Research Note

A Study on Eggshell Pigmentation: Biliverdin in Blue-Shelled Chickens

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ABSTRACT Biliverdin is an important pigment in the eggshell of chickens and other avian species. Determination of the biosynthesis site for biliverdin is essential for understanding the biochemical process and genetic basis of eggshell pigmentation. Either blood or the shell gland could be the biosynthesis site of eggshell biliverdin. A segregation population with full-sib sisters genotyped *Oo* and *oo*, which laid blue-shelled eggs and light brown eggs, respectively, was constructed in a native Chinese chicken breed. Ultraviolet spectrophotometry and HPLC were used to determine the biliverdin concentration in eggshells, blood, bile, excreta, and shell gland of both groups of chickens. Biliverdin content was significantly different between egg shells of blue-shelled and brown-shelled chickens ($P < 0.01$). Blood and bile were tested 3 to 4 h before oviposition, and excreta was tested randomly. Results showed no significant difference in biliverdin concentration in blood, bile, and excreta between the 2 groups. In the shell gland, the biliverdin contents for the blue-shelled and brown-shelled chickens were 8.25 ± 2.55 and 1.29 ± 0.12 nmol/g, respectively, which showed a significant difference ($P < 0.01$). Our results demonstrated that blood is not the biosynthesis site of the shell biliverdin. Biliverdin is most likely synthesized in the shell gland and then deposited onto the eggshell of chickens.

Key words: biliverdin-IX, blue-shelled chicken, *O* locus, pigmentation, shell gland

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INTRODUCTION

Kennedy and Vevers (1973) found that the blue eggs laid by Araucano contained biliverdin-IX, zinc biliverdin chelate, and protoporphyrin-IX in the eggshells. A more extensive survey among 108 avian species conducted by Kennedy and Vevers (1976), together with the research by Schwartz et al. (1980), showed that only biliverdin-IX and zinc biliverdin chelate could be detected in blue or green eggs of the domesticated fowl, whereas brown-shelled eggs contained large amounts of protoporphyrin. Biliverdin-IX is a derivative of heme, which results from heme oxygenase-1 activity in the porphyrin pathway and plays an important role in the biochemistry of all living systems (Lang and Wells, 1987). The bright colors may function as honest female signals of fitness, owing to the presence of the antioxidant pigments on the shells, and may affect male parental effort (Moreno and Osorno, 2003; Moreno et al., 2004). Therefore, studies on blue-shelled chickens are of great importance for biological understanding of the nature of avian eggshell pigmentation.

Oocyan, a dominant gene on chromosome 1, accounted for the blue shell appearance (Punnett, 1933 as cited in Kennedy and Vevers, 1973; Bitgood et al., 2000], and *O* (*oocyan*) locus was 2.3 cM away from *ALEV1* (avian leukemia endogenous virus element 1; Bartlett et al., 1996). In China, there remains a population of Dongxiang blue-shelled chickens similar to the Araucano with respect to specific shell color, and we demonstrated that eggshell color of Dongxiang blue-shelled chickens and Araucano resulted from the same genetic background by association analysis of molecular markers closely related to the *O* locus (Yang et al., 2003).

The shell gland pouch was considered to transfer the pigment and secrete the cuticular layer (Solomon, 2002). The experiments conducted in the brown-shelled chickens with protoporphyrin, of which all carbon and nitrogen atoms are from glycine and succinic acid, suggested that protoporphyrin was first synthesized in the shell gland and then secreted and deposited onto the eggshells (Baird et al., 1975). Biliverdin is derived by oxidation and ring opening of the prosthetic groups of haemoproteins (Hudson and Smith, 1975), which produce primarily biliverdin-IX in birds, amphibians, and fish and bilirubin in mammals as the end product in porphyrin pathway (Cornelius, 1991). Thus, in view of the different origins of the 2 main pigments of the eggshells, porphyrins were considered to be synthesized in shell gland epithelial cells, whereas biliverdin was postulated to be derived from either erythrocytes (Kennedy and Vevers, 1973; Lang and Wells, 1987) or from the shell gland (S. E. Solomon and M. M. Bain, 2005, personal communication). The present study was designed to compare biliverdin content in different tissues, which may provide evidence for the bio-

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synthesis site of eggshell biliverdin in blue-shelled chickens.

MATERIALS AND METHODS

Experimental Design and Sample Collection

A segregation population was constructed by crossing the heterozygotes (*Oo*) in Dongxiang blue-shelled hens, which were genotyped by progeny testing, with the White Leghorn males (*oo*). Only families with full-sib sisters of both blue-shelled layers (*Oo*) and brown-shelled layers (*oo*) were selected for further study. A total of 78 hens of both groups were individually caged and provided with commercial layer feed and an artificial lighting program of 16L:8D.

