence detection was performed on Spectrofluorimeter RF-1500 (Shimadzu, Japan) using 338 and 445 nm as the excitation and emission wavelengths, respectively (Plowman and Close, 1988).

Table 1. Factors for the central composite design for response surface methodology (RSM)

Factors	Actual			Coded		
	Low	Middle	High	Low	Middle	High
Time (h)	1	3	5	-1	0	1
Ethanol (%)	0	35	70	-1	0	1

Anti-glycation activity (AGE inhibition)

The anti-glycation activity of carnosine-containing extract from chicken breast (20- and 90-wk laying hen) was measured by % inhibition of AGE formation, according to the method reported by Li *et al.* (2008) but with a slight modification. The extract was mixed with BSA (50 mg/mL) and methylglyoxal (3 mM) in 50 mM sodium phosphate buffer (pH 7.4) and incubated at 25°C for 24 h under dark conditions. The formation of AGE was quantified by measuring the fluorescence intensity (excitation at 350 nm, emission at 450 nm). All the measurements were conducted in triplicate.

Anti-oxidation activity

The anti-oxidation activity of carnosine-containing extract from chicken breast (20- and 90-wk laying hen) on DPPH radicals was measured by Wu *et al.* (2002)'s method, with a slight modification. The samples were mixed with 0.2 mM DPPH (in 95% methanol) and kept for 30 min at room temperature; then, the absorbance at 517 nm was measured and the anti-oxidation activity was calculated. All the measurements were performed in triplicate.

Inhibition of lipid peroxidation (%)

The inhibition of lipid peroxidation of carnosine-containing extract from chicken breast was determined by a slight modification of the ferric thiocyanate method (Shimada *et al.*, 1992). To accelerate the oxidation, 190 mL of the extracted sample, 400 mL of 50 mM potassium phos-

phate buffer (pH 7.0), 200 mL of 2.5% linoleic acid, 200 mL of ethanol, and 10 mL of 10% Tween 20 were mixed and incubated for 24 h at 40°C. Various concentrations of ascorbic acid (0.001 to 10 mM) were used as the positive control. To 15 mL of the reaction mixture were added 1455 mL of 75% ethanol, 15 mL of 30% ammonium thiocyanate, and 15 mL of 20 mM ferrous chloride solution in 3.5% HCl. After the mixture was stirred for 3 min, the peroxide values were determined by monitoring the absorbance at 500 nm, and subsequently, the % inhibition of lipid peroxidation was calculated.

Results and Discussion

Optimizing extracting conditions and physiological activities

The extraction conditions for carnosine-containing mixtures with physiological activities such as anti-glycation activity, DPPH radical scavenging, and inhibition of lipid peroxidation was optimized to achieve the highest activities. Thirteen combinations of CCD were used with varying ethanol concentrations and lengths of time, and the resulting responses of four different factors were determined (Tables 2 and 3). Subsequently, multiple objective optimization was applied to identify the optimum conditions with the highest amounts of carnosine and anserine, along with maximized levels of physiological activities. Carnosine and anserine could be easily extracted within 2 h at low ethanol concentrations of less than 35%.

Table 2. Experimental design used in response surface analysis for optimization of extraction conditions from 20-wk chicken breast

Run	Factor 1 Ethanol (%)	Factor 2 Time (h)	Response 1 Carnosine+ anserine (mg/kg)	Response 2 AGE inhibition (%)	Response 3 DPPH radical scavenging (%)	Response 4 Inhibition of lipid peroxidation (%)
1	35.0	3.0	3733.6	32.8	48.6	64.1
2	70.0	3.0	2645.3	15.4	31.7	56.6
3	35.0	5.0	3249.8	16.8	46.1	56.4
4	35.0	3.0	3733.6	32.8	48.6	64.1
5	0.0	5.0	3093.8	34.2	51.3	62.3
6	70.0	1.0	3043.1	22.0	34.3	57.1
7	35.0	3.0	3733.6	32.8	48.6	64.1
8	35.0	3.0	3733.6	32.8	48.6	64.1
9	35.0	1.0	3454.0	26.3	48.4	61.9
10	70.0	5.0	2581.2	15.7	30.6	55.2
11	0.0	1.0	4080.3	49.0	51.9	62.9
12	0.0	3.0	3707.9	53.4	42.9	63.5
13	35.0	3.0	3733.6	32.8	48.6	64.1

Table 3. Experimental design used in response surface analysis for optimization of extraction conditions from 90-wk chicken breast

