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Determination of Immunoglobulin Y Concentration in Yolk Extract Prepared by Water Dilution Method: Comparisons among Three Strains of Chickens

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In avian species, immunoglobulin Y (IgY) is found abundantly in egg yolk as well as in blood. Although the concentration of IgY in yolk has been previously reported by many investigators, the reported IgY concentrations have varied from 1 to 25 mg/g yolk. In the present study, we determined yolk IgY concentration in three strains of chickens, taking into consideration the IgY recovery rate in yolk extract; in addition, we estimated the concentration factor of yolk IgY against plasma IgY. Egg yolk and plasma samples were collected from two commercial layers, Dekalb and Nagoya, and one inbred strain, PNP/DO. Extraction of yolk IgY was followed by the water dilution method reported by Akita and Nakai (Journal of Food Science 57, 629–634, 1992) with minor modification, and IgY concentration was measured by ELISA. The recovery of IgY from the yolk was in the 50 to 60% range, and no significant difference was observed among the strains. PNP/DO showed the highest concentration of IgY expressed as mg/g yolk (12.2), and Dekalb and Nagoya had almost the similar concentration of IgY (Dekalb, 6.2; Nagoya, 5.7). In all strains, the concentration of plasma IgY was proportional to that of yolk IgY, and the concentration factor of yolk IgY against plasma IgY was approximately 0.8. On the other hand, when the yolk IgY concentration was expressed as mg/mL water in yolk, the concentration factor of yolk IgY against plasma IgY represented approximately 1.7 regardless of the strains. Taken together, our findings suggest that the concentration of IgY in the yolk should be determined by considering the recovery rate during the process of IgY extraction; in addition, it raised the possibility that blood IgY might be concentrated in egg yolks of chickens.

Key words: chicken, egg yolk, IgY concentration, plasma, water dilution method

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Introduction

Avian egg yolk contains massive amount of immunoglobulin Y (IgY), the functional equivalent to mammalian IgG, which plays a central role in the protection of the newly hatched chick against infectious disease (Kowalczyk *et al.,* 1985). IgY is incorporated from circulating blood into developing ovarian follicles, and accumulates in oocyte cytoplasm as egg yolk. Although the exact mechanism by which IgY is incorporated into ovarian follicles has not been fully elucidated, the blood IgY is believed to be incorporated into ovarian follicles by a specific receptor existing in the ovarian follicular membranes (Roth *et al.,* 1976).

IgY concentration in the egg yolk of chickens has been measured by many investigators, but the reported IgY concentrations have varied from 1 to 25 mg/g yolk (Patterson *et al.*, 1962; Cutting and Roth, 1973; Rose *et al.,* 1974; Kowalczyk *et al.,* 1985; Li *et al.,* 1998; Carlander *et al.*, 2001; Hamal *et al.,* 2006). It seems likely that the scattering of the yolk IgY concentration data is caused by multiple reasons including differences in strains of chickens (Gross and Siegel, 1990) and daily fluctuation (Carlander *et al.*, 2001), but one of the main reasons is that the methods of preparing IgY yolk extract differed among the investigators. Egg yolk can be viewed as being an oil-in-water emulsion with the watery portion containing proteins and the dispersed portion comprising so-called yolk granules and lipid drops. Since yolk IgY is included in the water-soluble fraction, measuring its concentration requires the removal of the water-insoluble

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fraction from the yolk. Consequently, attempts have been made by a number of investigators to develop more efficient and convenient methods for isolation of IgY from the yolk. The most promising methods involve chloroform polyethylene (Polson, 1990), polyethylene glycol (Polson *et al.,* 1980) and water dilution (Akita and Nakai, 1992). The water dilution method provides a simple, rapid and efficient means of purifying IgY. Akita and Nakai (1993) compared the yield and purity of IgY between the water dilution method and three other methods using polyethylene glycol, dextran sulphate or xanthan gum. The water dilution method gave the highest yield of yolk IgY among the four methods, and the total yield of IgY by the water dilution method was about twice those of the other methods. This result implies that correction for the IgY recovery rate in the process of extraction is essential to measuring the IgY concentration in yolk. However, most of studies reporting yolk IgY concentration have not considered the IgY recovery rate from yolks.

