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≪Research Note≫

Changes in Carnosine and its Related Constituents during Embryonic Development in the Breast Muscle of Layer and Broiler Chickens

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Developmental changes in the levels of carnosine (β-alanyl-L-histidine), its derivative anserine (β-alanyl-1-methyl-L-histidine), and their constituents including β-alanine, L-histidine and 1-methylhistidine in the pectoralis muscle were investigated. Comparisons were made between broiler and layer type chickens at various embryonic stages (E14 and E18) and hatch (P0). Carnosine and anserine levels increased toward hatch and carnosine levels were higher in broilers than in layers. The β-alanine level was highest at E18 in both types of embryos. L-Histidine and 1-methylhistidine levels were comparable during embryogenesis, but 1-methylhistidine greatly increased at P0. No significant differences in dipeptide constituents were found between broilers and layers. In conclusion, concentration of carnosine was higher in broilers than in layers.

Key words: anserine, carnosine, chicken, embryo, skeletal muscle

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Introduction

Carnosine (β-alanyl-L-histidine) and its derivative anserine (β-alanyl-1-methyl-L-histidine) are present in high levels in the muscle of mammalian and avian species while almost negligible in plants (Biffo *et al.,* 1990; Kohen *et al.,* 1988; O'Dowd *et al.,* 1988; Tamaki *et al.,* 1976). These compounds are found at especially high levels in the breast muscle of chickens (Aristoy and Toldra, 2004). These dipeptides have antioxidant activities (Kohen *et al.,* 1988) and buffering capacities (Abe, 2000). Carnosine is synthesized from β-alanine and L-histidine by carnosine synthase, and anserine is mainly formed from carnosine by a methylation as shown in Fig. 1 (Boldyrev and Severin, 1990). As an alternative pathway, Horinishi *et al.* (1978) indicated that the purified enzyme from chicken muscle could use methylated histidine to generate anserine by direct synthesis (Fig. 1). Both carnosine and anserine are hydrolyzed to constitutive amino acids by carnosinase (Pegoya *et al.,* 2000). β-Alanine is synthesized in the liver as the final metabolite of uracil and thymine degradation (Matthews and Traut, 1987).

According to Fisher *et al.* (1977), the muscle of developing chick embryos contained 410 nmoles/g anserine while carnosine was present at levels of less than 30 nmoles/g at 15 days of embryogenesis. Levels of both dipeptides increased closer to hatch, the anserine and carnosine levels rising to 1000 nmoles/g and 225 nmoles/g, respectively in muscle of Columbian X New Hampshire cockerels. It was confirmed that the level of carnosine increased throughout embryonic development beginning with its appearance on the 15th day in breast muscles of chicken embryos from White Leghorns up to the 21st postnatal day (Boldyrev *et al.,* 1988). Therefore, it appears that the levels of carnosine and anserine depend upon strain and/or type of the chicken.

The present study was conducted to compare the carnosine and anserine content in breast muscle of broiler and layer type chickens during embryonic development. In addition, the levels of various constituents such as β-alanine, L-histidine and 1-methylhistidine were determined.

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Materials and Methods

Sample Preparation

Fertilized eggs were obtained from Mori hatchery, Fukuoka, Japan (broiler: Chanky) and GHEN corpora-