Run	Factor 1 Ethanol (%)	Factor 2 Time (h)	Response 1 Carnosine+ anserine (mg/kg)	Response 2 AGE inhibition (%)	Response 3 DPPH radical scavenging (%)	Response 4 Inhibition of lipid peroxidation (%)
1	35.0	5.0	2581.9	19.9	42.3	55.8
2	0.0	3.0	3469.1	52.4	52.9	68.5
3	0.0	5.0	2866.9	26.7	43.1	58.3
4	35.0	3.0	3168.3	29.6	43.4	59.8
5	70.0	5.0	2593.9	9.9	25.3	48.9
6	35.0	3.0	3168.3	29.6	43.4	59.8
7	35.0	3.0	3168.3	29.6	43.4	59.8
8	35.0	1.0	3174.1	44.0	43.9	58.7
9	0.0	1.0	2940.2	49.3	45.9	58.4
10	35.0	3.0	3168.3	29.6	43.4	59.8
11	35.0	3.0	3168.3	29.6	43.4	59.8
12	70.0	3.0	2629.8	13.7	23.8	50.2
13	70.0	1.0	2754.9	13.3	16.5	51.5

The AGE inhibition activity of the extract was sensitive to ethanol concentration, and higher activity was observed when the extraction was performed using water within 3 h. However, the DPPH radical scavenging activity was not significantly affected by the extraction time, and higher activity was observed with lower ethanol concentrations. Inhibition of lipid peroxidation was the highest when lower concentrations of ethanol were used with 3 h of extraction time. Further, the response of each factor followed the quadratic polynomial models as expected, thus, multiple objective optimization was clearly achieved, as envisioned. Fig. 1 clearly shows the successfully identified optimum conditions for carnosine and anserine

extraction from 20- and 90-wk old chicken breast. The optimum conditions were identified as water extraction for 1.60 h and 2.12 h, in 20-wk and 90-wk laying hen, respectively. Although carnosine and anserine are highly water soluble dipeptides, they can be easily isolated with water extraction within 2 h from chicken muscle. Table 4 shows the comparison of the estimated values under the optimum conditions with the experimental values to confirm the reliability of the method. In general, most of the responses under the optimum conditions were higher in the 20-wk laying hen than in the 90-wk samples, in accordance with the previous reports (Intarapichet and Maikhunthod, 2005; Kim *et al.*, 2012), which stated that the

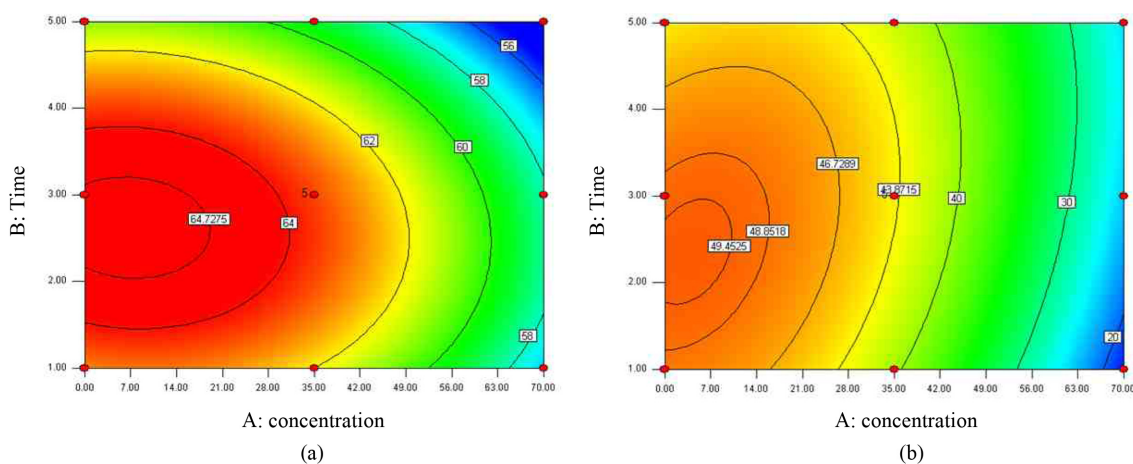


Fig. 1. Contour plots for simultaneous optimization of chicken breast extract with the highest physiological activities. A, 20-wk laying hen (25°C); B, 90-wk laying hen (25°C).

Table 4. Comparison of estimated and experimental values under the optimized extraction conditions

		Time (h)	Ethanol (%)	Carnosine and anserine concentration (mg/kg)	AGE inhibition (%)	DPPH radical scavenging effect (%)	Inhibition of lipid peroxidation (%)
Laying hen (20 wk)	Estimated	1.60	0.00	3944.53±140.42 ^b	51.97±4.12 ^b	48.69±1.20 ^a	64.12±1.39 ^c
	Experimental	1.60	0.00	4080.35±113.00 ^b	57.19±0.44 ^c	61.16±0.41 ^b	64.77±1.50 ^c
Laying hen (90 wk)	Estimated	2.12	0.00	3220.98±120.87 ^a	47.94±1.08 ^a	47.60±5.33 ^a	56.69±0.97 ^a
	Experimental	2.12	0.00	3469.09±25.82 ^a	52.39±3.05 ^b	52.86±0.43 ^a	62.13±0.39 ^b

