≪**Research Note**≫

Influence of Varying Dietary Protein Levels on Glycation of Albumin, Tryptophan and Valine in the Plasma of Chickens

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Glycation is a chemical reaction in which reducing sugars bind non-enzymatically to compounds containing amino groups. Avian species like chickens are hyperglycemic animals and have high body temperature compared to mammalian species, which enables avian species to accelerate the glycation of proteins and amino acids with glucose. Although varying dietary crude protein (CP) levels alter plasma concentrations of proteins and amino acids, the influence of varying CP levels on the glycation of plasma proteins and amino acids has not been studied so far. In the present study, therefore, glycation of albumin, tryptophan and valine in the plasma of chickens fed diets with varying CP levels (0, 10, 20, 40 and 60%) was examined. At the end of the experimental period, blood samples were collected and plasma concentrations of glycoalbumin, glycated tryptophan (tryptophan-Amadori product and (1R, 3S) - 1 - (D - gluco - 1, 2, 3, 4, 5 - pentahydroxypentyl) - 1, 2, 3, 4 - tetrahydro - β - carboline - 3 - carboxylic acid (PHP-THβC)), and valine-Amadori product were measured. Although plasma albumin concentration was reduced along with the decrease in dietary CP levels from 20% to 0%, glycoalbumin in the plasma was increased under such dietary conditions. Similar increase in the ratios of tryptophan-Amadori product to tryptophan and valine-Amadori product to valine in the plasma of chickens fed a protein-free diet was observed. These results suggest that dietary protein deficiency might enhance the non-enzymatic glycation of plasma proteins and amino acids in chickens.

Key words: Amadori product, chicken, glycation, glycoalbumin, tryptophan, valine

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Introduction

Glycation, also called Maillard reaction, is a chemical reaction in which reducing sugars bind non-enzymatically to compounds containing amino groups (Maillard, 1912). Glycation leads to formation of a Schiff base followed by re-arrangement into Amadori products. Amadori products are transformed into various advanced glycation end products (AGEs) through multi-stage reactions, i.e. oxidation and cleavage reaction, etc. The acceleration of glycation in hyperglycemia increases the production and accumulation of AGEs, which is associated with the gradual development of diabetic complications in diabetes mellitus (Brownlee, 2001).

Hemoglobin (Hb) Alc is a minor red cell constituent that comprises 5% of the total Hb in normal individuals but up to 15% in patients with diabetes mellitus (Trivelli *et al*., 1971). HbA1c, a dominant glycated hemoglobin, as well as fasting blood glucose concentration, has been a standard measure for diagnosis and monitoring the progression of diabetes

(Koenig and Cerami, 1980; Parrinello and Selvin, 2014). Because the lifespan of red blood cells is approximately 120 days, HbA1c therefore reflects average glycemia over the past 2 to 3 months (Goldstein *et al*., 2004). There has been recent interest in a nontraditional marker of hyperglycemia, including glycated albumin, as alternatives or adjuncts to standard measures. As the half-life of albumin is shorter than that of red blood cells, the measurement of glycoalbumin reflects average glycemia over a shorter duration, approximately 2 to 3 weeks (Armbruster, 1987).

Lately, the relation of various nutritional conditions to the extent of glycation has been investigated by many researchers (Chao *et al*., 2010; Davis *et al*., 2015; Li *et al*., 2015; Delgado-Andrade, 2016; Khangholi *et al*., 2016). For example, it was reported that dietary carboxymethyl-lysine (CML), which is the first discovered AGE (Ahmad *et al*., 1986), increased serum CML level in obese subjects only when dietary CML was fed a high-fat diet, not with a low-fat diet (Davis *et al*., 2015).

Chickens are hyperglycemic animals and have a high body temperature. In avian species, because of hyperglycemia and high temperature, glycation of proteins proceeds easily compared to mammalian species. In fact, the crosslinking fluo-

rescent AGE, pentosidine, was detected in the skin of broilers (Klandorf *et al.*, 1995; Iqbal *et al.*, 1997, 1999). In chickens, varying dietary CP levels affected plasma concentrations of glucose and total proteins (Kita and Okumura, 1998). This suggests that varying dietary CP levels may also change glycation because of simultaneous changes in precursors, glucose, and amino compounds; however, the influence of varying dietary CP levels at the extent of glycation of proteins and amino acids has not been clarified thus far. In the present study, therefore, glycation of albumin, tryptophan, and valine in the plasma of chickens fed diets with varying dietary CP levels (0, 10, 20, 40 and 60%) was examined.

