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RESEARCH ARTICLE

WILEY JEZ-B MOLECULAR AND DEVELOPMENTAL EVOLUTION

Fate of egg proteins during the development of *Columba livia domestica* embryo

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Abstract

The transfer of egg white into the yolk and consumption of yolk proteins by the embryo are largely unexplored in the pigeon *Columba livia domestica*. Here, we investigated the route of egg white transfer as well as the degradation and uptake of yolk proteins by the pigeon embryo. Initially, we tested the electrophoretic patterns of proteins in different egg compartments throughout development. Then, we used lysozyme as a reference protein to follow the egg white transfer, and we measured its activity using *Micrococcus lysodeikticus* as a substrate. Moreover, we determined the general protease activity during different developmental stages in the yolk using casein. Finally, we examined the expression of aminopeptidase-N (*APN*) and oligopeptide transporter *PepT1* genes in the yolk sac membrane (YSM) from incubation day 8 until day 17. Several electrophoretic bands of presumptive egg white proteins appeared in different egg compartments. Also, lysozyme activity was detected chronologically in the egg compartments. It appeared on day 12 in the amniotic and intestinal fluids and on day 14 in the yolk. Moreover, protease activity in the yolk increased significantly on day 14 and thereafter. *APN* expression was largest on day 8 and reduced generally afterward, whereas *PepT1* expression peaked between days 13 and 15 but then reduced substantially. Our results suggest that the egg white proteins move through the amnion and intestine into the yolk where they undergo degradation by the activated proteases. Furthermore, the YSM appears to have a role in protein consumption, and this role decreases toward hatch.

KEYWORDS

aminopeptidase-N, *Columba livia domestica*, egg white, egg yolk, lysozyme, oligopeptide transporter, protease, pigeon

1 | INTRODUCTION

Egg white and egg yolk are the two main sources of nutrients for the developing embryo inside the fertilized avian egg (Cotterill & Geiger, 1977). The egg white is composed of about 88.5% water, 10.5% proteins, 0.5% carbohydrates, and 0.5% of various minerals and vitamins (Gilbert, 1971; Stevens, 1991; Vieira, 2007). The egg yolk is made of approximately 50% water, 33% fat, 15% proteins, less than 1% carbohydrate, and the rest of different minerals and vitamins (Shenstone, 1968; Vieira, 2007). The egg white eventually reaches the yolk before being taken up by the embryo (Saito et al., 1965; Saito & Martin, 1966; Carinci & Manzoli-Guidotti, 1968; Baintner & Fehér, 1974; Sugimoto et al., 1984, 1989; Yoshizaki et al., 2002, 2004; Shbailat & Safi, 2015). It generally transfers through the amniotic fluid into the intestinal lumen, and then to the yolk (Carinci & Manzoli-Guidotti, 1968; Baintner & Fehér, 1974; Sugimoto et al., 1989; Yoshizaki et al., 2002; Shbailat & Safi, 2015), or directly moves to the yolk through the yolk sac umbilicus (Sugimoto et al., 1984, 1989). Alternatively, the egg white proteins can reach the entire body (Saito & Martin, 1966; Sugimoto et al., 1999) via the blood vessels at the extraembryonic cavity (Yoshizaki et al., 2002).

The transferred egg white and endogenous yolk proteins appear to undergo digestion by the activated proteases before being consumed by the developing embryo. However, the time of protease activation varies among different avian species. In the chicken *Gallus gallus* and turkey *Meleagris gallopavo*, the major transfer of egg white proteins through the amniotic sac and intestinal lumen into the yolk started on day 14 and day 17, respectively (Carinci & Manzoli-Guidotti, 1968; Shbailat & Safi, 2015). This transfer was followed by a rapid increase in the activity of proteases after day 14 in chicken (Sugimoto & Yamada, 1986), and on day 19 and thereafter in turkey (Shbailat et al., 2016). On the other hand, in the quail *Coturnix japonica*, the activation of protease between days 6 and 12 preceded the major transfer of egg white through the embryo

into the yolk, which occurred after day 12 (Yoshizaki et al., 2002, 2004).

The yolk mass is surrounded by a cell membrane at early stages of development, however, after the degeneration of this membrane, the yolk becomes completely surrounded by the yolk sac membrane (YSM) (Yoshizaki et al., 2004; Sheng & Foley, 2012). Previous studies showed that the YSM also has a role in the degradation of underlying yolk proteins during development. In quail, yolk proteins that were sequestered by the endodermal cells of YSM seemed to be broken down by the cysteine and aspartic proteases during the first week of embryogenesis, but after that, the membrane stopped metabolizing yolk (Gerhartz et al., 1997, 1999). In chicken, during early stages of development, the proteins, lipoproteins, and lipids were suggested to be taken via endocytosis by the apical surfaces of yolk sac endodermal cells. Inside cells, they underwent breakdown and re-synthesis, and the new products were exported from the basal surfaces of cells into the extraembryonic circulation, and then to the embryo (Mobbs & McMillan, 1979, 1981; Speake et al., 1998; Nakazawa et al., 2011). In the second half of chicken embryo incubation, the expression of aminopeptidase-N (*APN*) and oligopeptide transporter *PepT1* genes was investigated in the YSM (Yadgary et al., 2011; Speier et al., 2012). *APN* encodes for a membrane-bound zinc metallopeptidase that cleaves amino acids from the N-terminus of oligopeptides (Hooper, 1994), while *PepT1* encodes for a membrane transporter of di and tripeptides (Fei et al., 1994; Daniel & Kottra, 2004). The expression of both genes increased until day 15, and then decreased toward hatch (Yadgary et al., 2011; Speier et al., 2012). The expression of *APN* and *PepT1* was also examined in the YSM of the pigeon *Columba livia domestica* and was found to decrease after incubation days 14 and 12, respectively (Dong et al., 2012b).