The present study was conducted to determine yolk IgY concentration in three strains of chickens, two commercial layers (Dekalb and Nagoya; Nakamura *et al.,* 2006) and one inbred strain (PNP/DO; Mizutani, 2002). Extraction of yolk IgY followed by the water dilution method reported by Akita and Nakai (1992) with minor modification, and a corrected IgY concentration was determined by measuring DIG-labeled IgY recovery from yolk extract. Furthermore, we calculated the concentration factor of yolk IgY against plasma IgY to characterize the mechanism for IgY transport into ovarian follicles.

Materials and Methods

Experimental birds

All birds used for experiments were at 30–45 weeks of age. Single Comb White Leghorn type commercial layers were purchased from a local hatchery (Dekalb®; Chubu Kagaku Shizai Co., Ltd., Nagoya, Japan). Female PNP/DO and commercial Nagoya were provided from Nagoya University Avian Bioscience Research Center and Aichiken Agricultural Research Center (Nagakute, Japan), respectively. Animal care was in compliance with the guidelines of Nagoya University Policy on Animal Care and Use. The birds were individually maintained in steel cage with free access to water and commercial diet (S-seven, CP 17.5%, ME 2800 kcal/kg, Ca 2.7%; Nosan Corporation, Yokohama, Japan) under a daily light period of 14 h.

Sample collection

In Experiment 1, birds stably laying eggs were used for sample collection. Laid eggs were collected immediately after the laying, and stored at 4℃ until analysis. After the collection of an egg sample, a blood sample was collected from the wing vein into a heparinized plastic tube. A plasma sample was separated from the blood by centrifugation (5000×g, 10 min), and stored at −20℃. Egg samples were used for measurements of water content and IgY concentration in egg yolk, and plasma samples were used for measurement of IgY concentration.

In Experiment 2, to investigate the daily fluctuation of IgY concentrations in yolk and blood, Dekalb and PNP/DO were used. All laid eggs were collected for 10 days, and blood samples were collected 4 times, every 3 days (day 1, 4, 7 and 10). The egg and blood samples were analyzed for IgY concentration.

Determination of water content in yolk

The stored eggs were weighed, and then the egg yolk was separated from the egg white. After checking the egg yolk weight, the vitelline membrane was punctured, and 1 g of yolk was collected into a glass flask. The water content in the yolk was measured by the AOAC method (1995).

Quantitation of IgY in plasma

IgY concentration of the plasma sample was determined by Chicken IgG ELISA Quantitation kit (Bethyl laboratory, Montgomery, TX, USA).

Extraction of IgY from egg yolk and quantitation of IgY in yolk

Chicken IgY was obtained from Sigma-Aldrich (St. Louis, MO, USA), and was labeled with digoxigenin (DIG) by a DIG Protein Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. After labeling, the concentration of DIG-labeled IgY was determined by Chicken IgG ELISA Quantitation Kit (Bethyl laboratory).

Yolk IgY was extracted as described by Akita and Nakai (1992) with minor modification. Briefly, the follicular membrane was punctured and the yolk was allowed to pour into a glass dish. One g of the stirred yolk was replaced into a 50-mL polypropylene tube. Then, 720 ng of DIG-labeled IgY was added to the sample, which was diluted with 9 mL of distilled water acidified to pH 5.1 with HCl. After overnight storage at 4℃, the samples were centrifuged at 10000×g for 25 min at 4℃. The supernatant was collected, and an equal volume of saturated ammonium sulfate was added. After incubation for 4 h at room temperature, the samples were centrifuged at 12000×g for 30 min at 4℃. The pellet was resuspended in PBS (pH 7.4) and dialyzed at 4℃ overnight. The IgY concentration of the yolk extract was determined by Chicken IgG ELISA Quantitation Kit (Bethyl laboratory). To determine the recovery rate of IgY from the yolk, the DIG-labeled IgY concentration was measured by DIG-specific ELISA. Recovery of DIG-labeled IgY from the yolk was considered to represent total recovery of IgY. The details of DIG-specific ELISA are described below.

