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Original Article

Cysteine and Aspartic Proteases Underlie the Digestion of Egg Yolk Proteins during the Development of *Columba livia domestica* Embryo

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ABSTRACT

Yolk proteins undergo digestion either inside the egg yolk or in the surrounding yolk sac membrane (YSM) before being consumed by the developing avian embryo. However, the mechanisms underlying the digestion of yolk proteins during embryogenesis are largely unexplored in the pigeon *Columba livia domestica*. To better understand these mechanisms, the present study examined the classes of activated proteases in the egg yolk and the gene expression patterns of cathepsin B (*CTSB*) and cathepsin D (*CTSD*), which encode for lysosomal cysteine and aspartic proteases, respectively, in the YSM. We investigated the activated proteases by applying different types of protease inhibitors to yolk samples taken from incubation day 16. Then, we detected the mRNA levels of *CTSB* and *CTSD* in the YSM at incubation days 6, 8, 10, and 12-17. Both cysteine and aspartic proteases appeared to be activated in the egg yolk. Moreover, *CTSB* expression increased progressively and reached the maximum value on day 13; however, it decreased significantly on days 14 and 15 and further reduced toward hatching (day 17). In contrast, *CTSD* expression was weak and fluctuated insignificantly during development. Our results suggest that the degradation of yolk proteins at late developmental stages largely occurs in the egg yolk itself, probably by the activated cysteine and aspartic proteases. Furthermore, cathepsin B in the YSM seems to have a primary role in protein digestion, but this role decreases toward hatching.

INTRODUCTION

The avian egg is composed of four major constituents: a protective egg shell, a thin egg shell membrane, an egg white, and an egg yolk (Kovacs-Nolan *et al.*, 2005; Mahdavi *et al.*, 2021). Egg white and egg yolk are the primary reservoirs of nutrients in the laid egg. Egg white consists of approximately 88.5% water, 10.5% proteins, 0.5% carbohydrates, and the remainder of vitamins and minerals (Stevens, 1996; Mahdavi *et al.*, 2021). Egg yolk is roughly composed of 50% water, 30% lipids, 16% proteins, 4% carbohydrates, and traces of vitamins and minerals (Mahdavi et a., 2021). Egg white ultimately transfers into the yolk before being consumed by the developing embryo (Sugimoto *et al.*, 1984). The major route of egg white transfer is 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; Shbailat & Abuassaf, 2018; Shbailat & Aslan, 2018).

Initially, the egg yolk is surrounded by the cell membrane (Sheng & Foley, 2012). However, this membrane degenerates in the course of development, and the yolk contents become fully surrounded by the yolk sac membrane (YSM) which extends from the hind gut (Noble & Cocchi, 1990; Sheng & Foley, 2012). The egg yolk contents surrounded by

YSM form the yolk sac that becomes the predominant structure for nutrient supply to the developing embryo (Dong *et al.*, 2012; Yadgary *et al.*, 2013; van der Wagt *et al.*, 2020). Before their uptake by the embryo, yolk contents appear to undergo digestion either inside the egg yolk itself or in the endodermal cells of YSM. In the egg yolk of different avian species, the digestion of yolk proteins was concurrent with the activation of several classes of proteases, which suggests that the proteins are degraded by the activated proteases (Sugimoto & Yamada, 1986; Yoshizaki *et al.*, 2002, 2004; Shbailat *et al.*, 2016; Shbailat & Abuassaf, 2018; Shbailat & Aslan, 2018). The proteases were activated either before the major transfer of egg white into the yolk (Wouters *et al.*, 1985; Yoshizaki *et al.*, 2004) or after it (Sugimoto & Yamada, 1986; Shbailat *et al.*, 2016; Shbailat & Abuassaf, 2018; Shbailat & Aslan, 2018).

