EFFECT OF DIETARY METHIONINE ON METABOLISM OF CARNOSINE AND ANSERINE IN MUSCLES OF BROILER CHICKEN

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Abstract – Carnosine (Car; β-alanyl-L-histidine) and anserine (Ans; β-alanyl-1-methyl-L-histidine) are dipeptides mainly found in skeletal muscle and brain of vertebrates, and particularly high levels are found in chicken pectoral muscles. These peptides possess lots of functions such as antioxidant activity. Methionine (Met), one of the essential amino acid, plays a part in Car methylation into Ans as a methyl group donor. In this study, we examined effects of dietary methionine in different levels on carnosine and anserine levels in broiler muscle. The 14-days-old female Chunky strain broilers were given feeds containing three different levels of methionine; 75% (Low-Met), 100% (Control) and 200% (High-Met) of Met requirement according to NRC (1994). Chicks were fed experimental diets for 10 days. Free amino acids, Car and Ans were measured by HPLC. mRNA expression of two enzyme synthesizing IDP, Carnosine N-methyltransferase (CNMT) and Carnosine synthase (CS) of muscle, were measured by Real-time PCR. As a result, in High-Met group, Ans was increased significantly, while CNMT and CS expressions were decreased oppositely. In Low-Met group, no change was observed. From our data obtained suggests that the mechanism to enhance Ans levels in High-Met group would be direct synthesis from β-Ala and 1M-His, not methylation of Car.

Key Words – feed, imidazole dipeptide, methionine metabolism.

I. INTRODUCTION

Carnosine (β-alanyl-L-histidine: Car) and anserine (β-alanyl-1-methyl-L-histidine: Ans) are naturally occurring dipeptides which are present in the skeletal muscles and the brain of many vertebrates, particularly their high concentrations were observed in chicken pectoral muscle and brain (Bonfanti *et al.* 1999 [1]; Aristoy & Toldra 2004 [2]). These dipeptides sometimes call Imidazole Dipeptides (IDP). It has been reported that IDP would serve as an antioxidant agents (Kohen *et al.* 1988 [3]), proton buffering constituents (Abe 2000 [4]), and hypoglycemic effect (Yamano *et al.* 2001 [5]; Kubomura *et al.* 2010 [6]). It can be proposed that a chicken meat with these dipeptides would be beneficial and contribute for human health owing to their various functions.

Car is synthesized from histidine (His) and β-alanine (β-Ala) by carnosine synthase (CS; Drozak *et al*. 2010 [7]). Ans is considered to have two different synthesis pathways in chicken; firstly, methylation of Car to Ans by carnosine-N-methyltransferase (CNMT; McManus 1962 [8]; Bauer & Schulz 1994 [9]; Drozak *et al*., [10]), and secondly, directly synthesized from 1-methylhistidine (1M-His) and β-Ala due to the broad specificity of carnosine synthase (Horinishi *et al*. 1978 [11]; Drozak *et al*. 2010 [7]).

Several studies investigating the effect of dietary His or β-Ala, the constituent amino acids, on Car and Ans in muscles have been conducted in Mice (Takami *et al*. 1977 [12]; Quinn and Fisher 1977 [13]), Horses (Dunnett and Harris 1999 [14]) and broiler chickens (Tomonaga *et al*. 2006 [15], 2012 [16]; Kai *et al*. 2014 [17]). Nevertheless, the nutritional effects of non-constituent amino acids on IDP in chicken have not been well established.

We now focus on one of the essential amino acid, methionine (Met). As S‑adenosyl methionine, a metabolite of Met, is needed for Car methylation into Ans as methyl group donor (McManus 1962 [8]), we suspected that dietary Met levels in feed would affect to IDP levels, especially Ans levels, in muscles. Here, we report the effect of varying dietary Met levels on Car and Ans contents and gene expressions of two IDP synthesis enzymes, CS and CNMT in the muscles of broiler chickens.