Quantitation of DIG-labeled IgY by ELISA

Microtiter plates (96-well; Nalge Nunc International, Rochester, NY, USA) were coated with 100 μL/well of sheep anti-DIG IgG (1/500; Roche Diagnostics) in 0.05 M sodium carbonate at pH 9.6 for 60 min at room temperature. After incubation, the coating antibody solution was discarded and the plates were washed with washing buffer (0.14 M NaCl, 50 mM Tris-HCl at pH 8.0 with 0.05% (v/v) Tween 20). To block nonspecific binding, the plates were filled with 200 μL/well of blocking solution (0.14 M

NaCl, 50 mM Tris-HCl at pH 8.0 with 1% (v/v) BSA (catalog number A-7284; Sigma-Aldrich) and incubated for 30 min; then the plates were washed. Subsequently, the plates were incubated with standards or samples at 100 μL/well for 60 min. The standards were prepared by serial dilutions of DIG-labeled IgY solution with working buffer (blocking solution containing 0.05% (v/v) Tween 20). The plates were then washed and incubated for 1 h with 100 μL/well of horseradish peroxidase-conjugated sheep anti-DIG IgG Fab fragments (1/3000; Roche Diagnostics). The plates were washed and a colored reaction was initiated by adding *o*-phenylenediamine solution (0.05 M citric acid and 0.05 M Na_2HPO_4 at pH 5.0 containing an *o*-phenylenediamine tablet (Sigma-Aldrich) with 0.01% (v/v) H_2O_2) for 10 min. The colored reaction was terminated by adding 100 μL of 3 M H_2SO_4, and absorbance was measured at a wavelength of 490 nm with a microtiter plate reader.

Statistical Analysis

Statistical analysis was performed with the SAS 6.12 program (SAS Institute, Cary, NC, USA). Data were analyzed by one-way ANOVA, and the comparison between means was assessed by a Tukey-Kramer's test. Differences between means were considered to be significant at $P<0.05$.

Results

We sought to compare two types of chickens: commercial-type layers (Dekalb and Nagoya) and chickens with characteristically high or low yolk IgY concentration. Therefore, we examined plasma IgY concentration in five inbred strains of chickens maintained at Nagoya University Avian Bioscience Research Center. Among these inbred strains, PNP/DO had the highest plasma IgY concentration. Thus, the yolk IgY concentration of PNP/DO was predicted to be high compared with those of the other four strains, because the amount of IgY transported into the egg yolk has been shown to be proportional to maternal serum IgY concentration (Loeken and Roth, 1983). On the other hand, the other four strains represented similar plasma IgY concentration, and there was no strain exhibiting extremely low IgY concentration. Accordingly, we used PNP/DO as a model bird representing a high blood IgY concentration.

Experiment 1: Egg weight, yolk weight and yolk water content

To obtain basic data on collected eggs, egg weight, yolk weight and water content were determined (Table 1). Dekalb and Nagoya produced eggs similar in size, whereas PNP/DO produced approximately 20% smaller eggs than those of Dekalb and Nagoya. Although there was a statistical difference in yolk water content between Nagoya and the other two strains, all mean values were scattered within a narrow range (47–49%).

Experiment 1: IgY concentration in egg yolk

First, plasma IgY concentration was measured by ELISA (Table 2). Plasma IgY concentration of PNP/DO was the highest among the three strains, and there was no significant difference between Dekalb and Nagoya. In all three strains, IgY recovery was approximately 50–60%; no significant difference was observed among them. The IgY recovery rate was used for calculation of yolk IgY concentration, by dividing the IgY recovery rate into the IgY concentration of the yolk extract. When yolk IgY concentration was expressed as mg/g yolk, PNP/DO showed the highest concentration of IgY (12.2 ± 2.0, $n=5$), and Dekalb and Nagoya had almost the same concentration of IgY (Dekalb, 6.2 ± 0.4, $n=5$; Nagoya, 5.7 ± 0.6, $n=5$). Strain differences in yolk IgY concentration were similar to those in plasma IgY concentration. Therefore, the concentration factor of yolk IgY against plasma IgY was approximately 0.8 in all three strains. We further calculated yolk IgY concentration expressed as mg/mL water in yolk. Since IgY is a hydrophilic protein, use of yolk IgY concentration expressed as mg/mL water in yolk might be more suitable for comparison to plasma IgY concentration. PNP/DO represented the highest concentration of IgY (25.0 ± 4.2, $n=5$), and Dekalb and Nagoya had a similar concentration of IgY (Dekalb, 12.6 ± 0.9, n $=5$; Nagoya, 12.1 ± 1.3, $n=5$). Strain differences in the latter IgY concentration (mg/mL water in yolk) were similar to those of the former IgY concentration (mg/g yolk), as there was little difference in the egg yolk water content among the three strains (Table 1). The concentration factor of yolk IgY against plasma IgY represented approximately 1.7 regardless of the strain, and these values were nearly double the former calculated values.