The processing of yolk contents inside the endodermal cells of YSM can be summarized as follows, according to previously suggested models (Nakazawa *et al.*, 2011; Sheng & Foley, 2012; Bauer *et al.*, 2013; van der Wagt *et al.*, 2020): during the early stages of development, yolk processing starts with non-specific phagocytosis of proteins, lipoproteins, and lipids at the apical surfaces of endodermal cells (Bauer *et al.*, 2013). However, in the second half of incubation, yolk components are taken into the endodermal cells by receptor-mediated endocytosis (Bauer *et al.*, 2013). Inside the cells, the proteins and lipoproteins are digested by lysosomal proteases, while the lipid droplets undergo lipolysis into their main components (Bauer *et al.*, 2013). The digested yolk products are then used as substrates for the synthesis of new lipoproteins, and serum and regulatory proteins required by the developing embryo (Nakazawa *et al.*, 2011; Sheng & Foley, 2012; Bauer *et al.*, 2013). After that, the newly synthesized products are exocytosed from the basal surfaces of endodermal cells into the blood vessels in the extraembryonic splanchnic mesoderm, which deliver them to the embryo circulation (Nakazawa *et al.*, 2011; Sheng & Foley, 2012; Bauer *et al.*, 2013; van der Wagt *et al.*, 2020). It is worth mentioning that another route of nutrient delivery into the embryo, slightly before hatching, is the back flow of yolk from yolk sac through yolk stalk into the embryo intestine (Yadgary *et al.*, 2011; Dong *et al.*, 2012; Shbailat & Aslan, 2018; van der Wagt *et al.*, 2020).

Previous studies reported the activities of lysosomal proteases in the YSM of chicken and quail. In chicken, cathepsin D was purified, and its activity was found to be maximum on incubation day 10 (Wouters *et al.*, 1985). In quail, both cathepsin B and D were purified, and their activities were shown to be high during the studied period from incubation days 3 to 8 (Gerhartz *et al.*, 1997,1999). These findings support that the lysosomal proteases have roles in the digestion of yolk proteins and lipoproteins that were endocytosed by the endodermal cells. Furthermore, the genes encoding the oligopeptide transporter PepT1 and various amino acid transporters were demonstrated to be expressed in the YSMs of pigeons (Dong *et al.*, 2012; Chen *et al.* 2016; Shbailat & Aslan, 2018) and chickens (Yadgary *et al.*, 2011; Speier *et al.*, 2012; Yadgary *et al.*, 2014). Encoded PepT1 and other amino acid transporters seemed to participate in the transfer of protein products into the endodermal cells of YSM after their digestion in the egg yolk. The transferred peptides and amino acids probably contribute to the synthesis of new proteins and lipoproteins inside these cells.

Understanding the mechanisms that underly the extraembryonic digestion of yolk sac proteins will help us understand how egg proteins are processed before they are consumed by the developing avian embryos. In Neognathae birds, the processing of yolk sac proteins was largely studied in the chicken *Gallus gallus* (Wouters *et al.*, 1985; Sugimoto & Yamada, 1986; Nakazawa *et al.*, 2011; Yadgary *et al.*, 2011; Speier *et al.*, 2012; Bauer *et al.*, 2013) and the quail *Coturnix japonica* (Gerhartz *et al.*, 1997, 1999; Yoshizaki *et al.*, 2002, 2004). Both species belong to the Galliformes order of the Galloanserae basal clade (Hackett *et al.*, 2008; Braun & Kimball, 2021). However, the other orders belonging to the Neoaves clade (other neognaths; Hackett *et al.*, 2008; Braun & Kimball) are poorly investigated. In our previous study (Shbailat & Aslan, 2018), we took a step toward understanding the degradation of yolk sac proteins in one order of Neoaves, the Columbiformes, and we used the pigeon *Columba livia domestica* as a representative species of this order. We found that the general proteolytic activity in egg yolk started to increase significantly at late developmental stages. Nevertheless, the classes of activated proteases in the pigeon egg yolk are still unknown and, to the best of our knowledge, the gene expression patterns of lysosomal proteases are completely unexplored in the pigeon YSM. Here, we applied different types of protease inhibitors to yolk samples loaded in denaturing polyacrylamide gels in order to determine the classes of activated proteases in the pigeon egg yolk. We also used quantitative PCR at different developmental stages in the YSM to determine the mRNA levels of cathepsin B (*CTSB*) and cathepsin D (*CTSD*) genes, which encode for lysosomal cysteine and aspartic proteases, respectively.

MATERIALS AND METHODS

Egg Incubation and Embryo Staging

The males and females of *Columba livia domestica* were purchased from local farms in Amman. Fertilized eggs were collected immediately after laying and were kept in an egg incubator at a temperature of 38.5 ± 0.5°C and relative humidity of 65%. The eggs were subjected to an automatic see-saw motion every 2 h. The developmental stages of the embryo were measured from day 0 to day 17 (the day of hatching). They were identified according to the criteria published in Olea & Sandoval (2012) for pigeon, with reference to the classification of Hamburger & Hamilton (1951) for chicken. This 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.