II. MATERIALS AND METHODS

Birds and housing: One-day-old female Chunky strain broiler chicks were purchased from a commercial hatchery (Onuma, Niigata, Japan). All chicks were housed in a heated brooder from 0 to

14 days of age. At 14 days old, body weight was measured and chicks were assigned to three different experimental groups. After 14 days, cage temperature was 22 °C and lighting was automatically controlled on a regular schedule; light for 15 h from 04:00 to 19:00. Chicks were raised on a commercial starter diet before starting the experiments. All chicks were given feed and water *ad libitum*. All experimental protocols were approved by the Niigata University Animal Care.

Diets and experimental design: Low-Met, Control and High-Met groups contained dietary Lys at levels of 75%, 100% and 200%, respectively, of the recommended requirement (0.5% of diet) according to NRC (1994) [18]. CP, ME, amino acids, vitamins, and minerals also fulfilled the NRC requirements. Each diet was given to the chicks for 10 days.

Sample collection: Chicks were killed by cutting the carotid arteries. To determine the concentration of free amino acids, pectoral muscles were taken and immediately frozen in liquid nitrogen. Muscle samples were stored at −80 °C until analysis.

Measurement of free amino acids and IDP: Muscle extracts were prepared as described by Kai *et al.* (2014) [17]. The muscles were homogenized in 10% perchloric acid with a high-speed homogenizer (Ultra-turrax T25 basic, Ikawerke, Staufen, Germany). The homogenate was then centrifuged at 600 × g and the supernatant was neutralized with 10% (w/v) potassium hydrate. After removal of potassium crystals by filtration, the filtrate volume was adjusted to 50 mL using double-distilled water. The resulting samples were kept at −20 °C until analysis.

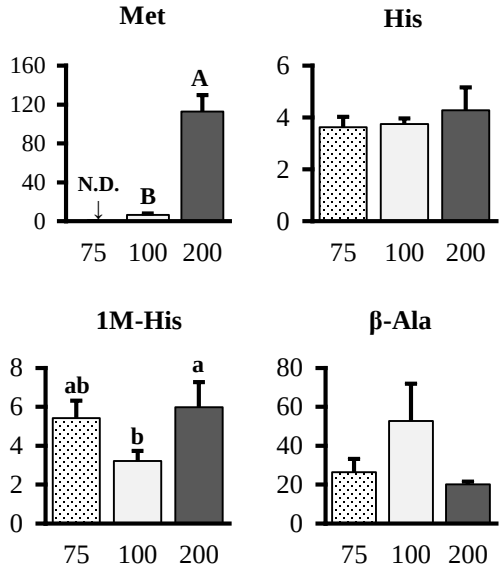
The levels of free amino acids in muscles were measured using an amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan) as described by Kai *et al*. (2014). The levels of Car, Ans and their constituent free amino acids, β-Ala, free His, 1M-His, and free Met, were determined.

Extraction of total mRNA and production of cDNA: Total mRNA was isolated from muscle using ISOGEN-LS (Wako Pure Chemical Industries, Osaka, Japan). The concentration of total mRNA was measured spectrophotometrically at 260 nm. Complementary DNA (cDNA) synthesis was performed as described by Shibata *et al*. (2006) [19]. PCR and determination of cDNA abundance was performed using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics, Basel, Switzerland) and real-time PCR (LightCycler 1.5, Roche Diagnostics, Basel, Switzerland). The primers for each enzyme were designed based on chicken (*Gallus gallus*) sequences archived in GenBank. Chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. All data were analyzed using LightCycler Software version 3.5 (Roche Diagnostics, Basel, Switzerland).

III. RESULTS AND DISCUSSION

The free amino acids in muscle are shown in Fig. 1. Free Met in Control group muscle was 6.6 ± 1.4 μg/g, and significantly increased in High-Met group ($P < 0.01$), while it was not detected in Low-Met group. 1M-His was also increased in High-Met group ($P < 0.05$), whereas no change was observed in Low-Met group.

