Identification and Characterization of the Precursor of Chicken Matrix Metalloprotease 2 (pro-MMP-2) in Hen Egg

SOPHIE RÉHAULT-GODBERT,*,[†] JOËL GAUTRON,[†] VALÉRIE LABAS,[‡]
MAYA BELGHAZI,[‡,§] AND YVES NYS[†]

INRA, UR83 Recherches Avicoles, Fonction et Régulation des Protéines de l'Oeuf,
F-37380 Nouzilly, France, and INRA, UMR85 Physiologie de la Reproduction et des Comportements,
Plate-forme de Protéomique Analytique et Fonctionnelle, Laboratoire de Spectrométrie de Masse,
F-37380 Nouzilly, France

Using zymography and mass spectrometry, we identified for the first time the precursor of chicken matrix metalloprotease 2 (pro-MMP-2) as a complex with TIMP-2 (tissue inhibitor of metalloproteinases) in egg white and yolk. Real-time polymerase chain reaction confirmed that MMP-2 and its inhibitors TIMP-2 and TIMP-3 were expressed all along the oviduct and in the liver of laying hens. We also demonstrated that the processing of pro-MMP-2 into mature MMP-2 by serine proteases does not occur in vivo, although purified pro-MMP-2 undergoes proteolytic maturation by these proteases in vitro. Moreover, the relative pro-MMP-2 activity assessed by gelatin zymography was shown to decrease in egg white during the storage of unfertilized or fertilized eggs. However, the mature form of 62 kDa MMP-2 could not be detected. The fact that MMP-2 is found as a proform in fresh eggs suggests that the activity of this metalloprotease is regulated under specific conditions during embryonic development.

KEYWORDS: Egg; metalloprotease; precursor; regulation; chicken development

INTRODUCTION

The protein components of egg white participate in embryonic development by providing essential nutrients for embryo growth (*1*) and by supplying potent antimicrobial protection against pathogens to the embryo. For humans, the egg is known for its high nutritive value, but it is also a source of numerous proteins with biological activities that are of major interest for different industrial areas related to animal and human health or agronomy (*2*).

Among the bioactivities that have been described, the antimicrobial activity of egg white proteins is the most widely documented. Some proteins indirectly act on bacterial survival by sequestering metals and vitamins that are essential for bacterial growth and survival. These molecules include ovotransferrin that binds iron (*3*) but also flavoprotein and avidin that possess a high affinity for riboflavin and biotin, respectively (*4*). Following their proteolytic digestion, these proteins are a source of vitamins for the developing chicken (*4*). In addition, two other systems participate in antibacterial defense: (i) the

lysozyme, which has a potent bacteriolytic activity (*5*), and (ii) the antiproteases that inhibit bacterial proteases. Four antiproteases are fully characterized in egg white to date: the serine protease inhibitors ovoinhibitor and ovomucoid; ovostatin, a broad-spectrum antiprotease; and cystatin, an inhibitor of cysteine proteases (*6*). However, the physiological proteases targeted by these four antiproteases have not yet been characterized. Despite the fact that ovalbumin belongs to the serine protease inhibitor (serpin) family, it lacks any inhibitory activity (*7*). This protein represents 50% of the proteins found in egg white, and its physiological function is still under investigation. However, it is probably a major source of nutrients for the embryo (*8*).

The recent use of 1-D PAGE followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and MS³ techniques revealed the presence of additional proteases and antiproteases in egg white (*9*). Proteases and their regulation by antiproteases are of major importance in numerous physiological and pathological processes. In humans, the azurophilic granules of neutrophils contains several proteases such as cathepsin G, elastase, and proteinase 3 that play an important role in the defense against pathogens and in the regulation of the inflammatory process (*10*). In *Gallus gallus*, proteases were shown to be essential for the ovulatory process from the development of the follicle to its atresia (*11*), for fertility and spermatozoa mobility (*12*), for coagulation and fibrinolysis (*13*),

* To whom correspondence should be addressed. Tel.: 33 2 47 42 78 39; fax: 33 2 47 42 77 78; e-mail: rehault@tours.inra.fr.
† UR83 Recherches Avicoles.
‡ UMR85 Physiologie de la Reproduction et des Comportements.
§ Current address: Centre d'Analyze Protéomique de Marseille, IFR Jean Roche, 51 Boulevard Pierre Dramard, 13916 Marseille cedex 20, France.

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and for embryonic chicken development (*14, 15*), including the intraoocytic processing of yolk proteins by cathepsin D for subsequent utilization by the embryo (*16*). Thus, proteases are key factors in many physiological mechanisms including antibacterial defense, reproduction, cell migration, and proliferation and are largely distributed among eukaryotes, prokaryotes, and viruses. The antiproteases present in egg white probably explain the difficulty in detecting protease activities in this fluid by conventional enzymatic methods. Moreover, proteases must be present as minor components since they have not been detected by classical biochemical and proteomic approaches (*17*) but required advanced and sensitive techniques for their recent identification (*9*).

To gain further insight into the role of egg proteases, we used gelatin zymography to detect for the first time gelatinase activity in egg whites of unfertilized and fertilized hen eggs. After purification followed by mass spectrometry, we identified this protease as the precursor form of matrix metalloprotease type 2 (pro-MMP-2). To explore its potential physiological function, we studied its activation in vitro and in vivo during the incubation or storage of eggs.

MATERIALS AND METHODS

Materials. Gelatin was purchased from Bio-Rad (Marnes-la-Coquette, France). Casein β, blotting grade buffer (nonfat dry milk), Triton X-100, EDTA, APMA, rabbit antihuman TIMP-2, and purified proteases (bovine trypsin, bovine chymotrypsin, human neutrophil elastase, human cathepsin G, and human pro-MMP-2) were from Sigma-Aldrich (Saint Quentin Fallavier, France). The Alexa Fluor 680 goat antirabbit IgG was from Molecular Probes (Cergy-Pontoise, France). Positive controls for TIMP-2 and MMP-2 (human fibroblasts) were purchased from Calbiochem (VWR, Fontenay-sous-Bois, France). Gelatin-sepharose 4B was obtained from Amersham Bioscience (GE Healthcare, Orsay, France). RNeasy RNA Extraction and Superscript II for reverse transcription were from Qiagen (Courtaboeuf, France) and Invitrogen (Cergy-Pontoise, France), respectively, and PCR primers were obtained from Eurogentec (Seraing, Belgium). All other chemicals were of analytical grade.