Isolation and Preparation of the Egg Yolk and Yolk Sac Membranes

The eggs were opened at their blunt ends at each developmental stage. The allantoic and amniotic fluids, if present, were withdrawn to prevent the contamination of underlying yolk sacs. YSMs were collected on incubation days 6 to 17. At each stage, a hole of 0.5 cm in diameter was made in the yolk sac and the yolk was sucked using a 5 mL syringe. Then, the membrane was washed several times with Diethylpyrocarbonate treated distilled water and immediately used for RNA extraction as described below. The absorption of yolk sac by the abdomen of embryo started on incubation day 16 and was completed on day 17 (Shbailat & Aslan, 2018). Therefore, during the last two stages of development, the yolk sac was removed outside the egg shell together with the embryo, and a hole was made in the embryo abdomen to pull the sac outside the body.

The egg yolk at incubation day 16 was further processed for use in biochemical analysis (Yoshizaki *et al.*, 2002; Shbailat & Safi, 2015). The yolk was diluted with five volumes of 0.87% NaCl solution, which contained 1 mM ethylenediaminetetraacetic acid-disodium, dihydrate ($Na_2EDTA.2H_2O$). The diluted sample was then homogenized using a tissue homogenizer (Omni International, Kennesaw, Georgia, USA), and the homogenate was centrifuged at 4,427 g for 30 min at 4°C. Following that, the supernatant was dialyzed against distilled water for 2 h. Then, the dialyzed solution was centrifuged twice at 4,427 g for 30 min at 4°C, and the supernatant was collected each time.

Denaturing Polyacrylamide Gel Electrophoresis

Egg yolk samples (15 µg each) were diluted (1:1) with citric acid–disodium hydrogen phosphate buffer ($HOC(CH_2CO_2H)_2$-Na_2HPO_4) at pH (3–7), disodium hydrogen phosphate-sodium dihydrogen phosphate (Na_2HPO_4-NaH_2PO_4) at pH (8), glycine-sodium hydroxide ($C_2H_5NO_2$-NaOH) at pH (8.8), borax-sodium hydroxide ($Na_2B_4O_7.10H_2O$-NaOH) at pH (10), and disodium hydrogen phosphate-sodium hydroxide (Na_2HPO_4-NaOH) at pH (11) (Yoshizaki *et al.*, 2002; Shbailat *et al.*, 2016). After that, the samples were incubated at 37°C for 24 h (Yoshizaki *et al.*, 2002; Shbailat *et al.*, 2016), and then they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the protocol described in Laemmli (1970), with slight modifications using 10% separating gels.

To test for the inhibition of protease activity, the egg yolk samples (15 µg each) were diluted (1:1) with citric acid–disodium hydrogen phosphate buffer (pH 3) mixed with a protease inhibitor at a final concentration of 0.4 mg/mL (Shbailat *et al.*, 2016). The mixture was incubated at 37°C for 24 h before loading in the polyacrylamide gel (Yoshizaki *et al.*, 2002; Shbailat *et al.*, 2016). The inhibitors used were trypsin inhibitor (Sigma-Aldrich, Saint Louis, Missouri, USA), aprotinin (Bioworld, Dublin, Ohio, USA), phenylmethylsulfonyl fluoride (PMSF; Bio Basic, Markham, Ontario, Canada), N-[N-(L-3-Trans-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E-64; Bioworld, Dublin, Ohio, USA), ethylene glycol tetraacetic acid (EGTA; Bio Basic, Markham, Ontario, Canada), $Na_2EDTA \cdot 2H_2O$ (Bio Basic, Markham, Ontario, Canada), and pepstatin A (Bioworld, Dublin, Ohio, USA). Broad-range marker proteins (17-245 kDa; iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea) were used as standard proteins, and for the construction of a standard protein curve to estimate the molecular weights of protein bands of interest. The gels were stained in Coomassie Brilliant Blue solution.