Materials and Methods

Animals and Experimental Procedures

Newly hatched single comb White Leghorn male chicks were purchased from a local hatchery (Koiwai Farm Co., Ltd, Shizukuishi, Iwate, Japan). They were allowed free access to water and a commercial chick mash (Toyohashi Feed Mills Co., Ltd. Toyohashi, Japan) for 10 days. All birds were raised in a chick brooder (Showa Furanki Co., Ltd. Saitama, Japan) and the temperature was kept at 29℃. At 10 days of age, 30 chickens were selected and divided evenly into 5 experimental groups ($n=6$). Five experimental diets with 5 dietary CP levels (0%, 10%, 20%, 40% and 60%) were prepared (Table 1). Experimental diets were given to chickens for 7 days. At the end of the experiment, blood samples were collected by heart puncture after light anesthesia with diethyl ether. Blood samples were placed on ice and then centrifuged at 4℃, 10,000×g for 20 min. After collecting plasma samples, they were frozen and stored at -20℃ until analysis. Animal care was in compliance with guidelines from the Iwate University Animal Care and Use Committee.

Measurement of Plasma Component

Measurement of plasma glucose was carried out according to the instructions of a commercial kit (Glucose CII-test, Wako, Osaka, Japan). Before measuring amino acid concentrations, plasma samples were mixed with an equal volume of 3% sulfosalicylic acid and stored at 4℃ for 1 h for deproteinization. The samples were then centrifuged at 4℃, 10,000

×g for 10 min. The supernatant was taken and passed through a 0.22 μm membrane filter. Deproteinized samples were stored at 4℃ overnight. After membrane filtration was repeated, samples were set on an automatic amino acid analyzer (All Automatic Amino Acid Analyzer, JEOL Ltd., Tokyo, Japan). Measurement of plasma albumin was carried out according to the instructions of a commercial kit (A/G-B test, Wako, Osaka, Japan).

Detection of tryptophan, valine, tryptophan- and valine-Amadori products, and PHP-THβC were performed using high performance liquid chromatography (HPLC) (NEXERA XR, SHIMADZU, Kyoto, Japan). The mobile phase A was the water containing 0.1% formic acid. The mobile phase B was 100% acetonitrile. The flow rate was 400 μl/min. The sample injection volume was 5 μl. Reverse phase HPLC column (100×2 mm I.D., Gemini 3 μm C18 110 Å, Phenomenex, Torrance, CA, USA) was used, and the column temperature was set at 25℃. Samples were measured using liquid chromatography-mass spectrometry (SHIMADZU, LCMS-2020, Kyoto, Japan) with Dual Ion Source (DUIS). Tryptophan, valine, tryptophan-Amadori product, (1R, 3S) - 1 - (D - gluco - 1, 2, 3, 4, 5 - pentahydroxypentyl) - 1, 2, 3, 4 - tetrahydro - β - carboline - 3 - carboxylic acid (PHP-THβC)), and valine-Amadori products were measured in positive ion mode using selected ion monitoring (SIM). The values for m/z of positive ion of tryptophan and glycated tryptophan compounds (tryptophan-Amadori product and PHP-THβC) were 205.10 and 367.15, respectively. The values for m/z of positive ion of valine and valine-Amadori product were 117.15 and 279.25, respectively. The vaporizer temperature was 250℃.

Statistical Analysis

Results are expressed as means±SE. Statistical analysis of data was performed by one-way ANOVA and Tukey's HSD test for multiple comparisons ($P<0.05$) using the General Linear Model Procedures of SAS (version 9.4) (SAS Institute, 2012).

Results

Body weight change and feed intake are shown in Table 2.