Sample Collection and Preparation. Fresh nonfertilized egg whites and yolks from laying hens (ISA Brown, Hendrix Genetics, St. Brieuc, France) were diluted 1:1 (v/v) in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, 0.02% NaN₃ and then gently homogenized and centrifuged at 14 000*g* at 4 °C for 10 and 20 min, respectively, to pellet viscous and insoluble proteins. The protein concentration of the clear supernatants was estimated from the absorbance at 280 nm using Nanodrop (Nanodrop ND-1000 spectrophotometer, Labtech, Palaiseau, France), which automatically calculates the protein concentration in mg/mL from the absorbance at 280 nm. Supernatants were kept at −20 °C until further use. Uterine fluids were collected as reported by Gautron et al. (*18*). All experiments, including all animal handling protocols, were carried out in accordance with the European Communities Council Directives of November 24,1986 (86/609/EEC) and the French decree 87848 of October 19, 1987 (revised on May 31, 2001).

Zymography. Gelatinolytic activities were assessed as previously described (*19*). Briefly, 40 *µ*g of proteins (from egg white or yolk) and 20 *µ*L of uterine fluid were diluted in 5X Laemmli buffer under denaturing but non-reducing conditions, loaded onto 10% acrylamide-bisacrylamide gels containing 0.3 mg/mL gelatin, and separated by electrophoresis. Protein renaturation was achieved by soaking the gels in 2.5% Triton X-100 for 1 h (2 × 30 min) at room temperature under constant shaking. Gels were then incubated in 50 mM Tris-HCl, 50 mM NaCl, 10 mM CaCl₂, pH 7.5, with or without 10 mM EDTA, for 20 h at 37 °C, followed by staining with Coomassie Blue. Hydrolytic bands, corresponding to degradation of gelatin by protease(s), were visualized as clear zones on a blue background (white zones on a black background in the figures).

Metalloprotease Purification. Yolk and egg white MMP-2 were purified by affinity chromatography using gelatin-sepharose (*19, 20*).

Gelatin beads were equilibrated in 50 mM Tris-HCl, 0.5 M NaCl, 10 mM CaCl₂, pH 7.5, 0.05% Brij 35, 0.02% NaN₃ and incubated overnight at 4 °C under constant shaking with the supernatant either from homogenized egg white or yolk (prepared as described previously). Beads were then loaded onto a polypropylene column and washed 3 times with 5 mL of incubation buffer. Elution was performed with 1 mL fractions of 50 mM Tris-HCl, 1 M NaCl, 10 mM CaCl₂, 0.05% Brij 35, 0.02% NaN₃ containing 5% DMSO. The activity of eluted samples was assessed by zymography as described previously. Active fractions were pooled, dialyzed for 20 h against diluted Tris buffered saline (0.2 × TBS), lyophilized, and stored at −20 °C.

Western Blot Analysis. Active gelatinase fractions from egg white and yolk were diluted in 5X Laemmli buffer under reducing conditions, boiled, and loaded on a 10% acrylamide-bisacrylamide gel. Proteins were separated by electrophoresis followed by electroblotting onto a nitrocellulose membrane. Membranes were blocked in TBS containing 10% nonfat dry milk for 1 h at room temperature under constant shaking and then incubated for 1 h at 37 °C with rabbit anti-hTIMP-2 (1:500 in TBS, 5% dry milk, 0.2% Tween 20). Membranes were washed 3 times for 10 min in TBS containing 1% dry milk, 0.2% Tween 20. Incubation with goat anti-rabbit Alexa Fluor 680-conjugated antibody (1:1000) was performed at 37 °C for 1 h followed by three washes in TBS containing 1% dry milk, 0.2% Tween 20 and a final wash in TBS. Immunodetection was performed using a Licor infrared Odyssey scanner (Odyssey, LI-COR Biosciences Inc., Lincoln, NE).

Protease Activation. Activation of pro-MMP-2 by exogenous proteases was performed by incubating pro-MMP-2 with cathepsin G (225 nM), neutrophil elastase (50 nM), trypsin (4 nM), or chymotrypsin (4 nM) in 50 mM Hepes, 0.1 M NaCl, pH 7.4 for 30 min at 37 °C. Reactions were stopped by the addition of non-reducing sample buffer, and samples were kept at −20 °C prior to analysis by gelatin zymography.

Effect of Storage. Nonfertile eggs were collected from 30 different laying hens of 33 weeks of age (ISA Brown, Hendrix Genetics, St. Brieuc, France). Eggs were incubated at 4, 20, and 37 °C for up to 8 days. Each day, whites from three eggs were separated from the yolks and diluted individually 1:1 (v/v) in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, 0.02% NaN₃. Homogenized egg whites and yolks were centrifuged at 14 000*g* for 10 and 20 min, respectively, and protein concentrations were estimated using absorbance at 280 nm (Nanodrop, ND-1000 spectrophotometer, Labtech, Palaiseau, France). As a control, egg whites sampled at day 0 were stored separated from yolks at 37 °C for 8 days. Gelatinase activity of egg whites (30 *µ*g) and yolks (60 *µ*g) at each time point was assessed by gelatin zymography. Quantification of hydrolytic bands was performed by quantitative enumeration of pixels (Odyssey Imager Software, LI-COR), and results were expressed as the relative activity by reference to the activity measured at day 0. All experiments were performed in triplicate.

Effect of Fertilization. Eggs were fertilized manually by several introductions of sperm into the oviduct of laying hens (ISA Brown, Hendrix Genetics, St. Brieuc, France). After collection, all eggs were incubated at 37.8 °C, beginning on the same day to allow embryonic development for up to 10 days. At day 0 (the day of lay), we collected egg whites daily from at least three different eggs (biological replicates) by perforating the shell at the smaller end of the egg, while the other eggs remained in the incubator for samplings on days 1, 2, 3, 4, 7, 8, 9, and 10 of incubation. Each egg white was independently processed by homogenization and centrifugation. The protein concentration of the supernatants was determined as described previously before analysis by zymography.