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, California, USA) per 100 mg of each tissue according to the manufacturer's protocol. 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,

Ipswich, Massachusetts, USA). Then, 1 μg of each RNA extract was reversed-transcribed by M-MuLV enzyme at 42°C for 1 h using Oligo (dT) adaptor primer following the manufacturer's procedure. The synthesized cDNAs were then used to perform quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using LINE GENE9600 PLUS real time PCR detection system (Bioer Technology, Hangzhou, Zhejiang, China) to determine the expression levels of *CTSB* and *CSTD* genes during different developmental stages. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control to which the fold changes in gene expression were normalized. qRT-PCR was carried out using PowerUp SYBR Green Master Mix (Applied Biosystems, Carlsbad, California, USA) and gene specific primers. The following primers were designed and used at a final concentration of 0.6 μM in 20 μL reaction: *CTSB* Forward 5'-CACTACGGCATCACATCCTAC-3', *CTSB* Reverse 5'-TAGACAATAAAGGCTCCTTCCAC-3', *CTSD* Forward 5'-GCTCCTCAAGTTCAAGCTAGG-3', *CTSD* Reverse 5'-CAATGCCAATCTCACCGTAGTA-3', *GAPDH* Forward 5'-GGTGGTGCTAAGCGTGTTAT-3', and *GAPDH* Reverse 5'-CAGGCAGTTAGTAGTGCAAGAG-3'. The PCR conditions used were: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s, 57°C for 15 s, 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 s, 60°C for 1 min, and 95°C for 15 s. Fluorescence emission was detected and relative quantification was calculated automatically using the software LINE GENE9600 PLUS real time PCR detection system.

Statistical Analysis

Data were analyzed using IBM SPSS statistics version 21 (SPSS INC., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) was used to assess the presence of any significant differences among the means of *CTSB* and *CTSD* gene expression levels in YSMs at different developmental stages. One-way ANOVA test was followed by a least significant difference (LSD) test to determine the groups that differ significantly from each other during the measurement of 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 $df_{between}$: degree of freedom between groups, df_{within}: degree of freedom within groups, MS_b: mean square between groups, MS_w: mean square within groups. The level of significance was set at $p \leq 0.05$.

RESULTS

Protease Activity in the Egg Yolk

Previously, we found that the activity of proteases in pigeon egg yolk increased significantly at late stages of development (Shbailat & Aslan, 2018). In order to determine the classes of activated proteases at late stages in the egg yolk, we initially tested the optimal pH range for protease activity. To achieve that, we diluted the samples with the same volume of buffer solutions at a pH range from 3 to 11 (as described under materials and methods), and then we loaded them in denaturing SDS polyacrylamide gel (Figure 1). One protein band with approximate molecular weight of 75.64 kDa was absent from the samples at pH 3 and 3.4 (Figure 1, lanes 1 and 2, respectively); however, it appeared at pH 4 (Figure 1, lane3) and became more intense from pH 5 to 11 (Figure 1, lanes 4 to10). Because the protein band was completely digested at pH 3 and 3.4, we chose the buffer at pH 3, mixed it with one of different types of protease inhibitors, and used it to dilute the yolk samples (Figure 2). Among the inhibitors used, E-64, an inhibitor of cysteine proteases, and pepstatin A, an inhibitor of aspartic proteases, largely inhibited the protease activity as revealed by the appearance of an obvious 75.64 kDa band after their addition (Figure 2, lanes 5 and 8, respectively; both lanes are similar to lane 9). On the other hand, the serine protease inhibitors: trypsin inhibitor, aprotinin, and PMSF (Figure 2, lanes 2 to 4, respectively) and the metalloprotease inhibitors: EGTA and $Na_2EDTA.2H_2O$ (Figure 2, lanes 6 and 7, respectively), did not abolish the proteolytic activity, as marked by the absence or great reduction of the 75.64 kDa band (like in lane1) despite their addition.

