Detection of Two Types of Glycated Tryptophan Compounds in the Plasma of Chickens Fed Tryptophan Excess Diets

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When tryptophan is glycated with glucose, it results in forming two types of glycated tryptophan compounds, glucose-tryptophan Amadori product and (1R, 3S)-1-(D-gluco-1,2,3,4,5-pentahydroxypentyl)-1,2,3,4-tetrahydro-\(\beta\)-carboline-3-carboxylic acid (PHP-TH\(\beta\)C). As hyperglycemia and high body temperature are the characteristics in avian species, these are supposed to elevate the concentration of glycated tryptophan compounds in the plasma of chickens. However, there was no attempt to detect two types of glycated tryptophan compounds in the plasma of chickens, so far. Therefore, young chickens were fed tryptophan-excess diets (0, 1, 2 or 3% excess) for 14 days, and glycated tryptophan compounds were detected using a liquid chromatograph mass spectrometer (LC/MS). In the present study, two types of glycated tryptophan compounds, glucose-tryptophan Amadori product and PHP-TH\(\beta\)C, were successfully detected in the plasma of chickens, and plasma levels of both glycated tryptophan compounds significantly correlated to plasma tryptophan concentration.

Key words: Amadori product, \(\beta\)-carboline, chickens, glycation, tryptophan


Introduction
Glycation, so-called Maillard reaction (Maillard, 1912) or amino-carbonyl reaction, causing dehydrating condensation between carbonyl group of reducing sugars and amino group of amino acids, is a non-enzymatic reaction that forms Schiff base. In case of glycation of glucose and tryptophan, Schiff base is followed by two different reactions, Amadori rearrangement and Pictet-Spengler reaction. Amadori rearrangement forms Amadori product, and Pictet-Spengler cyclization forms (1R,3S)-1-(D-gluco-1,2,3,4,5-pentahydroxypentyl)-1,2,3,4-tetrahydro-\(\beta\)-carboline-3-carboxylic acid (PHP-TH\(\beta\)C), which is one of natural \(\beta\)-carbolines consisting of an indole skeleton and various side chains (Röper et al., 1983; Fig. 1).

In healthy subjects, fasting blood glucose concentration is maintained under 110 mg/dl. On the other hand, chickens are known to be hyperglycemic animals and their blood glucose level is over 200 mg/dl (Hazelwood and Lorenz, 1959). Because of hyperglycemia and high body temperature in avian species, glycation is supposed to be easily proceeded compared to mammals (Klandorf et al., 1995; Iqbal et al., 1999). Amadori products generated by glycation undergo further complex reactions to form advanced glycation end products (AGEs), and it was reported that cellular actions of AGEs were mediated by their specific receptors of AGEs (RAGE) (Maillard-Lefebvre et al., 2009). Although the aspects of AGEs were investigated by some researchers, there has been little information of the physiological characteristics of Amadori products derived from glucose and amino acids in both mammalian and avian species so far.

In this study, therefore, we attempted to detect glycated tryptophan compounds, glucose-tryptophan Amadori product and PHP-TH\(\beta\)C, in the plasma of young chickens fed tryptophan-excess diets.

Materials and Methods

Animals and Experimental Procedures
Newly hatched single-comb White Leghorn male chicks were obtained from a local hatchery (Koiwai Farm Co., Ltd, Shizukuishi, Iwate, Japan). Chicks were fed a commercial chick mash diet (crude protein (CP) 207 g/kg, metabolizable energy (ME) 12.1 MJ/kg; Toyohashi Feed Mills Co., Ltd, Toyohashi, Aichi, Japan) from hatching until 8 d of age in...
electrically heated brooder. At this age, 48 birds were di-
vided into 24 cages of 2 birds each. These birds had free
access to an experimental control (0% dietary excess tryp-
tophan) diet (Table 1) for 3 d to become accustomed experi-
mental diet. At 11 d of age, 24 birds of uniform body weight
(average initial body weight ± SE, 111.5 ± 0.6 g) was se-
lected and divided evenly into 4 experimental groups of 6
birds each. The birds were placed in individual cages.
Continuous illumination was provided. According to Na-
tional Research Council (1994), the dietary protein require-
ment for Leghorn-type young chickens are 18.0%. In this
study, 18.0% CP was set as the dietary protein requirement.
From 11 d of age, the birds had free access to one of ex-
perimental diets with various supplementation levels (0, 1, 2
and 3%) of tryptophan for 14 days. Calculated ME of all
diets was set at 11.9 MJ/kg. The composition of experimen-
tal diets is shown in Table 1. At the end of experiment, body
weight and feed intake were measured. Thereafter, chickens
were anesthetized with diethylether and blood samples were
taken by heart puncture. Blood samples were centrifuged for
20 min at 5,000 × g, 4°C to separate plasma. Plasma samples
were stored at −20°C until analyzed. Animal care was in