Experiment 2: Daily fluctuation of IgY concentration in yolk and in plasma

Finally, daily fluctuation of IgY concentration was examined. PNP/DO exhibited higher concentrations of plasma IgY and yolk IgY (Table 2), but its egg production was lower than those of Dekalb and Nagoya (data not shown). This raised the possibility that IgY concentra-

Table 1. Egg weight, egg yolk weight and water content in three strains of chickens

	Dekalb	Nagoya	PNP/DO	*P*-value
Egg weight (g)	60.2 ± 0.7^{a}	61.1 ± 1.2^{a}	49.0 ± 1.1^{b}	<0.0001
Egg yolk weight (g)	16.9 ± 0.2^{b}	18.2 ± 0.2^{a}	14.2 ± 0.5^{c}	<0.0001
Water content (%)	49.0 ± 0.2^{a}	47.3 ± 0.2^{b}	48.6 ± 0.1^{a}	<0.0001

[a–c] Mean values within a row that have different letters are different at $P<0.05$.
Values are means$\pm$SEM of 5 samples.

Table 2. Plasma IgY and egg yolk IgY concentrations in three strains of chickens

	Dekalb	Nagoya	PNP/DO	*P*-value
IgY in blood				
IgY (mg/mL plasma)	8.93±1.01[b]	6.69±0.96[b]	14.29±1.46[a]	0.0018
Recovery of IgY				
DIG-labeled IgY recovered from yolk (%)[1]	61.7 ±6.0	58.6 ±4.8	52.7 ±5.1	0.5859
IgY in yolk				
IgY (mg/g yolk)	6.19±0.44[b]	5.73±0.64[b]	12.2 ±2.04[a]	0.0060
Ratio to plasma IgY	0.73±0.08	0.91±0.13	0.84±0.08	0.4571
IgY in yolk water fraction				
IgY (mg/mL water in yolk)	12.6 ±0.9[b]	12.1 ±1.3[b]	25.0 ±4.2[a]	0.0065
Ratio to plasma IgY	1.48±0.17	1.92±0.28	1.72±0.16	0.3630

[a–c] Mean values within a row that have different letters are different at $P<0.05$. Values are means±SEM of 5 samples.
[1] To measure IgY recovery rate in the process of extraction, DIG-labeled IgY (720 ng/sample) was added to egg yolk samples.

tions in plasma and egg yolk fluctuate depending on the timing of the sample collection. Thus, in PNP/DO and Dekalb, individual egg production was recorded for 10 days, and all laid eggs during the experimental period were analyzed for yolk IgY concentration. Plasma was collected every three days to avoid reduction of egg production caused by blood sampling. Egg production of PNP/DO ranged from 50 to 70%, whereas egg production of Dekalb ranged from 90 to 100% (Fig. 1). Both plasma IgY and yolk IgY of PNP/DO was approximately two-fold higher than those of Dekalb, which was similar to the result shown in Table 2. In both strains, the individual data of IgY concentrations in plasma and in yolk were distributed into a narrow range, and marked daily fluctuations were not observed.

Discussion

Reported yolk IgY concentrations have varied from 1 to 25 mg/g yolk (Patterson *et al.,* 1962; Cutting and Roth, 1973; Rose *et al.,* 1974; Kowalczyk *et al.,* 1985; Li *et al.,* 1998; Carlander *et al.,* 2001; Hamal *et al.,* 2006). By our measurements, the yolk IgY concentration of three strains of chickens ranged from 5.7–12.2 mg/g yolk, which represent intermediate values of the previously reported yolk IgY concentrations.

In the present study, we applied the water dilution method for extraction of IgY from yolk (Akita and Nakai, 1992), and the IgY recovery rate in its extraction step was determined. The resulting IgY recovery rate was 50–60% (Table 2), while Akita and Nakai (1993) reported that 91% of yolk IgY was recovered by the water dilution methods. The reason for the difference in the IgY recovery rate between their result and ours is unclear, but the present results suggest that correction by IgY recovery rate is essential to estimate IgY concentration accurately.