It is worth mentioning that the chicken, turkey, and quail are precocial birds that hatch in a mature and mobile form, and require no parental care (Starck & Ricklefs, 1998). In contrast, the altricial birds, like the pigeon, hatch in a form that is immature, incapable of moving around, and fully dependent on parents (Starck & Ricklefs, 1998). Although the developmental dynamics and nutrition consumption are well studied in precocial birds, further studies are required to be conducted in altricial birds. In pigeon, the transfer of egg white and degradation of yolk proteins by the activated proteases are still unexplored. Moreover, gene expression analyses that explored the role of YSM in the digestion and uptake of egg proteins by the developing embryo were only examined in one study (Dong et al., 2012b). However, more developmental stages are required to be sampled to further explore the contribution of YSM in protein consumption. In addition, understanding the contribution of YSM should be part of a comprehensive study that sheds light on the overall mechanisms underlying the transport, degradation, and absorption of egg white proteins. Here, we examined the route(s) of egg white transfer into the yolk in the fertilized pigeon egg. Then, we measured the protease activity in the egg yolk throughout out development. After that, we investigated the expression of *APN* and *PepT1* genes in the YSM during different developmental stages.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The study was approved by the Institutional Review Board (IRB) at The Hashemite University, and animal care, use, and all experimental protocols were carried out in accordance with the approved guidelines.

2.2 | Egg incubation and embryo staging

The males and females of *Columba livia domestic* (Arabian Trumpeter) were purchased from local farms in Amman. The laying hens were about 180 days old. Fertilized pigeon eggs were collected immediately after laying and kept in an incubator (Brinsea, Titusville, FL) at 38.5 ± 0.5°C and relative humidity of 60%. They were subjected to an automatic see-saw motion every 1 hr. The developmental stages of the embryo were measured from day 0 to day 17 (the day of hatch). They were identified according to the criteria published in Olea et al. (2012) for pigeon, with reference to the classification of Hamburger and Hamilton (1951) for chicken.

2.3 | Isolation and preparation of different egg compartments

The eggs were opened at their pointed ends at each developmental stage. The eggs were held horizontally above a light source in order to determine their pointed ends, which were opposite to the blunt ends where the air sacs were found. Different egg fluids were collected based on the methods published by Shbailat and Safi (2015). The allantoic fluid, if present, was carefully collected from the cavity that is surrounded by the chorio-allantoic membranes. The collection of the allantoic fluid was necessary to prevent the contamination of other egg fractions. The amniotic fluid was withdrawn directly from the amniotic sac using 1-ml syringe with a 25-G needle. After that, the embryo was carefully removed from the egg and dissected under the dissecting microscope. The complete gastrointestinal canal was removed outside the body, and then the whole intestine was separated, wiped with soft paper, and opened longitudinally using a fine blade. The intestinal fluid was gathered from the lumen using 10-μl micropipette and a fine spatula.

The egg yolk was separated into thin and thick portions (upper and lower, respectively). The thin portion was collected by puncturing the yolk sac using a 5-ml syringe with a 21-G needle. The thick portion was collected by making 0.5 cm in diameter hole in the yolk sac and sucking the egg yolk using 5-ml syringe without needle. The absorption of the yolk sac by the abdomen of the embryo started on incubation day 16 and was completed on day 17. Therefore, during the last two stages of development, the yolk sac was removed outside the eggshell together with the embryo, and a hole was made in the embryo abdomen to pull the yolk sac outside the body. Following the complete removal of the yolk sac and its contents, the underlying egg white was decanted from the eggshell and cleaned as thoroughly as possible from the adhering chorio-allantoic membranes.

The intestinal fluid, egg yolk, and egg white compartments were further processed for use in biochemical analyses (Yoshizaki et al., 2002; Shbailat & Safi, 2015). The intestinal fluid was centrifuged at maximum speed for 30 sec, and the supernatant was collected. The two yolk portions (thin and thick) were diluted with five volumes of 0.87% NaCl solution, which contained 1 mM ethylenediaminetetraacetic acid-disodium, dihydrate ($Na_2EDTA.2H_2O$). The diluted samples were then homogenized using a tissue homogenizer (Omni International, Kennesaw, GA), and each homogenate was centrifuged at 4,427 *g* for 30 min at 4°C. Following that, the supernatant was dialyzed against distilled water for 2 hr. The dialyzed solution was then centrifuged twice at 4,427 *g* for 30 min at 4°C, and the supernatant was collected each time. The egg white was diluted with five volumes of distilled water. The diluted sample was homogenized, centrifuged at 4,427 *g* for 30 min at 4°C, and then the supernatant was gathered.

2.4 | Denaturing polyacrylamide gel electrophoresis

The amniotic, intestinal, egg white, and thin and thick yolk fluids were diluted with distilled water before loading in the gel such that the final dilution factor was 48. The different proteins in different egg compartments were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis based on the protocol in Laemmli (1970) with slight modifications using 10% separating gels. The gels were stained in Coomassie Brilliant Blue solution. Chicken egg white ovalbumin and lysozyme were used as standard proteins in the denaturing gels, and broad-range marker proteins (10–250 kDa; New England BioLabs, Ipswich, MA) were used for the construction of a standard protein curve to estimate the molecular weights of several protein bands.

2.5 | Lysozyme activity assay

The lytic activity of lysozyme was measured using *Micrococcus lysodeikticus* bacteria (ATCC. 4698 lyophilized cells; Sigma–Aldrich, Saint Louis, MO) as a substrate following the protocol in Shugar (1952). The reaction mixture contained 0.0962 mg of freeze-dried bacteria in 0.962 ml of 66 mM potassium phosphate buffer (pH 6.24) at 25°C. The lysozyme solution (fluid from one egg compartment at certain developmental stage) at a volume of 0.038 ml was added to the mixture, and the decrease in absorbance at 450 nm was recorded every 30 sec for 5 min. The negative control contained 0.038 ml of 66 mM potassium phosphate buffer (pH 6.24) instead of lysozyme solution, while the positive control had chicken egg white lysozyme at a final concentration of 0.001 mg/ml. One unit of lysozyme activity was defined as 0.001 change in absorbance per min. The activity was estimated from the maximum linear line of absorbance that is plotted as a function of time, using a minimum of four absorbance points.