Table 1. Composition of experimental diets (g/kg)

<table>
<thead>
<tr>
<th>Excess dietary tryptophan level (%)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated soybean protein (CP84%)</td>
<td>214.0</td>
<td>214.0</td>
<td>214.0</td>
<td>214.0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.0</td>
<td>10.0</td>
<td>20.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>493.1</td>
<td>483.1</td>
<td>473.1</td>
<td>463.1</td>
</tr>
<tr>
<td>Cellulose</td>
<td>194.9</td>
<td>194.9</td>
<td>194.9</td>
<td>194.9</td>
</tr>
<tr>
<td>Corn oil</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Mineral mixture¹</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Vitamin mixture²</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Inositol</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

¹The composition of mineral mixture was as follows (kg of diet): CaHPO₄·2H₂O, 21.2 g; CaCO₃, 15.2 g; KH₂PO₄, 10.3 g; KCl, 3.1 g; NaCl, 6.2 g; MgSO₄, 3.1 g; FeSO₄·7H₂O, 513 mg; MnSO₄·5H₂O, 359 mg; KI, 2.7 mg; CuSO₄·5H₂O, 41 mg; ZnO, 64 mg; CoCl₂·6H₂O, 1.7 mg; Na₂MoO₄·2H₂O, 8.5 mg; Na₂SeO₃, 411 μg.
²The composition of vitamin mixture was described previously (Kita et al., 1996).
compliance with applicable guidelines from the Iwate University Animal Care and Use Committee.

**Preparation of Glycated Tryptophan Compounds**

The 2 M glucose - 5 mM tryptophan solution was sterilized, adjusted to pH 9, and incubated at 37°C for 3 d. To collect glycated tryptophan compounds from the mixture solution, a Sephadex G-10 column chromatography (300 mm x 10 mm I.D., GE Healthcare Japan, Tokyo) was applied. After 500 μl of mixture solution was loaded onto the column, fractions for 30 sec were collected into sample tubes. The mobile phase was distilled water and the flow rate was 2.0 ml/min. Tryptophan and glycated tryptophan compounds were detected by the absorbance at 220 nm. Glucose in fractions was detected by using a commercial kit (Glucose C II-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka). Fractions containing glycated tryptophan compounds were collected, which were diminished free glucose and tryptophan. Derived sample was used as a standard to decide the retention time of glycated tryptophan compounds in the plasma separated by high performance liquid chromatography (HPLC).

**Deproteinization and Delipidization of Plasma Samples**

Frozen plasma samples were thawed, and 200 μl of plasma was mixed with 800 μl of acetonitrile for deproteinization. After centrifugation for 10 min at 6,000 x g, 4°C, the supernatant was transferred to a fresh sample tube. The 100 μl of water/acetonitrile mixture (1:2, v/v) was added to remaining precipitate and mixed well. After centrifugation for 10 min at 6,000 x g, 4°C, the supernatant was transferred to a sample tube containing previous supernatant. The same procedure was repeated once. Collected supernatant was vacuum-dried with a centrifugal evaporator (CVE-2000, Tokyo Rikakikai Co., LTD, Tokyo). For lipidization of samples, 400 μl of water and 300 μl of chloroform were added to dried samples, and mixed vigorously. After centrifugation for 15 min at 1,000 x g, 4°C, the upper phase was transferred to a fresh sample tube. In this procedure, the contamination of lower phase should be avoided. Small volume of water was added into the remaining lower phase, and centrifuged for 15 min at 1,000 x g, 4°C. The upper phase was transferred into a tube containing previous upper phase. The same procedure was repeated once. Collected supernatant was vacuum-dried with a centrifugal evaporator. Dried samples were dissolved in 20 μl of water, and applied LC/MS to detect two types of glycated tryptophan compounds.