The present study showed that the yolk IgY concentration of PNP/DO was doubled compared with those of Dekalb and Nagoya, implying that yolk IgY concentration varies among genetic lines or breeds. At this point, our results are consistent with the previous reports of Schade *et al.* (2005) and Hamal *et al.* (2006). Of these, Schade *et al.* (2005) described that yolk IgY concentrations of Single Comb White Leghorn, SLU-1329 and Rhode Island Red were 2.2±0.4 mg/mL, 2.0±0.5 mg/mL and 1.7±0.5 mg/mL, respectively. Hamal *et al.* (2006) used two lines of broiler type chickens and determined their IgY concentration in yolk and in plasma; although there were significant differences in yolk and plasma IgY concentrations between the two lines, yolk IgY concentration was parallel to the plasma IgY concentration in both. The present study also indicated that the yolk IgY concentration is proportional to the plasma IgY concentration. These results suggest that strain differences in yolk IgY concentration are mainly attributable to variations in plasma IgY concentration.

The concentration factor of yolk IgY against plasma IgY should provide valuable information for characterizing the mechanism of IgY transport into ovarian follicles. Vitellogenin, which composes very-low-density lipoprotein as an apolipoprotein, is incorporated into yolk by receptor-mediated endocytosis (Stifani *et al.,* 1990). Cutting and Roth (1973) reported that phosvitin, a component of vitellogenin, was nine-fold concentrated in yolk relative to that in blood. In addition, riboflavin and its binding protein were also incorporated into the yolk by receptor-mediated endocytosis (Mac Lachlan *et al.,* 1994), and they are six-fold more concentrated in yolk than in blood (White *et al.,* 1986). If the IgY concentration in yolk is found to be higher than that in plasma, a mechanism by which IgY is actively transported into ovarian follicles might exist. However, it has been reported that yolk IgY concentration is less than or comparable to plasma IgY concentration (Cutting and Roth, 1973; Kowalcyzyk *et al.,* 1985; Hamal *et al.,* 2006). The same result was obtained in the present study; the concentration factor of yolk IgY against plasma IgY was 0.7–0.9

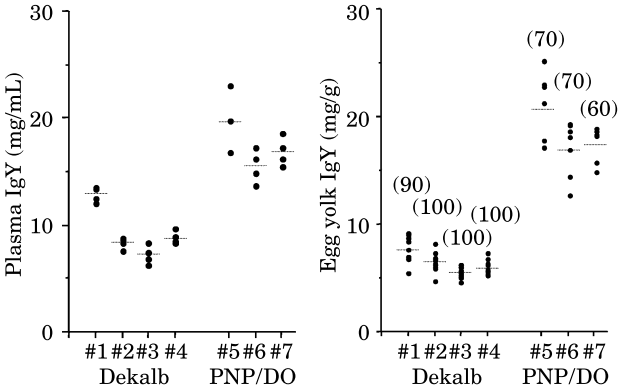

Fig. 1. Daily fluctuation of plasma and egg yolk IgY concentrations in individual chickens. All laid eggs from Dekalb and PNP/DO were collected for 10 days, and blood samples were collected for 4 times, every 3 days (day 1, 4, 7 and 10). #1–7 indicates ID number of chicken, and all measured values were plotted as dots. Horizontal bars represent means of the IgY concentrations in each bird. Values in parentheses indicate egg production during the experimental period.

when yolk IgY was expressed as mg/g yolk (Table 2). Apparently, the concentration factor of yolk IgY is lower than those of phosvitin and riboflavin. We further calculated the concentration factor by another method. Since yolk includes lipids at 30–35%, IgY is not uniformly distributed in the yolk. Therefore, IgY concentration expressed as mg/mL water in yolk may be more meaningful for calculation of a concentration factor against plasma IgY. The result showed that the concentration factor of yolk IgY against plasma IgY is approximately 1.7 regardless of strain (Table 2), suggesting that blood IgY is concentrated to some extent in egg yolks of chickens. Although it is uncertain whether this calculation is suitable for conceptualizing IgY concentration, this alternative result raised the possibility that IgY is actively transported into yolks of chickens.

In conclusion, our findings suggest that concentration of IgY in yolk should be determined by considering the recovery rate during the process of IgY extraction. When yolk IgY concentration was corrected by the recovery rate, it ranged from 5.7 to 12.2 mg/g yolk in the three strains of chickens. Our findings also show that strain difference of yolk IgY concentration is mainly attributable to a variation of plasma IgY concentration. Finally, we suggest that accurate determination of yolk IgY concentration may provide clues as to how ovarian follicles incorporate blood IgY into the yolk.

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