2.6 | Measurement of protease activity

General protease activity was measured according to the protocol in Shimogaki et al. (1991) and Cupp-Enyard (2008) with modifications. Briefly, 0.15 ml of the yolk samples were diluted with 0.35 ml of 20 mM Tris–HCl buffer (pH 7.2) containing 2 mM calcium acetate. The samples were added to 2.5 ml of substrate solution containing 0.65% casein in 20 mM buffers at different pH. The buffers used were sodium acetate-glacial acetic acid (CH_3COONa-CH_3COOH, pH 5.0 and 5.5), disodium hydrogen phosphate-sodium dihydrogen phosphate, dihydrate (Na_2HPO_4-$NaH_2PO_4.2H_2O$, pH 6–8), glycine-sodium hydroxide ($C_2H_5NO_2$-NaOH, pH 9.0), borax-sodium hydroxide ($Na_2B_4O_7.10H_2O$-NaOH, pH 10.0), and disodium hydrogen phosphate-sodium hydroxide (Na_2HPO_4-NaOH, pH 11.0). After 10 min incubation at 37°C, the reactions were terminated by the addition of 2.5 ml of 110 mM trichloroacetic acid. The mixtures were kept for 30 min at 37°C, and then filtered using a 0.45 μM polyethersulfone syringe filters. Afterward, 2.5 ml of 0.5 M sodium carbonate was added to 1 ml of each filtrate, followed immediately by the addition of 0.5 ml of 0.5 M Folin & Ciocalteu's phenol reagent. The mixtures were incubated for 30 min at 37°C, and after their filtration, the absorbance was measured using 660 nm wavelength. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per minute under the conditions described above.

2.7 | RNA extraction, cDNA synthesis, and gene expression analysis

Total RNA was extracted from YSM tissues during different developmental stages using 1 ml of TRIzol reagent (Ambion, Carlsbad, CA) per 100 mg of each tissue according to the manufacturer's protocol (Suppl. Figure S1). Different independent samples were used at each stage. After DNase-1 treatment and phenol/chloroform purification, the RNA extracts were reversed transcribed to cDNAs using ProtoScript Single Strand cDNA Synthesis Kit (New England BioLabs). Briefly, 1 μg of each RNA extract was reversed transcribed by M-MuLV enzyme at 42°C for 1 hr using Oligo (dT) adaptor primer following the manufacturer's procedure. The synthesized cDNAs were then used to perform quantitative real time reverse transcription PCR (qRT-PCR) using LINE GENE9600 PLUS real time PCR detection system (Bioer Technology, Hangzhou, Zhejiang, China) to determine the expression levels of *APN* and *PepT1* genes during different developmental stages. The expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control to normalize for initial variations in sample concentration and as a control for reaction efficiency. qRT-PCR was carried out using PowerUp SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and gene specific primers. The following primers were designed and used at a final concentration of 0.6 μM in 20 μl reaction: *APN* Forward 5′-CTTTCAACGAGGACGAGATCAA-3′, *APN* Reverse 5′-GTGAGGAAGTCAGAGAGCATTC-3′, *PepT1* Forward 5′-CTGTGCAGATCCCTCAGTATTT-3′, *PepT1* Reverse 5′-CAGCA CTGCCTTCATGTTAGA-3′, *GAPDH* Forward 5′-GGTGGTGCTAA GCGTGTTAT-3′, and *GAPDH* Reverse 5′-CAGGCAGTTAGTAGTGC AAGAG-3′. The PCR conditions used were: 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 sec, 57°C for 15 sec, and 72°C for 1 min. The specificity of amplification was verified by performing melting curve analysis under the following conditions: 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. PCR products were visualized using 1.5% agarose gels stained with ethidium bromide. Fluorescence emission was detected and relative quantification was calculated

automatically using the software of LINE GENE9600 PLUS real time PCR detection system.

2.8 | Statistical analysis

Data were analyzed using IBM SPSS statistics version 21 (SPSS INC., Chicago, IL). One-way analysis of variance (ANOVA) was used to assess the presence of any significant differences among the means of lysozyme or protease activities in certain egg fraction during different developmental stages. Also, it was used to assess the presence of any significant differences among the means of *APN* and *PepT1* gene expression levels in YSMs at different stages. One-way ANOVA test was followed by least significant difference test to determine the groups that differ significantly from each other during the measurement of the enzymatic activities or gene expression levels. The *F* value in the ANOVA test was calculated according to the following formula: $F\,(df_{between}, df_{within}) = MS_b/MS_w$, where $df_{between}$: the degree of freedom between groups, df_{within}: the degree of freedom within groups, MS_b: mean square between groups, MS_w: mean square within groups. The level of significance was set at $P \leq 0.05$.

3 | RESULTS

3.1 | Electrophoretic patterns of different egg compartments during different developmental stages

Egg white was collected on day 0 immediately after the eggs were laid by the pigeons until day 15, when only remnants of egg white remained. Figure 1A shows the different electrophoretic protein bands in the egg white samples at different developmental stages. The two bands marked with open arrowheads and have approximate weight of 53.17 and 48 kDa, as estimated from the standard curve, were assumed to be ovalbumin 1 and ovalbumin 2, respectively (Figure 1A, top to bottom). This assumption depended on a previous study that showed the presence of two types of ovalbumin with similar molecular weights in the pigeon egg white (Suzuki et al., 2001). The two pigeon ovalbumins are more glycosylated than their counterpart the chicken ovalbumin, and this increases their molecular weights (53.17 and 48 kDa) compared to the molecular weight of chicken ovalbumin (42.75 kDa; Figure 1A; Suzuki et al., 2001). The band represented with black arrowhead is a presumptive egg white lysozyme band because it has the same molecular weight (15 kDa) of that determined by a previous work in pigeon (Figure 1A; Gavilanes et al., 1982). Also, it has a molecular weight similar to the molecular weight of chicken egg white lysozyme (14,400 kDa; Figure 1A; Abeyrathne et al., 2013). The intensity of protein bands increased on day 4 and thereafter, however, it was highly reduced on day 15. The other egg compartments were collected at different developmental stages, and their electrophoretic patterns were analyzed in order to determine whether or not the egg white protein bands were transferred to these fluids.