carnosine and anserine concentrations would gradually decrease with aging in chicken muscle. The carnosine and anserine contents were 3944.53 mg/kg in the 20-wk sample and 3220.98 mg/kg in the 90-wk muscle sample. AGE inhibition activity was significantly higher in the 20-wk laying hen extract, which reflected the higher amounts of carnosine and anserine in younger muscles; the anti-oxidation activity, too, was also higher in the 20-wk muscle extract than in the 90-wk samples. Significantly, in the extract obtained from the 20-wk laying hen muscle, the experimental values of carnosine and anserine content were within the range of estimated values, while the AGE inhibition and DPPH radical scavenging activity were slightly higher than the expected levels. For the 90-wk laying hen muscle, although the estimated carnosine and anserine contents were comparable with the experimental values, inhibition of AGE formation and lipid peroxidation were slightly higher in the experimental measurements.

Effect of carnosine content on physiological activities

We successfully established the optimum conditions for the isolation of carnosine- and anserine-containing extracts and simultaneous maximization of three different physiological activities; further, we compared the relative contribution of carnosine and anserine to the activities (Fig. 2). Physiological activities of the extract were primarily affected by the amount of carnosine and anserine, and the ratio of carnosine seemed to be more critical on their activities. Carnosine content in 20 wk muscle sample (700.80 mg/kg) was significantly reduced in 90 wk muscle (537.60 mg/kg), thus AGE inhibition and anti-oxidation activities decreased in proportion to the carnosine ratio in the extract. Relative amount of carnosine was significantly decreased by aging with a loss in AGE inhibition and anti-oxidation activity. In anti-oxidation effect, DPPH radical scavenging activity was more influenced by a reduction of carnosine. Although carnosine and anserine are both

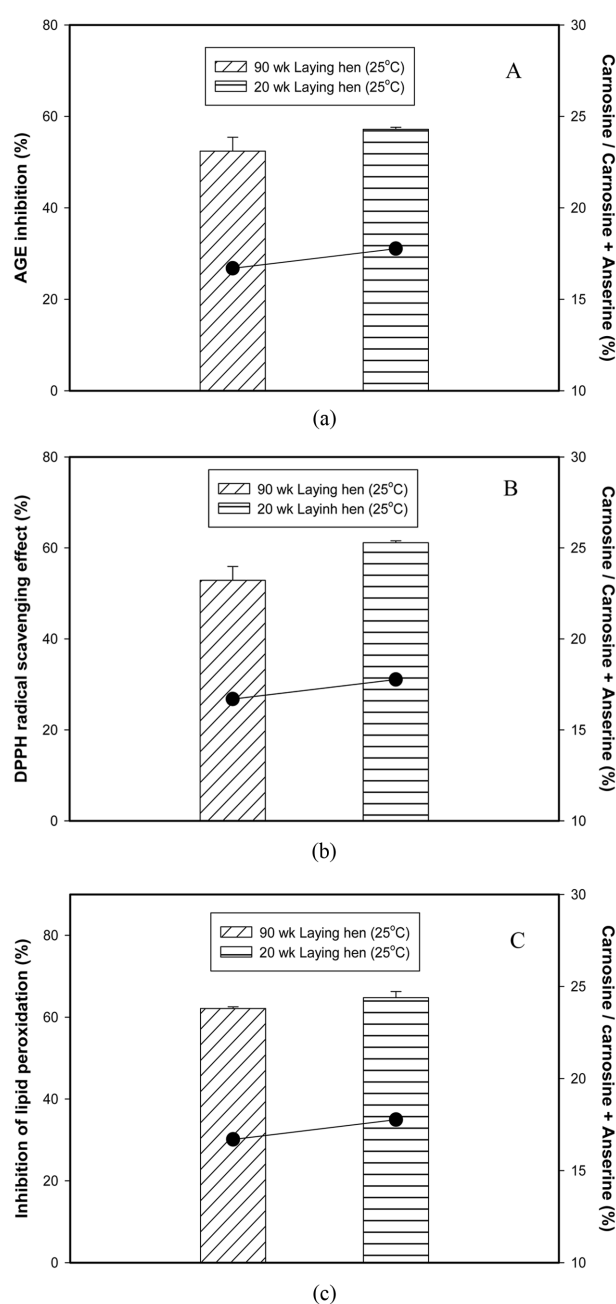


Fig. 2. Comparison of physiological activities of chicken extracts obtained under optimized conditions. A, AGE inhibition; B, DPPH radical scavenging effect; C, inhibition of lipid peroxidation.

effective antioxidants as extensively demonstrated by Wu *et al.* (2003), carnosine content would be more critical determinant for anti-oxidation. Thus, anti-glycation and anti-oxidation activity of carnosine and anserine extract from chicken muscle increased proportionally to the carnosine content as well as the relative amount of two peptides in the muscle.

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