Mass Spectrometry Analysis. The elution fractions from the gelatin-sepharose chromatography that possessed metalloprotease activity were further analyzed by SDS-PAGE under reducing conditions and stained by Coomassie Blue. Various bands were excised from the gel and digested in gel slices with bovine trypsin (Roche, Neuilly sur Seine, France, EC 3.421.4) as described by Shevchenko et al. (*21*). Digests were resuspended in 7 *µ*L of formic acid for analysis by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF).

MALDI-TOF. The matrix used was α-cyano-4-hydroxycinnamic acid at 10 mg/mL dissolved in 50% ethanol/50% acetonitrile. The sample and the matrix (1:1 v/v) were loaded on the target using the
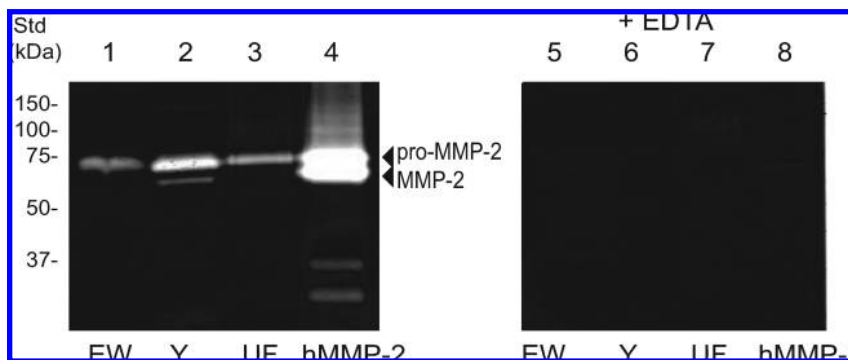
Figure 1. Identification of a 72 kDa gelatinase in egg white, yolk, and uterine fluid. Gelatinolytic activities of biological samples, egg white (EW, lanes 1 and 5), yolk (Y, lanes 2 and 6), uterine fluid (UF, lanes 3 and 7), as well as human matrix metalloprotease-2 containing zymogen and active form (hMMP-2, lanes 4 and 8) were assessed by gelatin zymography with (lanes 5—8) or without (lanes 1—4) EDTA, as described in the Materials and Methods.

dried droplet method. MALDI-TOF spectra of the peptides were obtained with a M@LDI L/R P/N mass spectrometer (Waters, Manchester, U.K.). The analyses were performed in positive ion reflector mode, with an accelerating voltage of 15 000 V. Spectra obtained were calibrated externally using $[M + H]^+$ ions from bovine serum albumin digest. For subsequent data processing, the MassLynx 4.0 software (Waters, Guyancourt, France) was used. MS data were searched against the nonredundant National Center for Biotechnology Information database using the ProFound software (http://prowl. rockefeller.edu) with a mass accuracy of 0.1 Da.

LC-MS/MS. Nanoscale capillary LC-MS/MS analysis of the digested proteins was performed using the CapLC system coupled to a hybrid quadrupole TOF mass spectrometer (Qtof-Global, Waters, Micromass, Manchester, U.K.) fitted with a Z-spray ion source. Samples were desalted and concentrated using an online precolumn (PepMap C_{18}, 0.3 mm i.d. × 5 mm length, Dionex). Peptide separations were conducted on a C_{18} column (Atlantis dC_{18}, 75 μm i.d. × 150 mm Nano Ease, Waters) running with a 180 nL/min flow. The gradient profile consisted of a linear gradient from 100% A (0.1% formic acid/2% acetonitrile/98% H_2O, v/v) to 60% B (0.1% formic acid/20% H_2O/ 80% acetonitrile, v/v) in 80 min.

Mass data were acquired using automatic switching between MS and MS/MS (fragmentation) modes: one MS survey scan was followed by MS/MS scans on the three most intense peptide ions detected. Only doubly and triply charged ions were allowed to be selected as precursors over a mass range of 400—1300 *m/z*. The collision energy was selected depending on the precursor ion mass and charge. The mass spectrometer was calibrated using the fragmentation spectrum of 500 fmol/μL Glu-fibrinopeptide (Sigma-Aldrich, Saint Quentin Fallavier, France) in 50% water/50% acetonitrile/0.1% formic acid.

The peptide and fragment masses obtained were matched automatically to proteins in a nonredundant database (NCBInr 20060318 with 3 479 934 sequences; 1196146007) using the MS/MS ion search option of Mascot software (http://www.matrixscience.com). Enzyme specificity was set to trypsin with two missed cleavages using carbamidomethylcysteine and methionine oxidation as variable modifications. The tolerance of the precursor ion was set to 0.3 mass units for both parent and fragment ion matches. All hits with a *p* value <0.05 were manually verified, and only those proteins containing at least two different peptide assignments were considered positively identified. Mascot indicates identity for ion scores >51.

Real Time Reversal Transcription Polymerase Chain Reaction (Real Time RT-PCR). Total RNA was extracted from different sections of the oviduct (magnum, white and red isthmus (proximal and distal isthmus), and uterus) and liver of 37 week laying hens (ISA Brown, Hendrix Genetics, St. Brieuc, France), using a commercial kit (RNeasy mini kit, Qiagen Sciences, Courtaboeuf, France) and simultaneously treated with DNase (RNase-free DNase set, Qiagen Sciences, Courtaboeuf, France). Total RNA samples (5 μg) were reverse transcribed using RNase H$^-$ MMLV reverse transcriptase (Superscript II, Invitrogen, Cergy-Pontoise, France) and random hexamers (Amersham, Orsay, France).