The amniotic fluid was collected on day 5 until day 15 (Figure 1B). No protein bands appeared from day 5 to day 11, however, they started to appear on day 12 and continued until day 15 (Figure 1B).

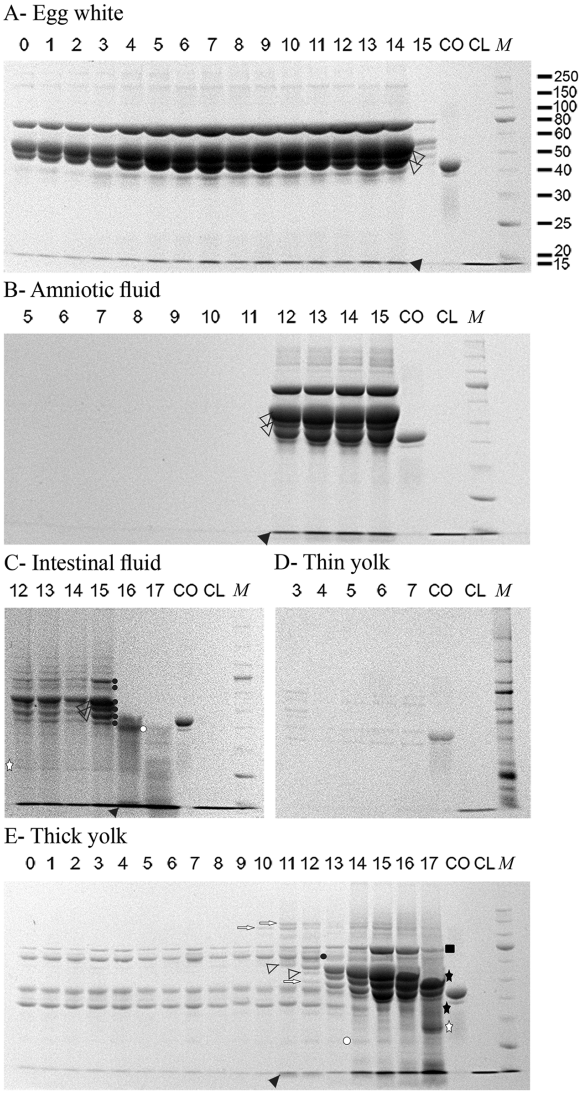

FIGURE 1 Electrophoretic patterns of different egg compartments during different developmental stages. Electrophoretic patterns of proteins in (A) egg white, (B) amniotic fluid, (C) intestinal fluid, (D) thin yolk, and (E) thick yolk. All samples of different egg compartments during different developmental stages were diluted with distilled water such that the final dilution factor was 48, and 10 μl of each sample was loaded in the gel. In all egg compartments, the developmental stages are shown on top of each gel. CO and CL stand for chicken ovalbumin and lysozyme, respectively. *M* represents the marker proteins, which have molecular weights of 250, 150, 100, 80, 60, 50, 40, 30, 25, 20, and 15 kDa. The two open arrowheads mark the presumptive ovalbumin 1 (53.17 kDa) and ovalbumin 2 (48 kDa) bands from top to bottom, while the black arrowhead represents the presumptive lysozyme band (15 kDa). In the intestinal fluid, white star shows the appearance of 26 kDa faint band from day 12 to day 15. On day 16, black circles represent the disappearance of 76.38, 65, 53.17, 48, 43.8, and 40 kDa bands from top to bottom, while the white circle marks the appearance of 35.29 kDa band. In thick yolk, white arrows reveal the appearance of 115, 131, and 43.8 kDa bands on days 10, 11, and 12, respectively. The black circle demonstrates the reduction of 65 kDa band after day 12, while white circle shows the appearance of 26 kDa band on days 14 and 15. On day 17, black square represents the reduction of 76.38 kDa band. Also, black stars mark the disappearance of ovalbumin 1 and 35.29 kDa bands from top to bottom, while white star represents the appearance of 28 kDa band

The intensity of bands in these stages was almost the same. After day 15, the amniotic fluid was completely taken up by the embryo. In the amniotic fluid, the protein bands including those of presumptive ovalbumin 1, ovalbumin 2 (Figure 1B; open arrowheads), and lysozyme (Figure 1B; black arrowhead) were similar to those in the egg white suggesting the transfer of egg white into the amniotic fluid.

The intestinal fluid appeared on day 12 and continued thereafter (Figure 1C). Distinct protein bands appeared from day 12 to day 15. Also, a faint small band with approximate weight of 26 kDa appeared on day 12 until day 15 (Figure 1C, white star). On day 16, several protein bands including those of presumptive ovalbumin 1 and ovalbumin 2 disappeared (Figure 1C, 76.38, 65, 53.17, 48, 43.8, and 40 kDa, black circles from top to bottom), while a large smear with 35.29 kDa band appeared (Figure 1C, white circle). Moreover, the presumptive lysozyme band became obvious with a co-migrated smear (Figure 1C, black arrowhead). On day 17, all the bands disappeared except the presumptive lysozyme band that became more obvious and surrounded with an extended smear (Figure 1C).