Table 1.   **Composition of experimental diets with varying dietary protein levels**

Ingredients (g/kg)	CP 0%	CP 10%	CP 20%	CP 40%	CP 60%
ISP	0.0	120.8	241.5	483.0	724.6
L-Cystine	0.0	1.1	2.2	4.4	6.6
L-Methionine	0.0	0.7	1.4	2.8	4.2
L-Threonine	0.0	1.0	2.0	4.0	6.0
Cornstarch	741.4	617.8	494.3	247.2	0.0
Cellulose	164.1	164.1	164.1	164.1	164.1
Corn oil	30.0	30.0	30.0	30.0	30.0
Mineral mixture[1]	60.0	60.0	60.0	60.0	60.0
Vitamin mixture[1]	2.0	2.0	2.0	2.0	2.0
Choline chloride	1.5	1.5	1.5	1.5	1.5
Inositol	1.0	1.0	1.0	1.0	1.0

ISP: Isolated soybean protein (CP 82.81%)
[1] Kita *et al.*, 1996.

Table 2. **Body weight change and feed intake of young chickens fed experimental diets with varying dietary protein levels**

	Diets				
	CP 0%	CP 10%	CP 20%	CP 40%	CP 60%
Body weight change (g/7 d)	-13.8 ± 0.7^{c}	37.2 ± 4.2^{a}	36.8 ± 3.5^{a}	25.8 ± 4.9^{ab}	14.5 ± 7.6^{b}
Feed intake (d/7 d)	85.0 ± 9.6^{b}	143.8 ± 6.2^{a}	109.7 ± 6.3^{ab}	90.17 ± 7.4^{b}	94.5 ± 14.4^{b}

[a–c] Means with different superscript letters are significantly different ($P<0.05$)
Values are means$\pm$SE. $n=6$.

Table 3. **Plasma concentrations of glucose, tryptophan (Trp), Trp-Amadori product, PHP-THβC, valine (Val), Val-Amadori product, total protein, albumin (ALB), glycoalbumin (GA), and the GA/ALB ratio in chickens fed diets with varying dietary protein levels**

Concentration and ratio	Diets				
	CP 0%	CP 10%	CP 20%	CP 40%	CP 60%
Glucose (mg/dl)	358 ± 26.6	308 ± 11.8	308 ± 2.1	316 ± 17.7	306 ± 8.5
Trp (μM)	$17.9\pm4.8^{b,*}$	36.0 ± 2.4^{a}	35.1 ± 5.4^{a}	33.7 ± 2.8^{ab}	41.7 ± 4.4^{a}
Trp-Amadori product (μM)	$8.6\pm1.1^{b,**}$	19.0 ± 1.1^{ab}	17.1 ± 1.7^{a}	14.6 ± 1.9^{a}	16.9 ± 1.7^{a}
PHP-THβC (μM)	$0.20\pm0.12^{b,**}$	0.76 ± 0.05^{a}	0.73 ± 0.07^{a}	0.45 ± 0.09^{ab}	0.53 ± 0.16^{ab}
Val (μM)	131 ± 13.1^{c}	156 ± 6.5^{c}	274 ± 12.3^{b}	$323\pm61.6^{b,*}$	476 ± 34.1^{a}
Val-Amadori product (μM)	$79.8\pm19.3^{b,**}$	73.0 ± 8.3^{b}	97.5 ± 8.7^{b}	93.6 ± 6.1^{b}	170.9 ± 22.9^{a}
Total protein (g/dl)	2.65 ± 0.2^{c}	2.97 ± 0.1^{bc}	3.36 ± 0.1^{ab}	3.32 ± 0.1^{ab}	3.46 ± 0.1^{a}
Albumin (ALB) (g/dl)	0.88 ± 0.1^{c}	1.43 ± 0.1^{b}	1.74 ± 0.1^{a}	1.95 ± 0.1^{a}	1.72 ± 0.1^{a}
Glycoalbumin (GA) (g/dl)	0.90 ± 0.1^{a}	0.53 ± 0.0^{b}	0.59 ± 0.0^{b}	0.54 ± 0.0^{b}	0.64 ± 0.0^{b}
GA/ALB (%)	101.9 ± 5.8^{a}	37.7 ± 2.0^{b}	34.3 ± 1.5^{b}	27.8 ± 1.7^{b}	37.1 ± 1.6^{b}

[a–c] Means with different superscript letters are significantly different ($P<0.05$).
Values are means$\pm$SE. CP 0%: $n=5$, CP 10–60%: $n=6$, * One missing value, ** Two missing values.