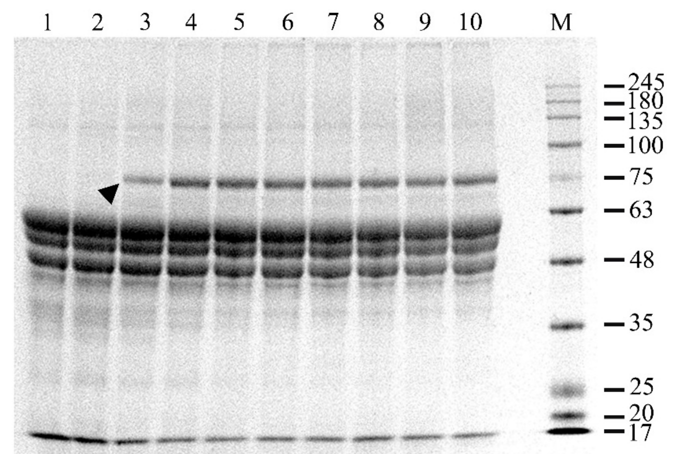

Figure 1 – Effect of pH on the patterns of yolk proteins during electrophoresis. Yolk samples from incubation day 16 were mixed (1:1) with different buffers at pH 3 (lane 1), 3.4 (lane 2), 4 (lane 3), 5 (lane 4), 6 (lane 5), 7 (lane 6), 8 (lane 7), 8.8 (lane 8), 10 (lane 9), and 11 (lane 10). Marker proteins (lane M) are shown in kDa at the right. The appearance of 75.64 kDa protein band is represented by an arrowhead.

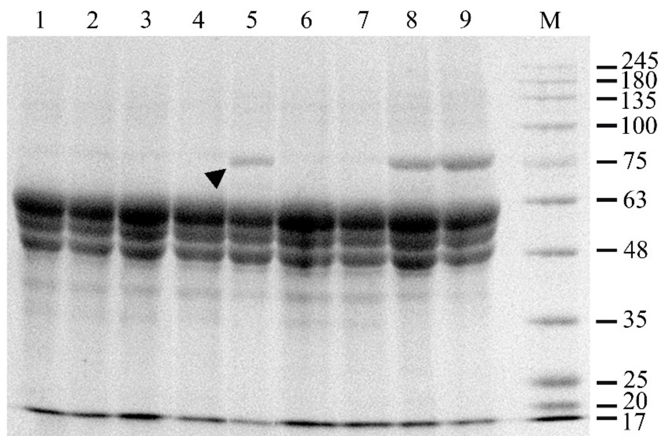

Figure 2 – Effect of protease inhibitors on the patterns of yolk proteins during electrophoresis. Yolk samples from incubation day 16 were mixed (1:1) with citric acid-disodium hydrogen phosphate buffer at pH 3. The buffer contained no inhibitor (lane 1; buffer--incubated, inhibitor-untreated control sample), trypsin inhibitor (lane 2), aprotinin (lane 3), PMSF (lane 4), E-64 (lane 5), EGTA (lane 6), $Na_2EDTA.2H_2O$ (lane 7), pepstatin A (lane 8), and no inhibitor and no incubation (lane 9; buffer-unincubated, inhibitor-untreated control sample). Marker proteins (lane M) are shown in kDa at the right. The appearance of 75.64 kDa protein band is represented by an arrowhead.

The Expression of *CTSB* and *CTSD* Genes in the YSM during Development

To uncover whether or not the genes encoding for lysosomal proteases are expressed in the pigeon YSM, we examined the expression of *CTSB* and *CTSD* genes in the YSM from day 6 of incubation to day 17 (Figure 3). The expression of both genes was measured relative to that of the GAPDH gene. *CTSB* expression increased gradually and reached its highest value on day 13. Then, it decreased dramatically on days 14 and 15, and became further reduced on days 16 and 17 (Figure 3A). The maximum expression of *CTSB* was significantly higher than the expression in all developmental stages, except on day 12 (Figure 3A). It was also 7.33 folds greater than the expression on day 6 (the reference stage; Figure 3A). On the other hand, although *CTSD* expression was detected throughout the studied stages, the expression was weak and fluctuated insignificantly among these stages (Figure 3B).

DISCUSSION

Previously, we found that during the development of pigeon embryos, the proteolytic activity in the egg yolk began to increase notably on incubation day 14 and continued toward the day of hatching (Shbailat & Aslan, 2018). This increase was concurrent with the obvious transfer of egg white proteins into the yolk. We also found dynamic changes in the electrophoretic bands of yolk proteins at late developmental stages (Shbailat & Aslan, 2018). These changes were marked