The thin yolk samples were collected on day 3 until day 7 (Figure 1D). In this portion, very faint protein bands were detected. On the other hand, the thick portion of yolk was collected throughout development and on the day of hatch (Figure 1E). The protein bands appeared at all stages. The intensity of bands was almost the same from day 0 until day 9. Following that, two new bands with approximate weights of 115 and 131 kDa appeared on day 10 and day 11, respectively, but the two bands disappeared after day 15 (Figure 1E, white arrows). The presumptive ovalbumin 1 band (53.17 kDa) appeared on day 11 until day 16 when it became partially hydrolyzed (Figure 1E; open arrowhead). Ovalbumin 2 (48 kDa) and another band (43.8 kDa) appeared as faint bands on day 12, however, they became obvious on day 13 and thereafter (Figure 1E; open arrowhead and white arrow, respectively). Moreover, the intensity of the presumptive lysozyme band increased on day 11 and became apparent on day 14 until the day of hatch (Figure 1E; black arrowhead). During this period, the band appeared with a co-migrated smear. One band with approximate weight of 65 kDa was highly reduced after day 12 (Figure 1E, black circle), and a small faint band (26 kDa) appeared on days 14 and 15 (Figure 1E; white circle). On day 17, a band with approximate weight of 76.38 kDa was reduced (Figure 1E; black square). Also, the presumptive ovalbumin 1 and 35.29 kDa bands disappeared (Figure 1E; black stars from top to bottom), while one new small band (28 KDa; Figure 1E, white star) and a large distinct smear appeared. Although several protein bands appeared on the egg yolk early in development, some of them before the growth of the embryo as shown on day 0, the intensity of bands started to increase on day 11 and became obvious on day 14 and thereafter.

3.2 | Lysozyme activity in different egg compartments during different developmental stages

The electrophoretic band that had a molecular weight of 15 kDa was suggested to be lysozyme. This band seemed to be transferred from egg white into the other egg compartments. In order to confirm the appearance and transfer of lysozyme, lysozyme activity assay using *M. lysodeikticus* as a substrate was performed. In the egg white (Figure 2A), the activity of lysozyme was found to be the largest among all egg compartments (Figure 2). It started on day 0 and continued throughout development until day 15, the last day of fluid appearance (Figure 2A). The activity of the enzyme increased, in general, with progress of development and reached its maximum value on day 7. Then, the activity fluctuated until day 15 when it was substantially reduced. The maximum activity was significantly higher than that on days 0, 1, 10, 12, and 15.

In the amniotic fluid, the enzymatic activity was detected from day 12 to day 15 (Figure 2B). The activity slightly increased with progress of development, however, this increase was statistically insignificant. In the intestinal fluid, the activity of lysozyme was initially detected on day 12 and continued until the day of hatch (Figure 2C). It increased significantly from day 12 to day 13 and reached the maximum value on day 14. Then, the activity was slightly reduced on day 15, but progressively slightly increased afterward.

In the thin portion of yolk, no lysozyme activity was detected throughout the fluid appearance. On the other hand, in the thick portion, the activity of lysozyme was initially detected on day 14 (Figure 2D), when the intensity of electrophoretic bands became obvious (Figure 1E). Then, the activity significantly increased and reached the maximum value on day 15, however, it was insignificantly reduced thereafter (Figure 2D).

3.3 | Protease activity in the egg yolk fraction during different developmental stages

To test whether or not the transferred egg white and endogenous yolk proteins are degraded before their transfer into the embryo, general protease activity assay was performed throughout development in the egg yolk. Initially, the optimal pH range for protease activity was determined (Figure 3). Protease activity was significantly high at pH 5.0 (1.09 unit/ml) and pH 5.5 (0.94 unit/ml), however, it decreased substantially at pH 6.0. Although the activity values fluctuated between pH 6.0 and pH 11.0, they remained relatively low.

Because the maximum protease activity was found at pH 5.0, the enzymatic activity was calculated in the egg yolk during different developmental stages using casein as a substrate in acetate buffer at pH 5.0 (Figure 4). In the thin yolk, no protease activity was detected throughout the fluid appearance. In contrast, in the thick yolk, the activity was basal between days 0 and 13. Then, it started to increase significantly on day 14 and reached the maximum value on day 15 (2.23 unit/ml). Following that, the activity largely reduced on day 16, but then obviously increased on day 17. Although the activity in the last two stages was significantly lower than that on day 15, it was significantly higher than the activity on other stages (Figure 4).

3.4 | The expression of *APN* and *PepT1* genes in the YSM during different developmental stages

To explore the role of YSM in the digestion and transportation of yolk proteins, the expression of *APN* and *PepT1* genes was investigated in the YSM from incubation day 8 until day 17. In general, the