MMP-2, TIMP-2, and TIMP-3 cDNA were amplified in real time using the qPCR Master mix plus for Sybr green I (Eurogentec, Seraing, Belgium) with the ABI PRISM 7000 apparatus (Applied Biosystems, Courtaboeuf, France). Six specific primers were chosen from the *G. gallus* MMP-2 (sense, 5′-CTCTTGGAGAAGGGCAAGTG-3′; antisense, 5′-CCATTGAACCAGAAGGGAAA-3′), TIMP-2 (sense, 5′-GGAACCCCATCAAGCGAAT-3′; antisense, 5′-GGCGCCGTGTA-GATGAATT-3′), and TIMP-3 (sense, 5′-TGGCCTGTGCAATTGGTATG-3′; antisense, 5′-AATCTTGCATCCACAGCCCAG-3′) cDNA sequences. The combination of MMP-2 sense/MMP-2 antisense primers, TIMP-2 sense/TIMP-2 antisense primers, and TIMP-3 sense/TIMP-3 antisense primers allowed the amplification of 112, 91, and 97 bp fragments, respectively. MMP-2, TIMP-2, and TIMP-3 mRNA levels were corrected relative to ribosomal 18S rRNA levels to account for variations in mRNA extraction and reverse transcription reaction between samples, using a TaqMan universal PCR master mix and predeveloped TaqMan assay reagents for human 18S rRNA (Applied Biosystems, Courtaboeuf, France), as described elsewhere (*22*).

The cycling conditions consisted of a uracil-*N*-glycosylase (UNG) preincubation step at 50 °C for 2 min, followed by a denaturation step at 95 °C for 10 min and 40 cycles of amplification (denaturation for 15 s at 95 °C and annealing and elongation for 1 min at 60 °C). A melting curve program was carried out from 60 to 95 °C for 20 min for each individual sample amplified with Sybr Green. Samples showed a single product with a specific melting temperature of 79.3, 78.3, and 77.9 °C for the combination of MMP-2 sense/MMP-2 antisense primers, TIMP-2 sense/TIMP-2 antisense primers, and TIMP-3 sense/TIMP-3 antisense primers, respectively. Each sample was diluted and then amplified in triplicate in the same run. Each run included triplicates of no template controls and triplicates of control cDNA corresponding to a pool of oviduct cDNA from laying hens sampled during eggshell formation (18 h post-ovulation) and egg white formation (3 h post-ovulation). The control cDNA was diluted from 1:50 to 1:156 250, and relative arbitrary quantities were defined. The threshold cycle (CT), defined as the cycle at which fluorescence rose above a defined baseline, was determined for each sample and control cDNA. A calibration curve was calculated using the CT values of the control cDNA samples; relative amounts of unknown samples were deduced from this curve. The PCR efficiencies for MMP-2, TIMP-2, TIMP-3, and 18S rRNA were 80.9, 98.2, 100, and 100%, respectively.

The ratio value was calculated for each sample with respect to 18S rRNA. The log of the ratio was used for statistical analysis using StatView software (SAS Institute Inc., version 5). A one-way analysis of variance (ANOVA) followed by a PLSD Fisher test was performed to detect significant differences in mRNA expression ($p < 0.05$) in the oviduct of birds at various physiological phases.

RESULTS

Detection of Gelatinolytic Activities in Different Egg Compartments. All samples displayed a 72 kDa gelatinolytic activity that was abolished by including the chelating agent

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EDTA in the activation buffer (**Figure 1**). The egg yolk sample also showed a lower molecular weight band that was similarly susceptible to EDTA (**Figure 1**, lanes 2 and 6). We also demonstrated that the 72 kDa activity was inhibited by the absence of calcium but was unaffected by the addition of heparin at 1000 U/mL (data not shown), which has been shown to modulate the activity of some metalloproteases (*23*). On the contrary, the reducing agent DTT suppressed the activity (data not shown), suggesting that the disulfide bonds of the molecule are essential for the enzymatic activity of the protease. Moreover, the 72 kDa protease displayed no activity on casein zymography (data not shown). Taken together, these results suggest that the 72 kDa protease was a metalloprotease with gelatinolytic activity. In these experiments, we also noticed high molecular weight bands showing very low gelatinolytic activities that did not seem to be affected by the absence of calcium nor by the presence of EDTA (data not shown).

Purification and Identification of the 72 kDa Protease. The 72 kDa protease was purified by affinity chromatography on a gelatin-sepharose column as generally used to purify metalloproteases with gelatinolytic activity (*19*). Most of the 72 kDa protease activity was recovered in the first four fractions of elution. These fractions were dialyzed and lyophilized, and the concentrated sample was analyzed by zymography. Besides the 72 kDa gelatinase activity, gelatinolytic bands of ∼90−95 kDa also were detected in purified fractions of pro-MMP-2 from yolk.

These samples were further analyzed by SDS-PAGE under reducing conditions. The band corresponding to 72 kDa was excised from the gel for MALDI-TOF mass spectrometry and LC-MS/MS analysis. This approach demonstrated that the metalloprotease that was isolated from egg white and yolk was the precursor form of matrix metalloprotease 2 (pro-MMP-2, EC 3.4.24.24). Eight peptides were assigned to MMP-2 in the fraction purified from egg white. Two of them were unambiguously identified within the pro-peptide sequence of pro-MMP-2 by mass spectrometry (APSPIIKFPGDSTPK and TD-KELAVQYLNK). Regarding egg yolk MMP-2, 16 peptides were matched with the MMP-2 sequence, and three were contained in the pro-region (TDKELAVQYLNK, CGNPD-VANYNFFPR, and FFGLPETGDLDQNTIETMK). The fraction of egg white pro-MMP-2 eluted from gelatin zymography was not pure, and LC-MS/MS analysis revealed the presence of protein contaminants (ovotransferrin BB type (BAE16337), fragments of ovalbumin (AAB59956), ovomucin α-subunit (BAB21488), ovostatin (CAA55384), similar to ovoinhibitor (XP_414557), and clusterin (NP_990231)). Pro-MMP-2 purified from yolk also was coeluted with minor proteins (similar to apolipoprotein B precursor (XP_419979), similar to fibronectin 1 isoform 3 preproprotein (XM_421869.1), and fragments of ovalbumin (AAB59956)). LC-MS/MS analysis detected two to 16 different peptides that corresponded to three coeluted proteins; because of the high heterogeneity of the samples, other proteins could not be detected unambiguously.