Clutch size of the birds was observed from 28 to 31 wk, and oviposition time was recorded 4 times each day at 0830, 1130, 1430, and 1700 h. Only the birds with fairly uniform laying intervals were selected for sacrifice 3 to 4 h before the next expected oviposition, as the shell pigmentation of domesticated chickens was considered to reach a peak about 2 to 3 h before oviposition. Birds were slaughtered at 0600, 0830, and 1100 h, respectively. Eggs and excreta were collected fresh 2 d before the birds were slaughtered, and bile samples were collected at 0600 h in the morning after the animals had been fasted for 24 h. The shell gland was weighed and collected, and the eggs in the shell gland were stored individually. All samples were protected from light and stored at –30°C until analysis. In addition, White Leghorns of similar age were selected for comparison, and eggs from these hens were sampled for further analysis.

Sample Preparation and Measurement Method

Standard biliverdin was purchased from Porphyrin Products Inc. (Logan, UT). Solvents and reagents for standard solutions and chromatography were methanol and acetonitrile (HPLC grade) from Fisher Scientific (Fair Lawn, NJ), ammonia acetate from Beijing Chemical Reagents Company (Beijing, China), hydrochloric acid (37%) from Beijing Fine Chemicals Co. Ltd. (Beijing, China), and Milli-Q grade water (Millipore, MA).

Blood samples of the killed birds were collected, and serum was transferred into Eppendorf tubes after centrifugation at 3,000 rpm for 5 min. Bile and fresh excreta of birds were vortexed in an Eppendorf tube for 15 s with 1.5 mL of mixed solution with 3N HCl:acetonitrile:water (3:3:2). The shell gland pouch was first grounded into powder in liquid nitrogen and then mixed with the mixed solution and vortexed. Bile, excreta, and shell gland samples were centrifuged for 10 min at 16,000 rpm, and 0.3 mL of supernatant was transferred to Eppedorf tubes for HPLC and ultraviolet (**UV**) spectrophotometry analysis. Eggshells were washed by Milli-Q water and then solubilized without shell membranes in the mixed solution at room temperature in the dark for 2 d. The colored extract was collected and subjected to UV spectrophotometry analysis with the eggshell extract of White Leghorn as a reference.

A spectral screen was performed on a Unicol UV spectrophotometer UV-2800 (Shanghai, China) from 190 to 1,100 nm to get the maximum absorption peak using the standard solution, and 2 peaks were detected at 374 and 670 nm, respectively. Both HPLC and UV spectrophotometric methods were performed in bile for comparison.

Biliverdin content in bile and excreta samples was analyzed by reversed-phase HPLC following the procedures of Mateo et al. (2004) using an Alliance 2695 system with Atlantis dC18 column (5 μM particle size, 4.6 mm × 100 mm) and was detected by UV-vis spectrometry at 374 nm. The column was maintained at 70°C, and the flow rate was 1.5 mL/min. The initial mobile phase of the solvent gradient was 25% methanol and 75% ammonium acetate (2.0 m*M*). The solvent gradient consisted of an 8-min linear change to 95% methanol and 5% ammonium acetate and was maintained in that condition for 2 min, after which the phase composition returned to the initial conditions. The total run time was 20 min.

The UV spectrophotometric method for the assay of biliverdin in serum, bile, shell gland, and eggshells was performed at 670 nm to avoid the disturbance of impurities in the prepared samples, and pigment measurement in excreta was subjected to HPLC. The quantification of samples was performed using calibration curves constructed with standard solutions. Calibrations were prepared as samples, and concentrations in the 5 calibration points were 56.5, 28.3, 14.1, 7.1, and 3.5 μ*M* with R values of 0.9998 and 0.9996 using HPLC and UV spectrophotometry, respectively. The percentage recovery of the extraction procedure was calculated by comparing standards and samples spiked with biliverdin ranging from 96.17 to 116.49% for the 5 types of samples. Precision of the samples varied from 0.18 to 1.51%.

Statistical Analysis

The correlation coefficient between biliverdin content in bile measured by HPLC and by UV spectrophotometry was calculated by means of Pearson correlations. Comparison of biliverdin content in different types of samples between blue-shelled and brown-shelled chickens was subjected to one-way ANOVA and Duncan's multiple range test. All of the analyses were performed with the SAS 8.2 package (SAS Institute, 1999).

RESULTS AND DISCUSSION

Because biliverdin existed in combination with cuticular material in eggshell and shell gland (Nakano et al., 2003), a UV spectrometry analysis was used to minimize the loss of biliverdin during the complex steps of pigment extraction and concentration. Pigment measurement in excreta was subjected to HPLC because of the interference of impurities in excreta when using the UV spectrometer.