Body weight gains of chickens fed diets containing 10% and 20% CP were higher than the other treatments. Above the requirement (CP19%) recommended by Japanese Feeding Standard for Poultry (National Agriculture and Food Research Organization, 2014), body weight gains decreased along with the rise in dietary CP levels from 20% to 60%. Body weight was reduced by feeding a protein-free diet. Feed intakes of chickens fed diets with excess CP levels, compared to the requirement, were significantly lower than those of birds fed the control (CP 20%) diet. Feed consumption of the protein-free diet was also lower than that of the control diet.

Plasma concentrations of glucose, tryptophan, tryptophan-Amadori product, valine, valine-Amadori product, total protein, albumin, glycoalbumin, and the ratio of glycoalbumin to albumin are shown in Table 3. No significant differences were found in plasma glucose concentrations among any dietary treatments. Plasma concentrations of tryptophan, tryptophan-Amadori product, and PHP-PHβC were almost halved by feeding a protein-free diet compared to those of chickens fed diets containing CP. Plasma valine concentrations increased along with an increase in dietary CP levels. Plasma concentration of valine-Amadori product of chickens fed a CP 60% diet was the highest of all. Plasma albumin concentrations increased along with the increment in dietary CP levels from 0% to 20% and remained constant above CP 20%. A linear elevation of plasma total protein of chickens was observed from 0% to 60% of CP. Plasma glycoalbumin concentration and the ratio of glycoalbumin to albumin of chickens given a protein-free diet was the highest of all.

The ratios of tryptophan-Amadori product to tryptophan, PHP-THβC to tryptophan and valine-Amadori product to valine in the plasma of chickens fed diets with varying dietary CP levels from 0% to 60% are represented in Fig. 1. The ratio of tryptophan-Amadori product to tryptophan in the CP 0% group was the highest and that in the CP 60% group was the lowest of all (Fig. 1A). There was no significant influence of varying dietary CP levels from 0% to 60% on the ratio of PHP-THβC to tryptophan in the plasma of chickens (Fig. 1B). The ratio of valine-Amadori product to valine in the plasma of chickens fed a protein-free diet showed the highest value and decreased gradually by rising dietary CP levels from 0% to 20%. Above the requirement, the ratio remained constant (Fig. 1C).

Discussion

Since CML was first discovered as an AGE, various types of AGEs have been identified and reported, and detailed information about AGEs has been accumulated thus far (Nagai et al., 2014; Ashraf et al., 2015; Nowotny et al., 2015). Information about Amadori products, which are recognized as early glycation products, has also been accumulated similar to AGEs. Amino compounds like proteins are the main precursors of Amadori products, and HbA1c and
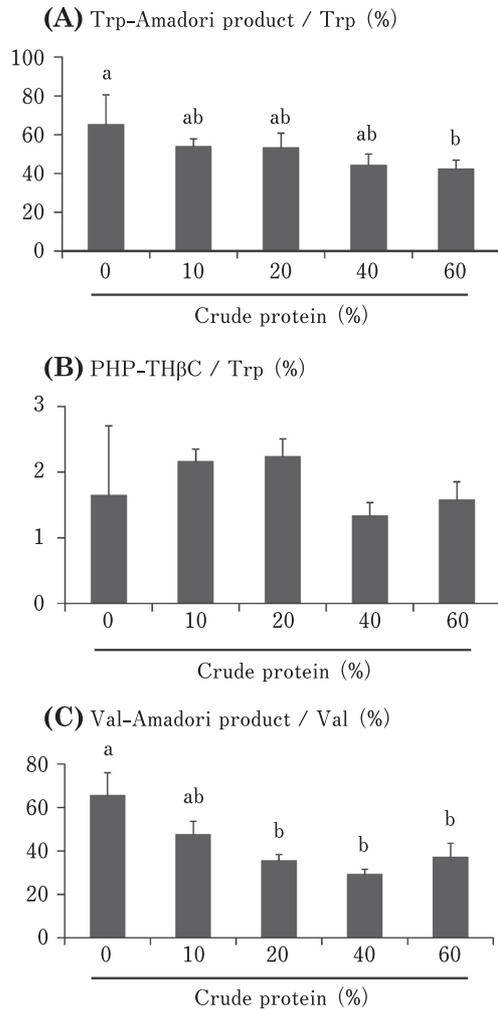
(A) Trp-Amadori product / Trp（%）