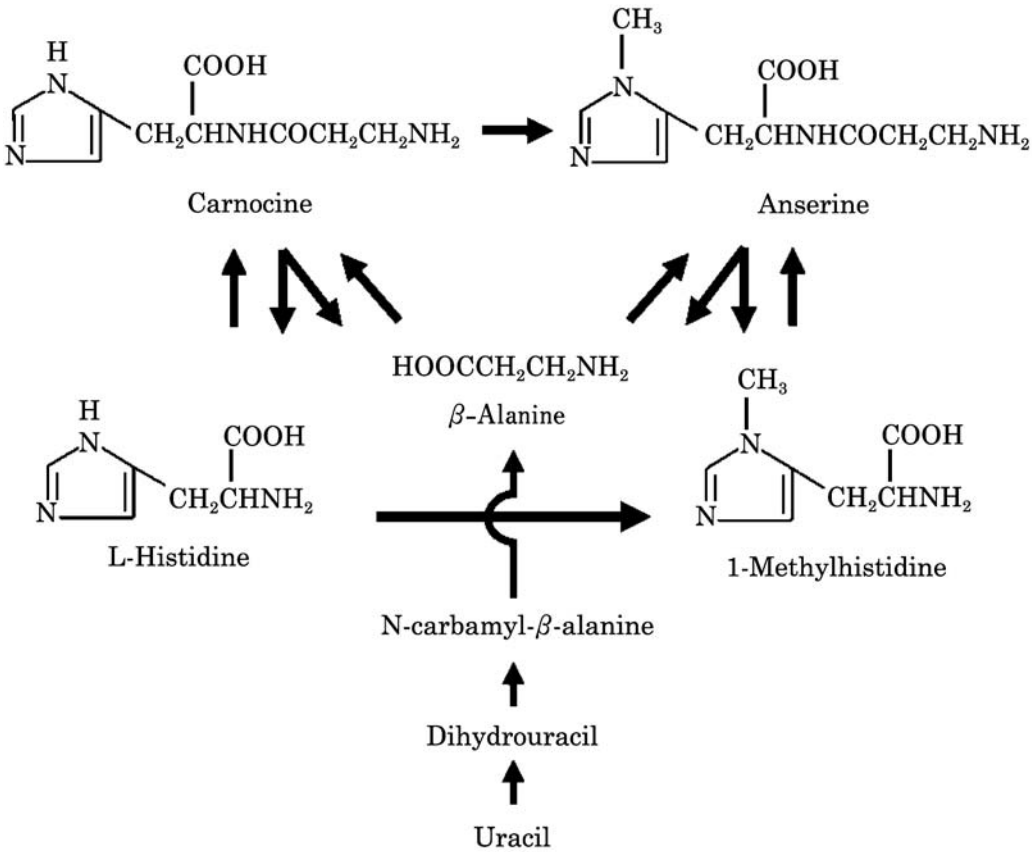

Fig. 1. **The synthesis and degradation of carnosine and anserine.**

tion Gifu, Japan (layer: Julia), and were incubated at 37.6℃ and a relative humidity of 58 to 68%. They were candled before incubation to remove chapped and broken eggs. Non-chapped and non-broken eggs were weighed individually. All experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and the guidance for Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Tissue

Samples were obtained at the following stages: embryonic day 14 (E14), E18 and within 2 h after hatching (P0). The pectoralis muscle was dissected rapidly, blotted, placed in liquid nitrogen, and stored at −80℃ until analysis.

Analysis of dipeptides and amino acids by High-Performance Liquid Chromatography (HPLC)

The pectoralis muscle was homogenized with distilled water, and then centrifuged at 10,000 g for 20 min. The supernatants were deproteinized by filtration through a 10,000 dalton molecular weight cut-off filter (Millipore, Bedford, MA, USA) via centrifugation at 10,000 g for 60 min. The samples (40 μl) were then completely dried under reduced pressure. Dried residues were dissolved in 10 μl of a 1 M sodium acetate-methanol-triethylamine (2: 2: 1) solution. Samples were re-dried, and dissolved in 20 μl of derivatization solution [methanol-water-triethylamine-phenylisothiocyanate (7: 1: 1: 1)]. A period of 20 min at room temperature was allowed for the reaction of phenylisothiocyanate with amino groups to produce phenylthiocarbamyl amino acid residues. The samples were dried again. The dried samples were dissolved in 100 μl of Pico-Tag Diluent (Waters, Milford, MA, USA). These diluted samples were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). The same method was applied to standard solutions prepared by diluting a commercially available L-amino acid solution (type AN II and type B; Wako, Osaka, Japan) with distilled water. These derivatized samples (skeletal muscle: 20 μl, and standard solution: 5 μl) were applied to a Waters HPLC system (Pico-Tag free amino acid analysis column (3.9×300 mm), Alliance 2690 separation module, 2487 dual-wavelength UV detector, and Millennium 32 chromatography manager; Waters, Milford, MA, USA). They were equilibrated with buffer A [70 mM sodium acetate (pH 6.45 with 10% acetic acid)-acetonitrile (975: 25)] and eluted with a linear gradient of buffer B [water-acetonitrile-methanol (40: 45: 15)] (0, 3, 6, 9, 40, and 100%) at a flow rate of 1 ml/min at 46℃. The absorbance at 254 nm was measured, and concentrations of free amino acids were determined. Triethylamine and sodium acetate trihydrate were