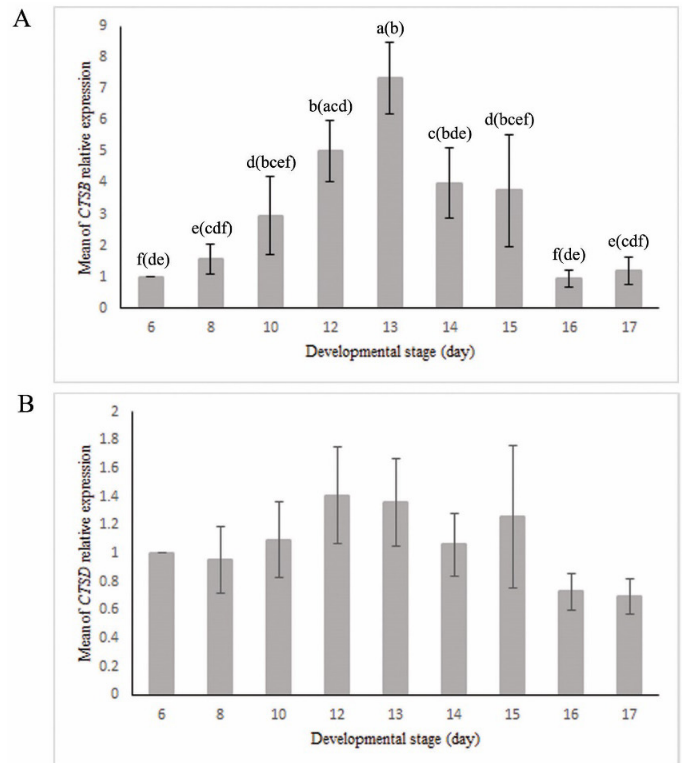

Figure 3 – Relative expression of *CTSB* and *CTSD* during different developmental stages.The expression of *CTSB* (A) and *CTSD* (B) was measured relative to *GAPDH* expression. The mean of relative expression of each gene at each stage was calculated from four different experiments using four different pools of cDNA, and each cDNA was read in duplicate. Stage 6 was used as a reference stage during quantification. Grey bars represent the means of *CTSB* and *CTSD* relative gene expression, while black lines mark the standard errors of the means. The F and *p* values of one-way ANOVA followed by LSD test are: F (8, 27) = 4.860, p = 0.001 and F (8, 27) = 0.848, p = 0.570 for *CTSB* and *CTSD* expression, respectively. Means of relative gene expression with different letters (a-f) differ significantly from each other.

by the disappearance or reduction of large molecular weight bands, and the appearance of small ones or a smear, which inferred the breakdown of large bands into smaller ones, probably by the activated proteases late in development (Shbailat & Aslan, 2018). In the present study, we further explored the proteolytic activity in the pigeon yolk sac. We found that: (1) the activated proteases in the egg yolk likely belonged to cysteine and aspartic classes; (2) the expression of both *CTSB* and *CTSD* genes was detected in the YSM; however, *CTSB* appeared to have the main role in protein digestion.

We suggested that the activated proteases in egg yolk were of cysteine and aspartic types because their activities were inhibited by the addition of the wide range cysteine and aspartic protease inhibitors, E64 and pepstatin A, respectively. Several possibilities can explain how these proteases are incorporated into the pigeon egg yolk. One possibility is that both proteases may be deposited together with other egg yolk precursors in the oocyte (yolky follicle) early during