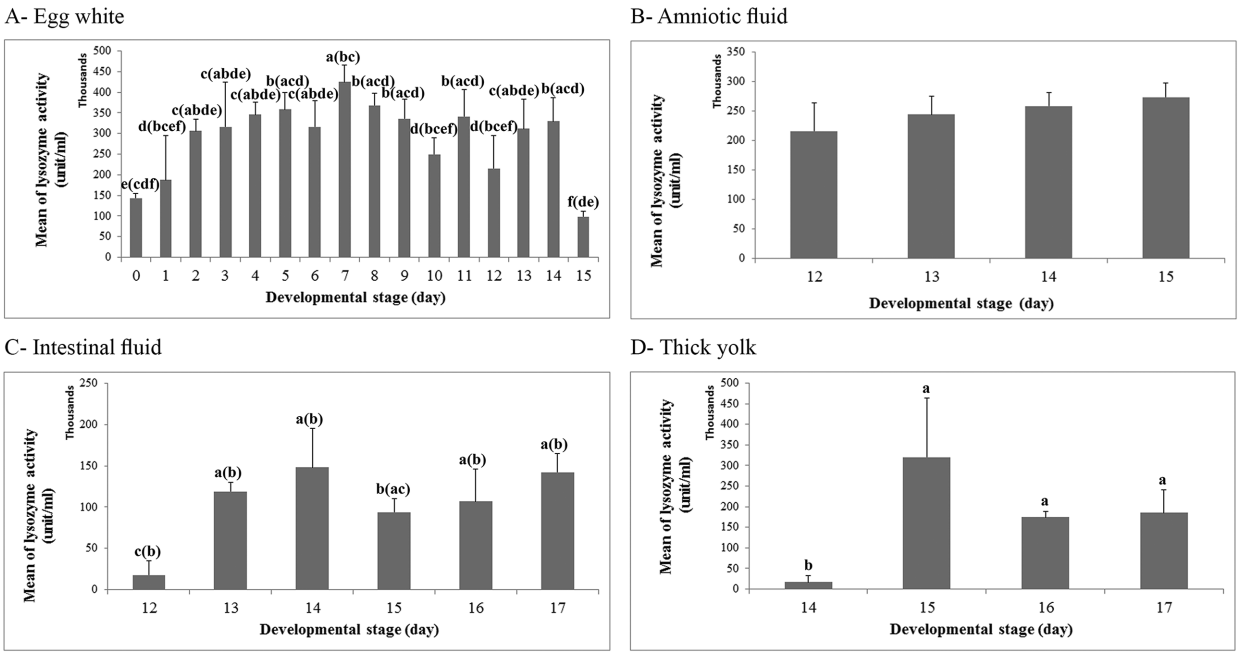

FIGURE 2 Lysozyme activity in different egg compartments during different developmental stages. Mean of lysozyme activity (unit/ml) is plotted as a function of developmental stage (day) in (A) egg white, (B) amniotic fluid, (C) intestinal fluid, and (D) thick yolk. The mean of lysozyme activity in each compartment at each stage is represented as a gray bar and the standard error of the mean is shown as a black line. The *F* and *P* values of one-way ANOVA test are: (F (15, 34) = 2.127, P = 0.034), (F (3, 11) = 0.476, P = 0.705), (F (5, 18) = 2.917, P = 0.042), and (F (3, 10) = 5.426, P = 0.018) in the egg white, amniotic fluid, intestinal fluid, and thick yolk, respectively. In all egg compartments, four independent samples were used at each developmental stage, and each sample was read in triplicate. Bars with different letters (a–f) differ significantly in the means of enzymatic activity

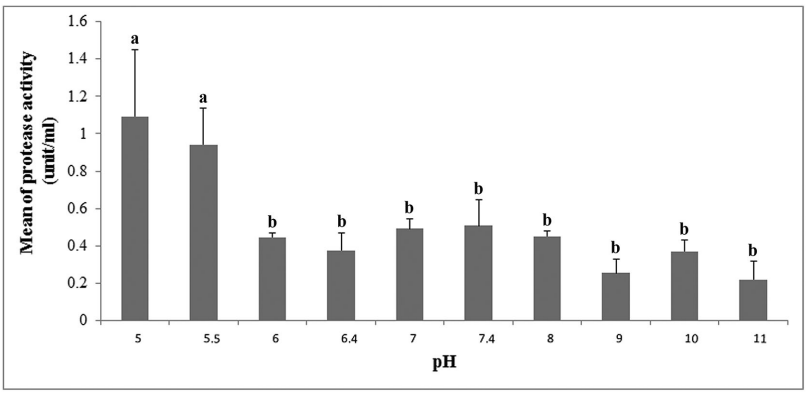

FIGURE 3 Effect of pH on protease activity. Mean of protease activity (unit/ml) in the yolk samples was measured using casein as a substrate in buffers at different pH. The mean of protease activity at each pH is represented as a grey bar and the standard error of the mean is shown as a black line. Protease activity was calculated for four independent samples at developmental stage 16, and each sample was read in triplicate at each pH. The *F* and *P* values of one-way ANOVA test are: F (9, 30) = 3.684 and P = 0.003. Bars with different letters (a or b) differ significantly in the means of enzymatic activity

expression of *APN* throughout development was low compared to that of *PepT1* (Figure 5). The maximum expression of *APN* was on day 8 (Figure 5A and B). Following that, the expression progressively decreased until day 12. On day 13, the expression slightly increased, but then it was reduced and reached its minimum value on day 16. Although the expression of the gene increased on day 17 compared to its expression on day 16, this increase was insignificant (Figure 5B). Therefore, the general pattern of *APN* expression was reduction toward the day of hatch. *APN* maximum expression was statistically higher than that on days 14 –17, while *APN* minimum expression was substantially lower than that on days 8–13 (Figure 5B). On the other hand, the expression of *PepT1* was drastically low on day 8, however, it increased progressively and reached its maximum value on day 13 (Figure 5C and D). Then, the expression of the gene slightly decreased on day 14, but increased again on day 15. After that, it was considerably reduced on days 16 and 17. The period between days 13 and 15 showed the maximum expression of *PepT1*, which was significantly higher than that at early (days 8 and 9) and late (days 16 and 17) stages (Figure 5D).

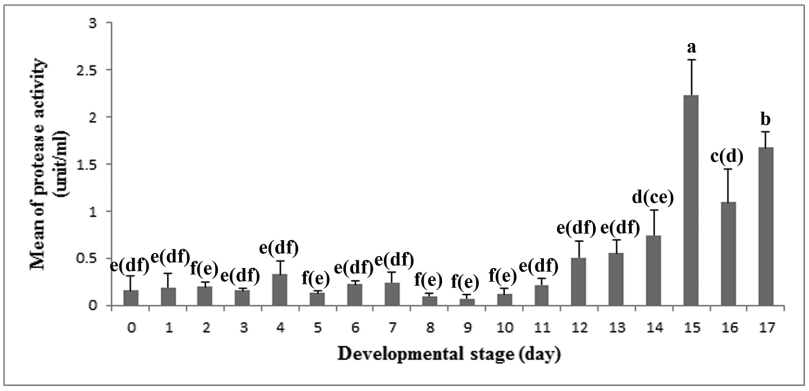

FIGURE 4 Protease activity in the egg yolk during different developmental stages. Mean of protease activity (unit/ml) in the thick yolk is plotted as a function of developmental stage (day). The mean of protease activity at each stage is represented as a grey bar and the standard error of the mean is shown as a black line. Three independent samples were used at each developmental stage, and each sample was read in triplicate. The *F* and *P* values of one-way ANOVA test are: $F(17, 43) = 10.930$ and $P = 0.000$. Bars with different letters (a–f) differ significantly in the means of enzymatic activity

4 | DISCUSSION

In the present study, we took a step toward exploring the fate of egg proteins during the development of pigeon embryos. We found that: (1) the major route of egg white transfer was through the embryo into the yolk; (2) the major egg white transfer was simultaneous to the increase in general proteolytic activity in the yolk; and (3) the expression of *APN* and *PepT1* genes decreased toward hatch.