Figure 1
Effect of dietary methionine levels on free amino acids in muscle.
(μg/g muscle)

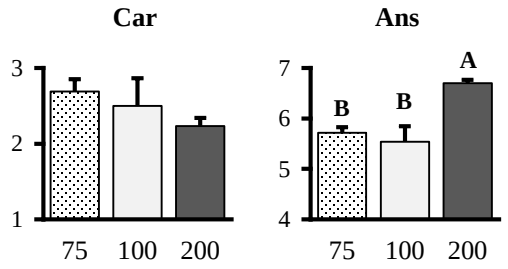


Values are means ± SE. N.D means not detected. Values in each row with different superscripts are significantly different at $^{A-C}P < 0.01$, or $^{a-c}P < 0.05$.

Results of IDP in muscle are shown in Fig.2 The Car and Ans level in Control group muscle were 2.5 ± 0.4 and 5.5 ± 0.3 mg/g, respectively.

These values were in agreement with those of a previous study by Aristoy and Toldra (2004) [2]. In High-Met group, Ans levels was increased significantly ($P < 0.05$) by 21 % approximately. On the other hand, no significant change was confirmed in Car.

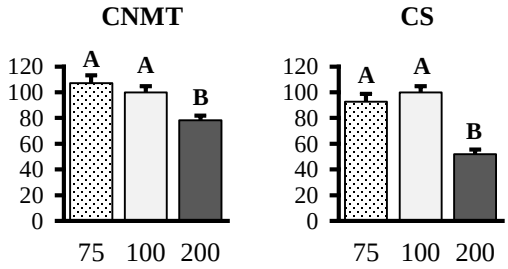
Figure 2
Effect of dietary methionine levels on Car and Ans in muscle. (mg/g muscle)



Values are means ± SE. N.D means not detected. Values in each row with different superscripts are significantly different at $^{a\text{-}c}P < 0.05$.

S-adenosyl methionine, a metabolite of Met, acts as a methyl group donor in Car methylation into Ans. From our results in Ans, we suspected that added Met would promote the Ans synthesis via the methylation of Car, therefore, enhance of mRNA expression of CNMT, the methylation enzyme, was expected in High-Met group. Fig. 3 shows the results in mRNA expression of CS and CNMT, the enzymes to synthesize IDP.

Figure 3
Effect of dietary methionine levels on relative mRNA expression of CNMT and CS (vs % of Control) in muscle.



Values are means ± SE. Values in each row with different superscripts are significantly different at $^{A\text{-}C}P < 0.01$.

In High-Met group, mRNA expression of CNMT was significantly decreased as opposed to our predictions (Kai *et al*., manuscripts in preparation). Ans is considered to possess two synthesis pathways; (1) methylation of Car to Ans, (2) directly synthesized from 1M-His and β-Ala. Although the former has been regarded as main Ans synthesis pathway, our results in this study indicate that the increase of Ans by added Met in feed would be involved in the latter pathway. The increased levels of 1M-His in High-Met group would support this hypothesis. On the other hand, mRNA expression of CS, the other enzyme, was also decreased in High-Met group. As this result was difficult to concern, we did not conclude the reason why. However, this decrease of CS might be engaged in the Ans increase. Overall, our results indicate a possibility to enhance Ans levels in muscle of broiler chicken. Ans is reported to possess stronger antioxidant activity than Car (Kohen *et al*., 1988 [3]). Therefore, chicken meats fed Met supplementation feed would possess higher antioxidant capacity, which provide some beneficial effects for human health.

IV. CONCLUSION

Met rich feed (200% of NRC) increased Ans levels in muscle of broiler chicken. Our data in mRNA expression indicate that dietary added Met facilitate direct synthesis of Ans from β-Ala and 1M-His, not methylation of Car.

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