oogenesis. This assumption depends on previous studies in chicken, which reported that most egg yolk precursors were synthesized in the liver of laying hens, secreted into the blood, and then transferred into the developing oocyte in ovary (Bourin *et al.*, 2012a,2012b; Cui *et al.*, 2020). In a hepatic transcriptome profiling of laying hens versus pre-laying pullets, the researchers found that most of the top ten over expressed genes encoded for components of egg yolk or perivitelline membrane (Bourin *et al.*, 2012a). Among the top overexpressed egg yolk constituents was cathepsin E-A-like/similar to nothepsin which is an aspartic protease (Bourin *et al.*, 2012a). Cathepsin E-A-like/similar to nothepsin was predicted to assist cathepsin D, another aspartic protease found in oocyte, in the processing of two key egg yolk precursors, very low-density lipoprotein (VLDL; containing apolipoprotein B) and vitellogenin, to produce the final yolk components that were used late by the developing embryo (Retzek *et al.*, 1992; Bourin *et al.*, 2012a, 2012b). The transcriptome analysis also revealed that among the differentially expressed genes in the liver of sexually mature hens were those that encode for proprotein convertase subtilisin/kexin type 6 (serine protease), ubiquitin carboxyl-terminal hydrolase 3 (ubiquitinyl hydrolase), and OUT domain-containing protein (cysteine protease; Bourin *et al.*, 2012a). The authors suggested that the three encoded proteases could be potentially secreted into the blood to be transferred into the oocyte (Bourin *et al.*, 2012a). Taking together all the above findings, we can propose that in the laying pigeon female, cysteine and aspartic proteases help in the processing of egg yolk precursors after their deposition in the oocyte. Another possibility is that the presumptive cysteine and aspartic proteases in the pigeon egg yolk may have been synthesized in tissues other than the liver, such as the theca and granulosa layers of ovarian follicle. This prediction is based on two proteomic analyses of egg yolk from unfertilized chicken eggs (Mann & Mann, 2008; Farinazzo *et al.*, 2009), which showed that several proteases and other yolk components that were revealed by proteomics were not identified in the transcriptome profiling in the liver of laying hens (Bourin *et al.*, 2012a). The proteases that were only detected by proteomics include similar to transmembrane protease serine 13, similar to transmembrane protease serine 9, and similar to ubiquitin specific protease 42 (Farinazzo *et al.*, 2009). It is worth pointing out that cathepsin D which was previously purified from the chicken egg yolk (Wouters *et al.*, 1985) was not identified in any of the two proteomic studies (Mann & Mann, 2008; Farinazzo *et al.*, 2009), even though, similar to nothepsin was revealed in both. Alternatively, the aspartic protease in the pigeon egg yolk may be embryonic pepsinogen that is synthesized in the proventriculus, transported through the intestine, and reached the egg yolk together with other transferred egg white proteins. Pepsinogen may not be activated unless it reaches the egg yolk due to its presence as a precursor, or due to inappropriate environmental conditions, or the co-existence of an inhibitor (Yoshizaki *et al.*, 2002). Indeed, we previously found that protein digestion was not evident in the intestinal lumen of pigeon embryo; however, the digestion became apparent on days 16 and 17, after the back flow of yolk contents (Shbailat & Aslan, 2018) (probably including pepsinogen) into the intestine. Furthermore, although embryonic pepsinogen showed peaked activity on days 12 and 13 in quail (Yasugi & Mizuno, 1981), obvious digestion seemed to occur in the intestinal lumen only one day before hatching (Yoshizaki *et al.*, 2002).

The activation of proteases in the egg yolk during development was also examined in different avian species. In chicken, two acidic aspartic proteases that belonged to the cathepsin D class were purified from the egg yolk (Wouters *et al.*, 1985). The activity of proteases increased progressively at early stages until it reached the highest value on day 7, and decreased thereafter (Wouters *et al.*, 1985). Furthermore, Sugimoto & Yamada (1986) found that the activity of a neutral protease and a neutral benzoyl-L-tyrosine ethyl ester hydrolase in the egg yolk increased quickly after incubation day 14 and became maximum on day 18. One difference between pigeon and chicken is in the classes of proteases that became highly activated late in development after the major transfer of egg white into the yolk, which started on day 11 in pigeon (Shbailat & Aslan, 2018), and day 14 in chicken (Carinci & Manzoli-Guidotti, 1968). While both cysteine and aspartic proteases were activated in pigeon, neutral protease and neutral hydrolase were activated in chicken (Sugimoto & Yamada, 1968). Moreover, the time of aspartic protease activation differed between the two species, as this activation occurred early before the major transfer of egg white in chicken (Wouters *et al.*, 1985). In the turkey *Meleagris gallopavo* and the duck *Anas platyrhynchos domestica*, aspartic proteases appeared to be activated in the egg yolk at late stages (Shbailat *et al.*, 2016; Shbailat & Abuassaf, 2018), after the major transfer of egg white proteins into the yolk, which began on day 17 and day 18 in turkey (Shbailat & Safi, 2015) and duck (Shbailat & Abuassaf, 2018), respectively. In addition, weak

activity of cysteine protease(s) was also suggested to be present in turkey egg yolk at late developmental stages (Shbailat *et al.*, 2016). On the other hand, in quail, the aspartic protease cathepsin D was activated between incubation days 6 and 12 (Yoshizaki *et al.*, 2004), before the large transfer of egg white into yolk, which started after day 10 (Yoshizaki *et al.*, 2002). Our results in pigeon are consistent with those from turkey and duck in the time and class of activated proteases (aspartic class). Nevertheless, unlike in pigeon egg yolk, cysteine protease(s) was not activated in duck yolk. In contrast, although aspartic proteases were activated in both pigeon and quail, the time of their activation differed between the two avian species. Moreover, the activity of cysteine protease was not detected in quail egg yolk.