**Detection of Glycated Tryptophan Compounds in the Plasma Using LC/MS**

Separation of two types of glycated tryptophan compounds was performed by using HPLC (PU-2080, JASCO Corporation, Tokyo, Japan). The mobile phase was the mixture of methanol and water (3:1, v/v) containing 5 mM of ammonium formate. The flow rate was 400 μl/min. The sample injection volume was 3 μl. HPLC column (100 mm x 2 mm I.D., Gemini 3 μm C18 110Å, Phenomenex, Torrance, CA, USA) was used, and the column temperature was set at 25°C. The retention time for glycated tryptophan compounds was set by using a standard collected from the mixture of glucose and tryptophan. Samples were measured using a double-focusing magnetic sector mass spectrometer JMS-700 MStation (JEOL Ltd., Tokyo, Japan) with an atmospheric pressure chemical ionization (APCI). Two types of glycated tryptophan compounds, Amadori product and PHP-THC, were detected in positive ion mode using selected ion monitoring (SIM). The value for m/z of positive ion of glycated tryptophan compounds was 367.1505. The vaporizer temperature was 500°C. The amount of glycated tryptophan compounds in the plasma was described as the relative value with arbitrary unit.

**Measurement of Amino Acid Concentrations in the Plasma**

Frozen plasma samples were thawed, and 300 μl of plasma was mixed with equal volume of 3% (w/v) sulfosalicylic acid for deproteinization. After centrifugation for 10 min at 10,000 x g, 4°C, the supernatant was transferred to a fresh sample tube. Samples were passed through 0.45 μm membrane filter, and amino acid concentrations in the plasma were determined by an automatic amino acid analyzer (JLC-500 V2, JEOL Ltd., Tokyo, Japan).

**Statistical Analysis**

All data are presented as the mean ± SE. Statistical analysis of data was performed by one-way ANOVA and Duncan’s multiple range test (P<0.05) using the General Linear Model Procedures of SAS (SAS/STAT version 6, SAS Institute, 1999). Correlation equations were also calculated by GLM Procedure of SAS.

**Results**

**Growth Performance**

Body weight gain, feed intake and feed efficiency of chickens fed experimental diets with various dietary excess tryptophan levels are shown in Table 2. Body weight gain of chickens fed a diet containing 1% excess tryptophan was not different from that of birds fed a control diet (P>0.05). The increase in dietary excess tryptophan levels from 1% to 3% significantly and gradually decreased body weight gain. Feed intake of chickens fed the diet containing 1% excess dietary tryptophan was the highest of all (P<0.05). Feed intake of birds fed a control diet was not different from that in the 2% dietary excess tryptophan group (P>0.05). The increment in dietary excess tryptophan levels from 1% to 3% significantly decreased feed intake. When dietary excess tryptophan levels varied from 0% to 2%, feed efficiency was not significantly different among experimental groups. There was no significant difference in feed efficiency of chickens fed experimental diets containing dietary excess tryptophan from 1% to 3%.

**Plasma Concentrations of Glucose and Tryptophan**

Plasma concentration of glucose was not affected by varying dietary excess tryptophan levels (P>0.05; data not shown). The average of plasma glucose concentration was 15.4±0.53 (SE) mM. Plasma concentration of tryptophan is shown in Fig. 2. Plasma tryptophan concentration increased as elevating dietary excess tryptophan levels (P<0.05).
Plasma Levels of Two Types of Glycated Tryptophan Compounds

The SIM at 367.1505 of m/z revealed two peaks of glycated tryptophan compounds (data not shown). Two peaks were corresponded to glucose-tryptophan Amadori product and PHP-THβC. As shown in Fig. 3, the peak at former retention time increased by an increment of dietary excess tryptophan levels ($P<0.05$). Although the peak at latter retention time showed similar tendency to the former peak (Fig. 4), only the peak of the 3% dietary excess tryptophan group was the highest of all ($P<0.05$).