We showed the appearance of presumptive egg white protein bands in different egg fractions during different developmental stages. We used lysozyme as a reference egg white protein, and we detected the chronological appearance of its activity in different egg fractions. The first appearance of lysozyme activity was on day 12 in the amniotic and intestinal fluids and on day 14 in the thick yolk. Therefore, the major route of egg white transfer in pigeon can be through the amniotic fluid into the intestinal lumen, and then to the yolk. Our results in pigeon are consistent with those previously published in chicken (Carinci & Manzoli-Guidotti, 1968; Baintner & Feher, 1974; Sugimoto et al., 1989), quail (Yoshizaki et al, 2002), and turkey (Shbailat & Safi, 2015) and showed that the major transfer of egg white in the fertilized eggs followed a similar route. In addition, small amount of egg white seemed to be directly transferred into the yolk as several protein bands were detected in the egg yolk early in development. This direct transfer was also suggested during early stages in chicken (Sugimoto et al., 1984, 1989) and turkey (Shbailat & Safi, 2015).

We found that in the egg white, the activity of lysozyme increased progressively during the initial stages of development. This increase might be due to the formation of thin yolk by the water flow from egg white into the yolk (Ar, 1991), and the consequence increase in the concentration of egg white proteins including lysozyme that showed simultaneous increase in its activity. After that, the activity of the enzyme fluctuated until day 15, when it was obviously reduced as remnants of egg white proteins remained. The thin yolk, which showed very faint electrophoretic protein bands, did not show any lysozyme activity. The appearance of these bands might be due to the solubility of small amount of egg white proteins in the water that transferred into the yolk (Shbailat & Safi, 2015), or due to slight contamination of the thin yolk by the underlying thick yolk (Baintner & Feher, 1974). During the period between days 12 and 15, the egg white proteins including lysozyme apparently transferred into the amniotic fluid. Large transfer seemed to occur throughout this period because the intensity of electrophoretic protein bands was large and insignificant change in lysozyme activity was observed. In the intestinal fluid and thick yolk, the first appearance of lysozyme activity on day 12 and day 14, respectively, was significantly lower than that on the next stages. Following that, the activity changed but insignificantly among stages. The initial reduction in the enzymatic activity can be due to the reduction in the concentration of the enzyme, the initial exposure to different environmental cues such as different pH or ionic strength, or the presence of an inhibitor or absence of certain cofactor at these stages.

In order to explore the fate of transferred egg white and endogenous yolk proteins, we performed general protease activity assay in the egg yolk throughout development. We found that the proteolytic activity started to increase significantly on day 14, and this increase was simultaneous to the obvious transfer of egg white proteins into the yolk. We also found dynamic changes in the yolk protein bands at late developmental stages, which further supported the presence of high proteolytic activity. These changes are marked by the disappearance (131, 115, 53.17, and 35.29 kDa) or reduction (76.38 and 65 kDa) of large bands, and the appearance of small ones (26 and 28 kDa) or a smear, which suggest the degradation of the large molecular weight bands into smaller ones. Furthermore, the appearance of obvious lysozyme band with an extended smear on day 14 and thereafter might result from the co-migration of degradation products with this band. Taken together, our results propose that the transferred egg white and endogenous yolk proteins are mostly degraded by the activated proteases late in development. Our findings are similar to those from chicken (Sugimoto & Yamada, 1986) and turkey (Shbailat et al., 2016), which showed elevation of protease activity after the major transfer of egg white into the yolk. In contrast, protease activity was detected before the transfer of egg white proteins in quail (Yoshizaki et al., 2002).