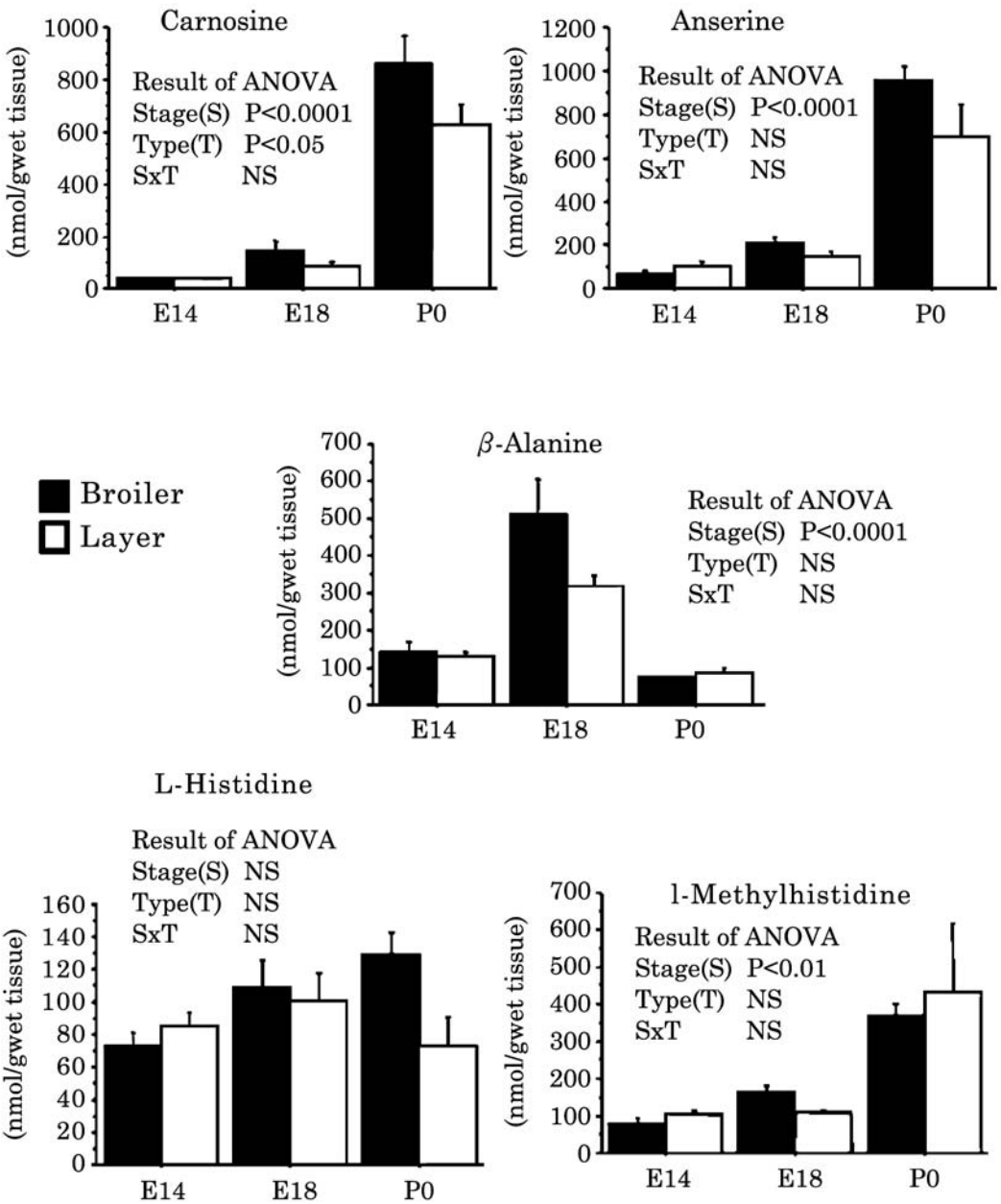

Fig. 2. **Changes in carnosine, anserine, β-alanine, L-histidine and 1-methylhisidine concentrations in the skeletal muscle between broiler and layer type chickens.** The values are means±S.E.M.

purchased from Wako (Osaka, Japan), while other drugs for which no manufacturer is noted were purchased from Sigma (St. Louis, MO, USA).

Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) with respect to types (broiler and layer) and developmental stages (E14, E18 and P0). Statements of significance were based on $P<0.05$. Data were expressed as means±S.E.M.

Results

Changes in the concentrations of carnosine, anserine, β-alanine, L-histidine and 1-methylhisidine in the pectoralis muscle of broiler and layer embryos are shown in Fig. 2. For carnosine, significant effects of stage ($P<0.0001$) and type ($P<0.05$) were detected. No significant ($P>0.05$) interaction was detected between stage and type. Carnosine was found at E14 and E18, but the levels were low. At hatch, the carnosine level greatly increased. Broilers had higher levels of carnosine compared with layers. Anserine followed a similar pattern as seen with carnosine. Anserine increased with age ($P<0.0001$), but there were no differences between broilers and layers ($P>0.05$). There was no significant ($P>0.05$) interaction between

stage and type. The levels of anserine were somewhat higher than those of carnosine. β-Alanine showed a different response, being highest at E18 ($P<0.0001$). No significant ($P>0.05$) effect of type was detected. The interaction between stage and type was nearly significant ($P=0.051$), since the value for broilers tended to be higher compared with layers at E18. No significant effects were detected in L-histidine (stage ($P>0.05$); type ($P>0.05$) nor stage x type ($P>0.05$)). 1-Methylhistidine increased with age ($P<0.01$), but there were no differences between broilers and layers ($P>0.05$). There was no significant ($P>0.05$) interaction between stage and type. The values for L-histidine and 1-methylhistidine were comparable at E14 and E18, but 1-methylhistidine was higher than L-histidine at P0.