To examine the role of YSM in the digestion of yolk proteins before their transfer into the developing pigeon embryo, we investigated the mRNA expression of *CTSB* and *CTSD* genes during different developmental stages. We found that *CTSB* expression increased progressively and reached maximum value on day 13; however, the expression decreased significantly after that and reached minimum values on days 16 and 17. The initial increase of *CTSB* expression in YSM cells may reflect the need for production of cathepsin B enzyme during this period, probably to participate in the lysosomal digestion of proteins and lipoproteins that are endocytosed by the endodermal cells of YSM. Following that, the decrease in *CTSB* expression was concurrent with the increase in the activity of yolk proteases which started on day 14 and became maximum on day 15 (Shbailat & Aslan, 2018). Our results largely support that after the considerable transfer of egg white into the yolk, extensive protein degradation occurs in the egg yolk itself, probably by cysteine and aspartic proteases. The digested protein products may then transfer into the endodermal cells of YSM. Consistent with this suggestion, we previously found that the expression of the oligopeptide transporter *PepT1* in YSM increased gradually and reached highest values between days 13 and 15 (Shbailat & Aslan, 2018), and then the expression decreased toward hatching (Dong *et al.*, 2012; Shbailat & Aslan, 2018). The reduction in the expression of both *PepT1* (Dong *et al.*, 2012; Shbailat & Aslan, 2018) and *CTSB* (this work) was similar to the reduction in the expression of other nutrient transporters and digestive enzymes in the pigeon YSM toward hatching (Dong *et al.*, 2012; Shbailat & Aslan, 2018). This reduction was simultaneous with the shift in the route of nutrient transport to the embryo from YSM to yolk stalk when the back flow of yolk into the intestine occurred (Dong *et al.*, 2012; Shbailat & Aslan, 2018). In fact, the YSM started to internalize into the embryo intestine on day 16 and then degenerate; probably due to the induction of apoptosis, as has been found in chicken (Reno *et al.*, 2022). With regard to *CTSD*, we showed that the expression of the gene was weak allover development and did not change significantly across stages. Therefore, we propose that the encoded cathepsin D has a minor role in yolk protein digestion inside the endodermal cells of YSM. It is unknown, however, whether the other lysosomal aspartic protease cathepsin E (Patel *et al.*, 2018) is involved in protein digestion. Furthermore, as far as we know, the gene expression patterns and functions of other lysosomal cysteine proteases like cathepsin C, L, and H (Patel *et al.*, 2018) and serine proteases such as cathepsin A and G (Patel. *et al.*, 2018), are still unexplored in the pigeon YSM. Future studies are awaited to uncover the action of different cathepsins in yolk protein degradation during the development of pigeon embryo.

Finally, the expression of proteases was also revealed in the chicken YSM using a temporal transcriptome analysis. Yadgary *et al.* (2014) showed that 3500 genes in the membrane exhibited a significantly changed expression across embryonic days 13, 15, 17, 19, and 21. Among the 50 highest expressed genes were *CTSB*, cathepsin L2 (*CTSL2*), and cathepsin A (*CTSA*). The two former genes encode for cysteine proteases, whereas the last one produces a serine protease (Patel *et al.*, 2018). Regarding the expression pattern of cathepsin B, it was upregulated in the period between embryonic days 13 and 17; however, the expression was downregulated toward hatching (Yadgary *et al.*, 2014). In agreement with previous study, we showed that the expression of cathepsin B in pigeon YSM was downregulated toward hatching after reaching the maximum value on day 13.

CONCLUSION

In conclusion, our results showed that after the considerable transfer of egg white into the yolk during the development of pigeon embryo, the degradation of yolk proteins appeared to occur largely in the egg yolk itself, probably by the activated cysteine and aspartic proteases. Furthermore, *CTSB* in the YSM appeared to have an essential role in protein digestion; however, this role decreased toward hatching as the whole membrane started to degenerate and the main route of nutrient transfer was shifted to yolk

stalk. Future studies should investigate the expression patterns of proteases and determine their classes and proteolytic activities in the yolk sacs of different avian embryos that belong to other orders of Neoaves. This will help to better understand whether or not similar mechanisms underlying yolk protein processing are evolved in basal (Galloanserae) and derived (Neoaves) clades of Neognathae (Hackett *et al.*, 2008; Braun & Kimball, 2021).

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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