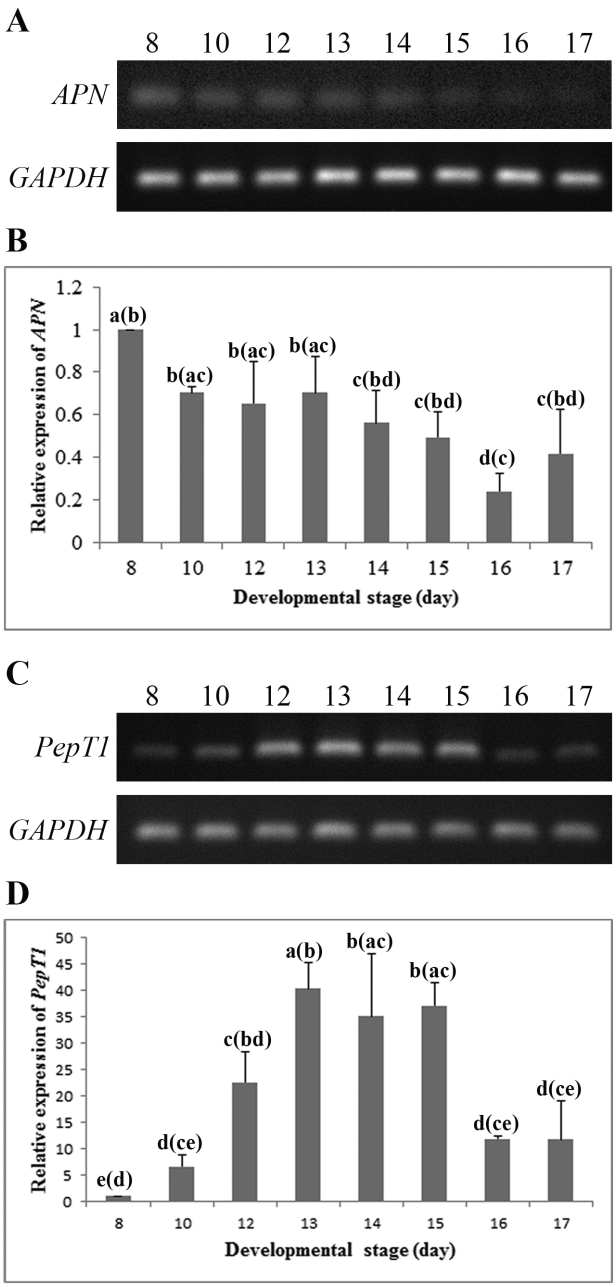

FIGURE 5 Expression of *APN* and *PepT1* during different developmental stages. (A and C) Representative agarose gel electrophoresis for (A) *APN* (109 bp) and (C) *PepT1* (110 bp) gene products compared to *GAPDH* (132 bp) product. The developmental stages are shown above the electrophoretic patterns. (B and D) Mean of (B) *APN* and (D) *PepT1* relative expression was normalized to the corresponding mean of *GAPDH* relative expression at each developmental stage. In the relative quantification settings, stage 8 was used as a reference stage. The mean of relative gene expression is represented as a grey bar and the standard error of the mean is shown as a black line. The experiment was repeated four times using four different pools of cDNA for each gene at each stage, and each cDNA was read in duplicate. The *F* and *P* values of one-way ANOVA test are: (F (7, 24) = 2.638, P = 0.036) and (F (7, 24) = 6.713, P = 0.000) for *APN* and *PepT1* expression, respectively. Bars with different letters (a–e) differ significantly in the means of relative gene expression

To test whether or not the YSM has a role in the degradation and transport of yolk proteins into the embryo, we investigated the expression of *APN* and *PepT1* genes in the membrane during different developmental stages (days 8, 10, 12, 13, 14, 15, 16, and 17). The expression of *APN* was largest on day 8, and then it generally decreased toward the day of hatch. Dong et al. (2012b) also examined *APN* expression in the pigeon YSM and reported that the expression was generally reduced until hatch. However, in the previous study, the maximum expression of the gene was on day 14. The expression of *PepT1* increased progressively and reached maximum values in the period between days 13 and 15. Then, it was substantially reduced on days 16 and 17. On the other hand, Dong et al. (2012b) found that *PepT1* expression reached maximum value on day 12. After that, it was progressively reduced and reached minimum value at the day of hatch. Although our results are similar to the previous results (Dong et al., 2012b) with respect to *APN* and *PepT1* expression on days 16 and 17, the difference in the expression patterns before that can be due to different reasons. In our study, we investigated more developmental stages (day 8, 10, 12, 13, 14, 15, 16, and 17) than those investigated by the previous study (day 12, 14, 16, and 17). Indeed, stage 8, which exhibited maximum *APN* expression in our work, and stages 13 and 15, which showed peak of *PepT1* expression, were not examined in the previous work (Dong et al., 2012b). Moreover, Speier et al. (2012) showed that the expression of several enzymes and membrane transporters including *APN* and *PepT1* in chicken YSM, varied according to the breed and age of laying hens. In our study, we used Arabian Trumpeter breed that is probably different from the breed used by Dong et al. (2012b) in China. Also, we used younger laying hens (180 day old) compared to those (280 day old) used by the other researchers (Dong et al., 2012b).

The decrease in *APN* and *PepT1* expression in the YSM on days 16 and 17 (present work; Dong et al., 2012b) was simultaneous to the reduction in the yolk sac weight and backflow of yolk into the small intestine (Dong et al., 2012b). These results are in agreement with those from chicken that showed the reduction of *APN* and *PepT1* expression in the YSM toward hatch (Yadgari et al., 2011; Speier et al., 2012), the concurrent decrease in the YSM weight (Yadgari et al., 2010), and the transfer of yolk via yolk stalk into the small intestine (Esteban et al., 1991; Noy et al., 1996).

The intestine and YSM are closely associated structures, as the YSM develops from the hindgut (Noble & Cocchi, 1990). Therefore, it is reasonable to assume that the intestine also participates in the digestion of egg white proteins as well as back-flowed yolk proteins. Previous studies in pigeon strongly supported this assumption. Dong et al. (2012b) showed that *APN* expression in the intestine increased quadratically from embryonic day 12 to post-hatch day 16. In another study, they found that the activity of the brush border enzyme, *APN* as well as the pancreatic enzymes, trypsinogen and chymotrypsinogen increased from the day of hatch (day 17) until post-hatch day 3 (Dong et al., 2012a). Our results are consistent with these findings. We showed the appearance of a small band (26 kDa) in the intestinal fluid from day 12 until day 15. However, apparent dynamic changes in the electrophoretic patterns occurred on days 16 and 17 following the backflow of yolk into the intestine. These changes are marked by the disappearance of almost all protein bands and the appearance of an

extended smear. After the digestion of egg proteins, the oligopeptides and amino acids are probably absorbed by the intestinal enterocytes because the expression of *PepT1* and $b^{0,+}$*AT*, *EAAT3*, y^{+}*LAT2*, and *LAT4* amino acid transporters was found to be high during the last stages in the intestinal cells (Dong et al., 2012a; Chen et al., 2015).

In conclusion, the present study is the first to explore the fate of proteins in the fertilized pigeon egg from the time of its deposition until the day of hatch. We showed that the egg white was transferred through the amnion and intestinal lumen into the yolk, similar to what has been found in chicken, quail, and turkey. The three avian species belong to the basal order of Neognathae, the Galliformes, whereas the pigeon belongs to a more derived order, the Columbiformes (Livezey & Zusi, 2007; Hackett et al., 2008; Zelenitsky et al., 2011). Therefore, it will be interesting to sample more species that belong to other basal and derived orders, to determine whether the egg white transfer through the embryo into the yolk is a basal trait in Neognathae, or whether it evolved independently in certain orders. Our results also suggested that after the egg white transfer, the yolk contents are probably degraded by the activated proteases late in development. Moreover, the YSM appear to have a role in the digestion of yolk proteins and absorption of oligopeptides. Future studies should further investigate the type of activated proteases in the egg yolk, and examine the expression of different types of amino acid transporters in the pigeon YSM. This will deepen our knowledge about the development of altricial birds in general.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

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