Western blotting conducted with anti-human TIMP-2 (tissue inhibitor of metalloprotease type 2) on purified fractions of MMP-2 demonstrated that pro-MMP-2 from yolk and egg white both copurified with TIMP-2 (**Figure 2**) as already was reported for mammalian pro-MMP-2 (*20, 24*). The presence of TIMP-2 was corroborated by LC-MS/MS analysis of a 20 kDa protein contained in purified pro-MMP-2 from egg white and was barely perceptible by SDS-PAGE with Coomassie Blue staining. TIMP-2 was identified with a Mascot score equal to 53 with two different peptides EVDSGNDIYGNPIKR (sequence 54−68)
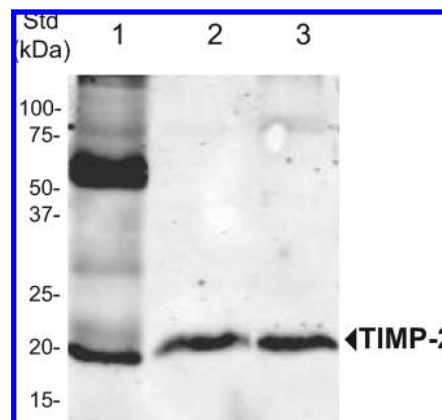

Figure 2. Western blot analysis of purified pro-MMP-2 from egg white and yolk with anti-human TIMP-2 antibodies. Lanes were loaded as follows: lane 1, fibroblast lysate (positive control for MMP-2 and TIMP-2, 5 μL) and purified pro-MMP-2 from egg white and yolk (lanes 2 and 3, respectively).

and GMAPPKQEFLDIEDP (sequence 206−220). The MS/MS spectrum visually was verified to confirm that the four ion fragments followed a minimum. In this gel band, we also identified lysozyme (630460A) and quiescence-specific protein or ex-FABP (NP_990753). The concentrations of pro-MMP-2 in yolk and egg white were difficult to estimate because of the presence of coeluted proteins. Moreover, the band corresponding to pro-MMP-2 on SDS-PAGE was faintly detectable after purification, suggesting that pro-MMP-2 is a minor protein in egg white and yolk.

Real time RT-PCR performed with different segments of the oviduct using specific primers for MMP-2, TIMP-2, and TIMP-3 (tissue inhibitor of metalloprotease type 3) revealed no significant difference between the two stages studied, 3 and 18 h after ovulation, corresponding, respectively, to the secretion of egg white proteins and eggshell formation. Therefore, data originating from the experiments using tissues harvested at 3 and 18 h after ovulation were grouped together to increase the significance between tissues. Results showed that all three proteins were expressed in the oviduct (**Figure 3**): magnum (egg white deposition), white isthmus (or proximal isthmus, where eggshell membranes are added), red isthmus (or distal isthmus, where mineralization is initiated at the origins of the mammillary knobs), and uterus (eggshell formation), in addition to the liver, which expresses proteins found in egg yolk. MMP-2 expression was shown to be significantly higher in red isthmus as compared to magnum ($p < 0.005$), white isthmus ($p < 0.0005$), liver ($p < 0.0005$), and uterus ($p < 0.05$). Regarding TIMP-2, we observed a substantial expression of TIMP-2 in liver in comparison to oviduct tissues ($p < 0.0001$). Moreover, we showed that expression of TIMP-2 in the oviduct followed a similar pattern as the expression of MMP-2, with a higher expression in red isthmus as compared to expression in white isthmus ($p < 0.05$) and uterus ($p < 0.01$). Although the expression of TIMP-2 in the magnum was not statistically different from expression in the white and red isthmus, it was, however, significantly higher as compared to the uterus ($p < 0.05$). TIMP-3 displayed a higher expression in magnum and red isthmus as compared to expression in the uterus ($p < 0.0001$), white isthmus ($p < 0.001$), and liver ($p < 0.0001$).

Activation of pro-MMP-2. The mercuric derivative APMA is known to be a potent activator of metalloproteases and generates a 62 kDa active form from the 72 kDa human pro-MMP-2 (*25*). However, purified pro-MMP-2 from chicken egg
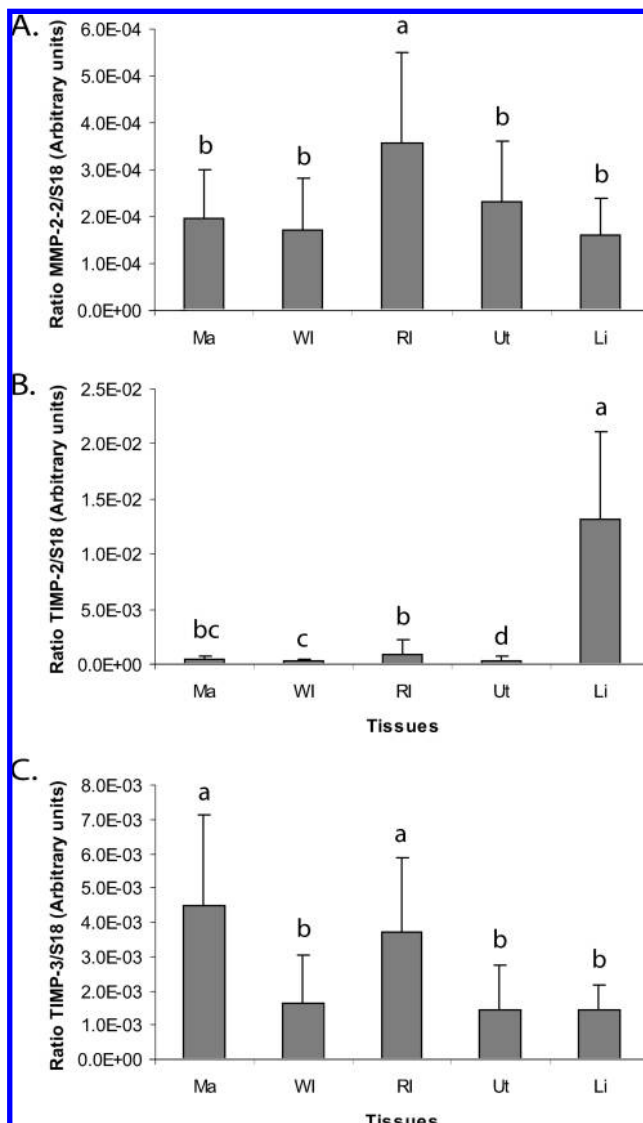
Figure 3. Analysis of mRNA expression of (**A**) MMP-2, (**B**) TIMP-2, and (**C**) TIMP-3 by Real time RT-PCR. Ma, magnum; WI, white isthmus; RI, red isthmus; Ut, uterus; and Li, liver. Mean standard deviations that are indicated with the same letter are not significantly different from each other ($p < 0.05$).