Table 1. Comparison of biliverdin content in different tissues

Sample[1]	Method	Unit	Biliverdin concentration		
			Blue shell	Brown shell	Contrast
Serum	UV[2] spectrometry	μmol/L	5.48 ± 2.81	5.28 ± 2.87	NS
Bile	HPLC	nmol/g	865.59 ± 220.55	907.55 ± 224.44	NS
	UV spectrometry	nmol/g	761.68 ± 276.62	878.37 ± 274.67	NS
Excreta	HPLC	nmol/g	22.88 ± 8.95	21.99 ± 6.86	NS
Shell gland	UV spectrometry	nmol/g	8.25 ± 2.55	1.29 ± 0.12	$P < 0.01$
Eggshell[3]	UV spectrometry	nmol/g	2.60 ± 1.15	0.14 ± 0.01	$P < 0.01$
Eggshell[4]	UV spectrometry	nmol/g	3.59 ± 1.31	0.15 ± 0.03	$P < 0.01$

[1]n = 10.
[2]UV = ultraviolet.
[3]From eggs collected in shell gland.
[4]From eggs laid.

Comparison between HPLC and UV spectrometry was conducted in bile pigment content measurement and the correlation coefficient between the 2 methods was 0.826 ($P < 0.001$), suggesting the reliability of the UV spectrophotometric method.

Results of biliverdin content between different groups in serum, bile, excreta, shell gland, and eggshells are listed in Table 1. There was no significant difference in the pigment concentration in serum, bile, and excreta between blue-shelled and brown-shelled chickens. In both groups, biliverdin content in serum was much lower than that in bile and excreta. Biliverdin content in the shell gland of blue-shelled individuals was much higher than that of brown-shelled chickens when the eggs were in the shell gland ($P < 0.01$).

Studies with mammals have shown that bilirubin, which is reduced from biliverdin during porphyrin metabolism, as the end product, was mainly synthesized from mature erythroid cells and liver cells, and then entered into gall bladder and was excreted as by-products (Ponka, 1997). Lacking biliverdin reductase, chickens mainly excreted biliverdin (Cornelius, 1991). Previous studies implied that either blood or shell gland could be the origin of the shell biliverdin. However, there was no experimental evidence to support either of the hypotheses. In the current study, biliverdin concentration comparison was conducted from serum and 2 major excretory sites of blood pigment, bile and excreta; serum and bile were measured 3 to 4 h before oviposition, and excreta was randomly collected before animals were slaughtered. Similar pigment concentration in blue-shelled and brown-shelled birds demonstrated both directly and indirectly that blood was not the origin of shell biliverdin.

As expected, the current results showed a higher biliverdin content in the shell gland of blue-shelled individuals (8.25 ± 2.55 nmol/g) compared with that of brown-shelled chickens (1.29 ± 0.12 nmol/g). Turchini (1924 as cited in Kennedy and Vevers, 1973) reported that the lining of the uterus of the fowl, unlike the mammalian uterus, contained porphyrin granules in the epithelial cells. Hijmans van den Bergh and Grotepass (1936 as cited in Kennedy and Vevers, 1973) considered that protoporphyrin was formed at the same time as haemoglobin, rather than as a breakdown product of the blood pigment. Tamura and Fujii (1965, 1967 as cited in Schwartz et al., 1980) found pigment granules containing porphyrin in the apical cells together with larger periodic acid-Schiff-positive granules that contributed to the organic matrix, and the granules increased gradually until cuticle deposition when both granule numbers declined rapidly. In the present study, the shell biliverdin content of blue-shelled chickens was 2.60 nmol/g when the eggs were in the shell gland, 0.72 times that when the eggs were laid, which suggested that significant amounts of eggshell biliverdin had been deposited onto the eggshells 3 to 4 h before the oviposition. This work, together with that of Polin (1957 as cited in Lang and Wells, 1987), Poole (1966 as cited in Lang and Wells, 1987), Yamada (1972), and Baird et al. (1975) lends strong support for the hypothesis that the avian shell gland is most likely to be the site of biosynthesis and secretion of both eggshell porphyrins and biliverdin.

Trace amounts of coprotoporphyrin as well as biliverdin, zinc biliverdin chelate, and an increased quantity of protoporphyrin were found in the eggshells of the Araucano fowl (Kennedy and Vevers, 1973; Schwartz et al., 1980), which implied that the product of the porphyrin pathway was boosted in the shell gland of blue-shelled chickens by the *O* gene. In Japanese quail, *ce* (*celadon*) was responsible for the glossy pale blue shell, which resembled the eggshell of the Araucano hen both in the external appearance and pigment content (Ito et al., 1993). According to the survey by Kennedy and Vevers (1976), many other species, including the Purple heron, Little egret, Buzzard, Knysna lourie, and others had blue eggshells containing biliverdin. Therefore, the present work lays a foundation for future research on the blue-shelled gene and also on the physiological and histological study of the avian shell gland.

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