(B) PHP-THβC / Trp（%）

(C) Val-Amadori product / Val（%）

Fig. 1. **The ratios of Trp-Amadori product to Trp (A), PHP-TH*β*C to Trp (B) and Val-Amadori product to Val (C) in the plasma of chickens fed diets with varying dietary CP levels from 0% to 60%.** Values are means± SE. n: See Table 3. [a,b] Means with different superscript letters are significantly different ($P<0.05$).

glycoalbumin are known to be typical Amadori products derived from plasma proteins, hemoglobin and albumin, respectively. Recently, glycoalbumin has been focused on as a non-traditional marker of hyperglycemia because the ratio of glycoalbumin to albumin reflects average glycemia over a shorter duration (approximately 2 to 3 weeks) than that of HbA1c (Armbruster, 1987). As shown in Table 3, the ratio of glycoalbumin to albumin of chickens given a protein-free diet was the highest in all dietary treatments. A similar change in plasma glycoalbumin concentration was observed in chickens fed the CP 0% diet, whereas plasma albumin concentration was decreased by protein-deprivation (Table 3). It is well-known that plasma levels of chicken albumin are easily affected by changes in dietary CP levels (Takahashi *et al*., 1995; Poosuwan *et al*., 2010), which is in agreement with the results derived in the present study. In addition, as

there was no change in plasma glucose concentrations from feeding a protein-free diet, the increase in the ratio of gly-coalbumin to albumin might be due to both an increase in plasma glycoalbumin and a decrease in plasma albumin by feeding the CP 0% diet. A similar phenomenon was reported where low level of albumin was associated with an increase in plasma HbA1c (Bhonsle *et al*., 2012), which supports the results derived from the present study.

We have recently established the analytical methods to measure plasma concentrations of tryptophan-Amadori product and PHP-TH*β*C (Kita *et al*, 2013; Makino *et al*., 2015a) and valine-Amadori products (Takahashi and Kita, 2016). As shown in Table 3, Amadori products of trypto-phan and valine were successfully measured, and the derived results revealed that approximately 60% of tryptophan and 40% of valine were glycated, even when chickens were fed on the control (CP 20%) diet. In addition, it was clarified that valine-Amadori product and PHP-TH*β*C did not have nutritional value as precursors of protein synthesis for chicken embryo myoblasts (Makino *et al*., 2105b). These results suggest that not all amino acids in the plasma are used as body constituents and energy production because a con-siderable proportion of plasma amino acids is glycated.

As shown in Table 3, when chickens were fed a protein-free diet, plasma concentrations of tryptophan and valine were lower than those of birds given the control (CP 20%) diet. Similar decrease in plasma amino acid concentrations was observed in broilers fed low-protein diets (Kiode *et al*., 1992), which suggested that amino acids synthesized from glucose would less contribute to maintain the plasma amino acid concentration of chickens fed high-carbohydrate, low-protein diets. It is well established that body constituents are turned over (Schoenheimer and Rittenberg, 1935; 1936; Rittenberg and Schoenheimer, 1937; Schoenheimer, 1937; Rittenberg *et al*., 1938), and this concept suggests that plasma concentrations of nutrients and metabolites are also regulated by inflow (intake, synthesis) and outflow (degrada-tion, excretion) of compounds. Therefore, the decrease in plasma glycated amino acids resulting from feeding a protein-free diet may be brought about by decreasing inflow (intake and/or synthesis) of glycated amino acids and in-creasing outflow (degradation and/or excretion) of glycated amino acids. On the other hand, the ratios of tryptophan-Amadori product to tryptophan and valine-Amadori product to valine of chickens fed the CP 0% diet were the highest of all (Fig. 1). The increased ratios suggest that the extent of decrease in glycated amino acids was lower than that of reduction of originated amino acids.

In conclusion, dietary protein deficiency might enhance the non-enzymatic glycation of plasma proteins and amino acids in the plasma of chickens.

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