white and yolk did not undergo a marked activation by APMA after 2 h of incubation at 37 °C (data not shown).

We investigated the activation of pro-MMP-2 by exogenous serine proteases, including trypsin (T), chymotrypsin (CT), cathepsin G (CG), and elastase (HNE) since the latter proteases are potent activators of human pro-MMP-2 (*26*). Cathepsin G, elastase, trypsin, and chymotrypsin are themselves capable of degrading gelatin and appeared as gelatinolytic bands of ~25 kDa for cathepsin G (**Figure 4A,B**, lane 1) and elastase (and a higher molecular band of ~100 kDa, **Figure 4A,B**, lane 2) and 18 and 25 kDa for trypsin and chymotrypsin, respectively (**Figure 4A,B**, lanes 3 and 4). Pro-MMP-2 from egg white and yolk after being submitted to the various steps of purification was unstable over time and was shown to autoactivate into MMP-2 during a 30 min incubation at 37 °C (**Figure 4A**, lanes 6 and 11), as mentioned elsewhere (*27*). This mature MMP-2 is also present in yolk (**Figure 1**, lane 2), but the mechanism by which it is partly activated in yolk was not elucidated. In the controls (**Figure 4A**, lanes 6 and 11), the relative activity between pro-MMP-2 and MMP-2 was equal to 2 and 1.42 for purified MMP-2 from egg white and purified MMP-2 from egg

yolk, respectively, which demonstrates that pro-MMP-2 is the major form in these fractions. All proteases promoted the processing of pro-MMP-2 into the mature protein (MMP-2), which appeared with a molecular mass of 62 kDa (**Figure 4A**, lanes 7−10 vs pro-MMP-2 purified from egg white without proteases, lane 6 and **Figure 4A**, lanes 12−15 vs pro-MMP-2 purified from yolk without proteases, lane 11). The ratio between pro-MMP-2 and MMP-2 forms varied from 0.3 to 0.61 according to the protease, emphasizing the fact that pro-MMP-2 disappeared in favor of mature MMP-2 after incubation with the proteases. Additionally, all proteases hydrolyzed pro-MMP-2 into smaller fragments of ~40 and/or 32 kDa that still showed gelatinolytic activities (**Figure 4A**, lanes 7−10 and 12−15).

When crude egg whites or yolks were incubated with proteases, no apparent variation of the pro-MMP-2 activity or further production of mature MMP-2 could be observed (**Figure 4B**, lanes 7−10 vs crude egg white without proteases, lane 6 and lanes 12−15 vs crude yolk, lane 11). The incubation of egg white with elastase also produced a gelatinolytic band of 80 kDa that we could not explain in this work (**Figure 4B**, lane 8). The proteolytic fragments of 40 and 32 kDa generated by the proteases from purified pro-MMP-2 (**Figure 4A**, lanes 7−10 and 12−15) were not detected in egg white or yolk after incubation with either proteases (**Figure 4B**, lanes 7, 10, 12, and 15). In egg white, the presence of a large amount of ovalbumin (MW of 40 kDa) on SDS-PAGE under non-reducing conditions interferes with the detection of proteolytic fragments in this size range. However, in egg yolk where such a major protein is not present, these potential MMP-2 fragments can be revealed, but they were, however, not detected. Interestingly, the endogenous gelatinolytic activity of elastase, trypsin, and chymotrypsin tended to disappear when the proteases were incubated with crude egg whites or yolks (**Figure 4B**, lanes 8−10 and 13−15 vs lanes 2−4). Altogether, these data suggest that both fluids contain components that are able to inhibit and inactivate these specific exogenous proteases.