### Discussion

It has been well known that non-enzymatic amino-carbonyl reaction is the first step of glycation (Maillard, 1912) and leads to form Amadori product. But it has not been clarified the aspect of Amadori products generated from amino acids so far. In the present study, therefore, we attempted to detect two types of glycated tryptophan compounds, glucose-tryptophan Amadori product and PHP-THβC. As shown in Figs. 3 and 4, two types of glycated tryptophan compounds were successfully detected in the chicken plasma, which was the first measurement of glucose-tryptophan Amadori product and PHP-THβC.

As represented in Fig. 2, plasma concentration of tryptophan increased as elevating dietary excess tryptophan levels. It is known that feeding a tryptophan-excess diet caused reduction of feed efficiency in young chickens (Edmonds and Baker, 1987; Baker et al., 1996), which was in good agreement with the results observed in the present study. Similar response to varying dietary excess tryptophan

<table>
<thead>
<tr>
<th>Excess dietary tryptophan level (%)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/14 d)</td>
<td>146.5±6.8</td>
<td>146.6±10.2</td>
<td>108.7±11.3</td>
<td>74.1±7.2</td>
</tr>
<tr>
<td>Feed intake (g/14 d)</td>
<td>367.5±15.1</td>
<td>436.9±15.7</td>
<td>327.3±14.3</td>
<td>286.9±20.8</td>
</tr>
<tr>
<td>Feed efficiency (%)</td>
<td>40.0±1.7</td>
<td>33.4±1.6</td>
<td>32.9±2.8</td>
<td>26.5±3.0</td>
</tr>
</tbody>
</table>

Table 2. Body weight gain, feed intake and feed efficiency of young chickens fed tryptophan excess diets
level was observed in plasma concentrations of glycated tryptophan compounds (Figs. 3 and 4). In order to clarify the relation of varying plasma tryptophan concentrations to plasma glycated tryptophan level, the correlation coefficient was calculated. The correlation coefficients derived from statistical analyses were 0.764 ($P<0.001$) on the former peak of glycated tryptophan and 0.881 ($P<0.001$) on the latter peak of glycated tryptophan. The positive and significant correlations between plasma tryptophan concentration and plasma glycated tryptophan level suggested that the elevation of plasma tryptophan concentration resulted in the increase in glycated tryptophan compounds level in the plasma.

Hyperglycemia is commonly observed in avian species like diabetes mellitus, which offers class aves the great advantage in providing animal models for diabetology (Klandorf et al., 1995; Iqbal et al., 1999). The preeminent traits of this class are as follows: 1) High blood glucose concentrations typically 2–3 times higher than human, which should accelerate amino-carbonyl reaction and generate high concentration of AGEs; 2) An elevated basal body temperature (about 3°C higher than mammals), which should contribute to the non-enzymatic attachment of glucose to proteins and amino acids. During last half of 1990’s, it was reported that the relationship between dietary factors and tissue pentosidine, which is one of representative AGES formed by the non-enzymatic glycation of lysine and arginine residues, in broiler breeder hens (Iqbal et al., 1997; 2000). In these studies, pentosidine in collagen, which is the main protein of connective tissue and the most abundant protein in animals (Di Lullo et al., 2002), of skin and tendon was determined in chickens. As represented in Figs. 3 and 4, we successfully detected two types of glycated tryptophan compounds, which might be involved in hyperglycemia in chickens. Although hyperglycemia was also observed in diabetic mammals like streptozotocin–induced type-1 diabetic mice and rats, whether glycated amino acid compounds would be detected in the plasma or not. The study solving this issue should be conducted in the future.

Acknowledgments

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References