Discussion

Histidine-dipeptides such as carnosine and anserine are beneficial components of chicken muscle, since these dipeptides have antioxidant activities (Kohen *et al.,* 1988) and buffering capacities (Abe, 2000). However, no clear data are available for changes in muscular contents of both dipeptides between meat- and layer-type chicken during embryogenesis. The present data demonstrated that carnosine contents were higher in broilers than in layers. During embryonic stages broilers grow faster than layers, suggesting that muscle growth is improved by genetic selection (Sato *et al.,* 2006a, b). The results reveal that selection for meat production, as has occurred in broilers, has increased carnosine production. Lower carnosine concentration in layers would be explained by antioxidant activities of carnosine (Kohen *et al.,* 1988), since oxygen consumption was higher in layers than in broilers (Sato *et al.,* 2006a). Carnosine may be highly used in layers.

Fisher *et al.* (1977) reported that the anserine content was much higher than carnosine at 15 days of embryogenesis. However, such a difference was not confirmed at E14 and E18 in either layers or broilers in the present study. The carnosine level is reported to be higher in the pectoralis than leg muscles (Davies *et al.,* 1978). The study reported by Fisher *et al.* (1977) used a mixture of the pectoralis and leg muscles. This may explain why the carnosine levels in the present study may be higher than those of Fisher *et al.* (1977). According to Boldyrev *et al.* (1988), both dipeptides similarly increased the observation period beginning with their appearance on the 15th day in breast muscles of chicken embryos. Our results corresponded closely with the results of Boldyrev *et al.* (1988). Furthermore, our data suggested that increases of carnosine during embryogenesis differed in chicken types, being higher in broilers. Anserine level showed a similar tendency with carnosine, but the difference did not reach significance between two types. Anserine as well as carnosine as antioxidants may be required for the embryo with growth.

β-Alanine is not a proteinogenic amino acid. Thus, production of β-alanine does not depend on protein degradation, but is dependent upon synthesis in the liver as the final metabolite of uracil and thymin degradation (Matthews and Traut, 1987). The level of β-alanine greatly decreased at P0. It is suggested that β-alanine as a constituent for carnosine and anserine was utilized for carnosine and anserine synthesis. Uptake of β-alanine and synthesis of carnosine and related dipeptides could be demonstrated in primary muscle cell cultures derived from embryonic chick pectoral muscle (Bakardjiev and Bauer, 1994; Bauer and Schulz, 1994). However, the present results indicate that the uptake and synthesis of carnosine was low during embryogenesis.

For anserine synthesis, two ideas are reported: one was synthesized through carnosine and another was directly synthesized with 1-methylhistidine and β-alanine. Horinishi *et al.* (1978) indicated that the purified enzyme from chicken muscle of 2 to 3 week old White Leghorn chickens could use methylated L-histidine to generate anserine by direct synthesis. On the other hand, Bauer and Schulz (1994) reported that the kinetics of dipeptide formation indicated that anserine is not formed directly by these cells, but as a secondary product via the methylation of carnosine in embryonic chick pectoral muscle. These facts suggest that to synthesize anserine directly from 1-methylhistidine and β-alanine the level of 1-methylhistidine may be important. The level of 1-methylhistidine may be age dependant. In the present study, the levels of L-histidine and 1-methylhistidine were comparable at E14 and E18, but the level of 1-methylhisitidine was much higher at P0. According to Horinishi *et al.* (1978), synthesis of carnosine was competitively inhibited by 1 -methylhistidine with a *Ki* value which was at least 40 times the *Km* value for L-histidine. Furthermore, the activity for synthase may be rapidly enhanced after hatch. However, lower level of L-histidine at P0 implies that L-histidine is easily incorporated into the dipeptide compared with 1-methylhistidine.

In conclusion, both carnosine and anserine were comparably synthesized in the pectoralis muscle of both broiler and layer embryos and were increased toward hatch. Carnosine concentration in the breast muscle was higher in broilers than in layers during embryogenesis.

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