Effect of Egg Storage and Fertilization. To further analyze the regulation of pro-MMP-2 in yolks or egg whites, we studied the effect of storage of unfertilized eggs at 4, 20, and 37.8 °C for up to 8 days. No change in activity of pro-MMP-2 in egg white or yolk was observed when eggs were incubated at 4 and 20 °C (data not shown). In yolks at 37.8 °C, the intensity of pro-MMP-2 gelatinolytic activity remained essentially the same during this period, whereas no band corresponding to mature MMP-2 could be detected (**Figure 5A**). On the contrary, the relative pro-MMP-2 activity in egg white from stored eggs at 37.8 °C decreased drastically from day 3 to day 4, leading to the absence of any detectable activity after day 4 (**Figure 5A**). In contrast, the activity in the egg white sampled at day 0 and stored independently of other compartments of the egg at 37.8 °C remained stable (data not shown). These results suggest that pro-MMP-2 in egg whites is rapidly and irreversibly inactivated, by inhibition or by proteolytic degradation, during storage of the whole egg at 37.8 °C. Similarly, the relative pro-MMP-2 activity in egg whites of fertilized eggs was observed to decline rapidly up to day 4 but then remained stable up to at least day 10, and no further processing of the 72 kDa zymogen form could be observed (**Figure 5B**, zymogram). Interestingly, this gelatinolytic activity disappeared completely from day 4 in non-fertilized eggs. We did not include egg yolk analysis in this study of fertilized eggs because it undergoes major changes during embryonic development and was difficult to collect without contamination by other fluids.
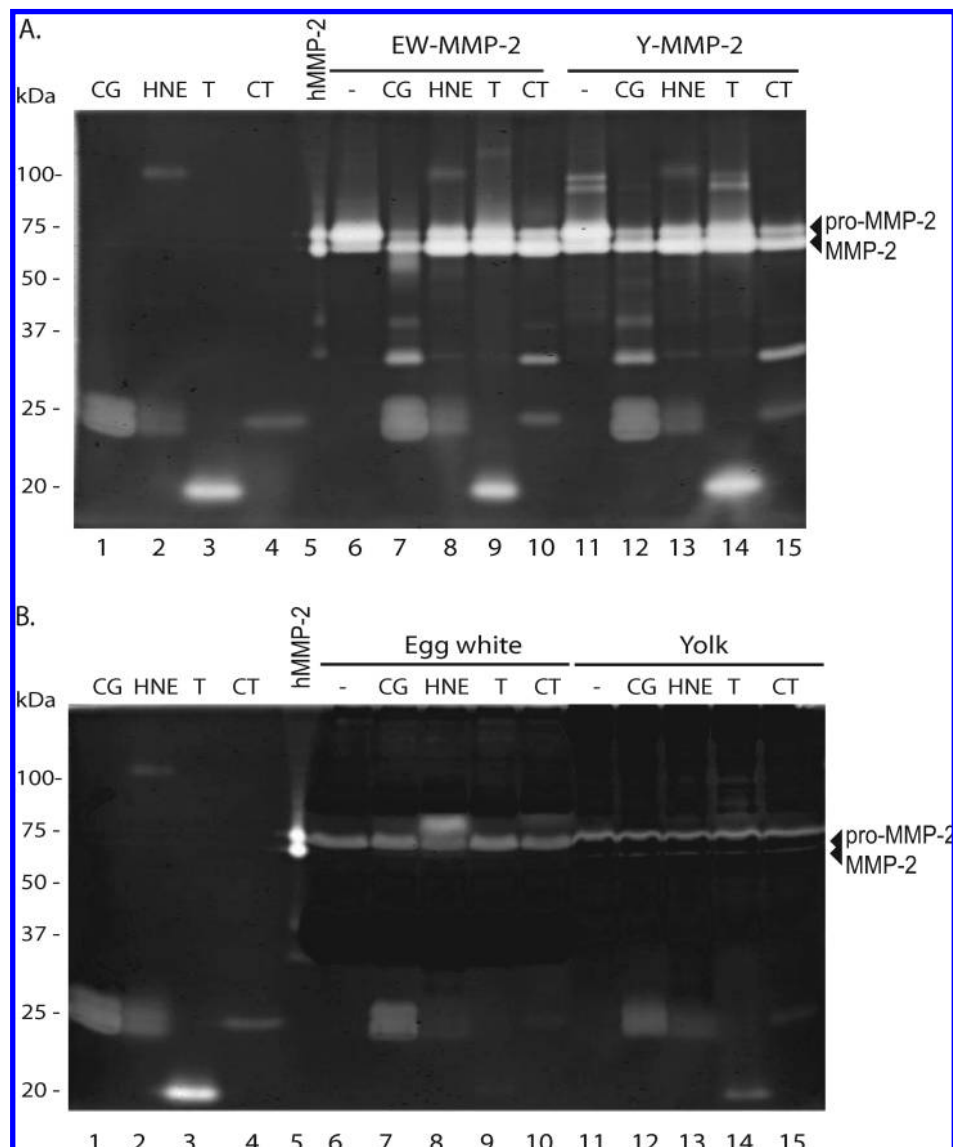
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Figure 4. Gelatin zymography to detect activation by serine proteases of the precursor of pro-MMP-2. (**A**) Activation of pro-MMP-2 purified from egg white and yolk by proteases. Pro-MMP-2 purified from egg white (EW-MMP-2, lanes 6−10) or from egg yolk (Y-MMP-2, lanes 11−15) was incubated without protease (lanes 6 and 11, respectively) or with cathepsin G (CG, lanes 7 and 12), elastase (HNE, lanes 8 and 13), trypsin (T, lanes 9 and 14), and chymotrypsin (CT, lanes 10 and 15) for 30 min at 37 °C. Controls for proteases alone are in lane 1 (cathepsin G, CG), lane 2 (elastase, HNE), lane 3 (trypsin, T), lane 4 (chymotrypsin, CT), and lane 5 (human MMP-2, hMMP-2). (**B**) Activation of endogenous pro-MMP-2 in egg white or yolk by proteases. Similarly, egg white or egg yolk was incubated with proteases as described previously to assess the activation of endogenous pro-MMP-2. Gelatin zymography was then performed to identify the gelatinolytic bands.

DISCUSSION

In this study, we demonstrated that pro-MMP-2 is expressed in the oviduct and the liver of laying hens and that this metalloprotease precursor is present in various compartments of the egg. The physiological function of MMP-2 in mammals has been largely reviewed (*28*). Matrix metalloprotease 2, also called 72 kDa type IV collagenase or gelatinase A, is capable of degrading proteins of the extracellular matrix and is involved in all processes requiring tissue remodelling (*28*). Its role is established in major physiological mechanisms including embryonic development, morphogenesis, angiogenesis, fertility, and reproduction (*29, 30*) but also in pathological situations such as cancer and metastasis (*31*).

Mammalian and avian MMP-2 is secreted as a zymogen or proform (pro-MMP-2) that is enzymatically inactive. This precursor is characterized by the presence of an 80 amino acid propeptide at its N-terminal sequence in which the N-terminal

cysteine residues interact with zinc ions contained in the active site thus interfering with substrate or inhibitor access to this enzymatic domain (*32*). In this study, we identified for the first time chicken pro-MMP-2 as a minor component of egg white, yolk, and uterine fluid. Levels of pro-MMP-2 in egg white seem to be very low, which probably explains as to why the proteomic analyses of egg white reported recently did not detect this specific protein (*9, 17, 33*). Proforms of proteins are inactive in vivo but display activity on gelatin zymography due to the presence of denaturing SDS that removes the propeptide from the active site (*34*).

We have shown that pro-MMP-2 from egg white and yolk copurifies with its specific inhibitor TIMP-2 on gelatin affinity chromatography. Goldberg et al. (*35*) demonstrated that human TIMP-2 did not have any affinity for gelatin-sepharose and suggested that the coelution of bovine TIMP-2 with pro-MMP-2 indicates a tight complex between TIMP-2 and proform of
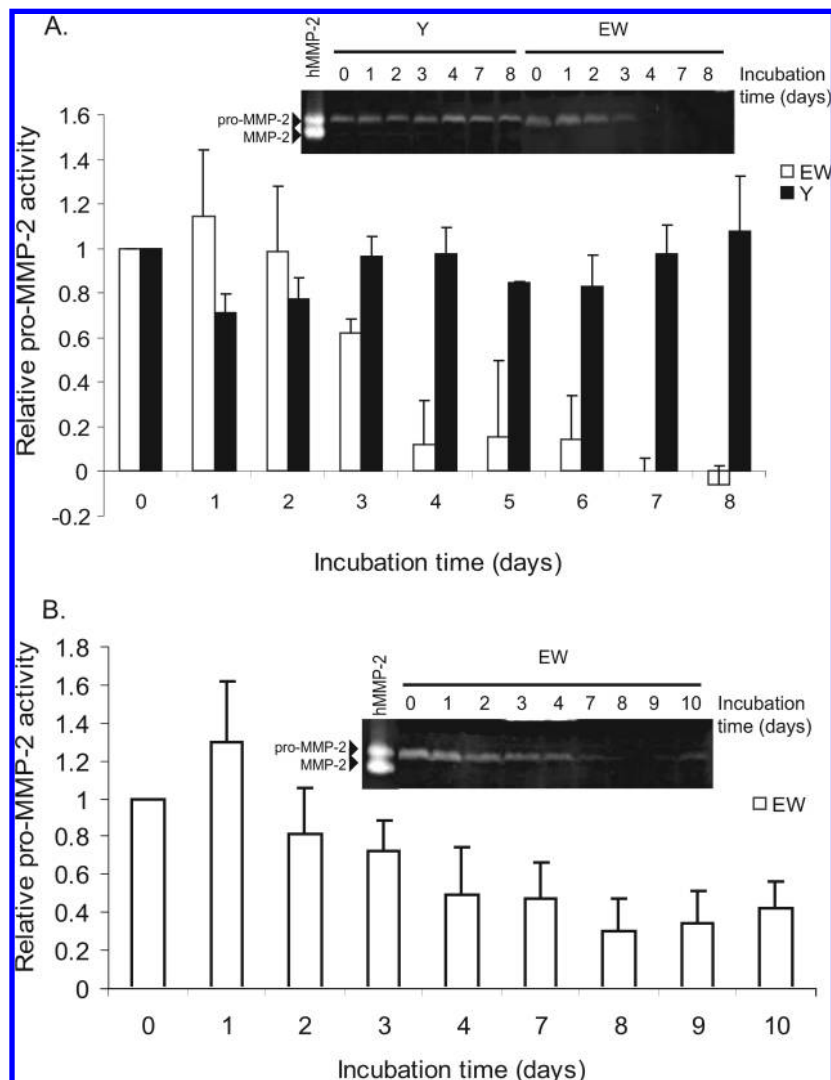
Figure 5. Study of regulation of pro-MMP-2 precursor activity during incubation and fertilization. For each day of incubation, the yolk was separated from the white, and 60 μg of yolk proteins and 30 μg of egg white proteins were loaded on 10% acrylamide gels containing gelatin for analysis by zymography. The graphs illustrate the remaining pro-MMP-2 intensity measured by densitometry (Odyssey) by reference to day 0. Results are expressed as the average of at least three independent experiments. Inset: gelatin zymography of one representative experiment of a series of eggs incubated at 37.8 °C. (**A**) Pro-MMP-2 activity in yolks and egg whites of unfertilized eggs incubated for 8 days at 37.8 °C. (**B**) Pro-MMP-2 activity in egg whites of fertilized eggs incubated for 10 days at 37.8 °C. hMMP-2, human fibroblasts lysate (pro-MMP-2 and mature MMP-2); Y, yolk; and EW, egg white.

matrix metalloprotease 2 (*20*). However, because of the presence of the propeptide, TIMP-2 does not bind to the active site of pro-MMP-2 but rather to the C-terminal extremity of the so-called hemopexin domain of pro-MMP-2 (*35, 36*). Bound TIMP-2 is essential for pro-MMP-2 activation as demonstrated using a model of TIMP-2-deficient mice (*37*) or antisense TIMP-2 oligonucleotides (*38*). Therefore, TIMP-2 allows for the activation of the precursor of MMP-2 by binding to the hemopexin domain of pro-MMP-2, and it also acts as an inhibitor of mature MMP-2 by binding to the active site of the metalloprotease. Some authors have suggested that the presence of TIMP-2 complexed to pro-MMP-2 could contribute to its stability (*39*) and might have a protective effect against proteolytic degradation by physiological proteases (*40*). RT-PCR further demonstrated that TIMP-2 and TIMP-3 were expressed all along the oviduct and in liver with significantly higher expression in some of the tissues tested (**Figure 3**). The biological significance of this tissue distribution pattern needs further investigation.

Activation of pro-MMP-2 in mammals is thought to be mainly triggered by membrane type-matrix metalloproteases (MT-MMP) anchored to the cell membrane and more specifically by MT1-MMPs (or MMP-14) that form dimers or multimers at the cell surface (*28, 41*). In *G. gallus*, the mechanism by which pro-MMP-2 is activated has not yet been elucidated. No protein similar to the mammalian MT1-MMP has been described so far. However, Yang et al. (*42*) reported the identification of a membrane-type matrix metalloprotease that was 89% identical to human MT3-MMP. This MT-MMP was shown to be expressed during early development of the chicken embryo and was found to be colocalized with MMP-2. This MT-MMP might be a candidate for the activation of pro-MMP-2 originating from egg white or yolk during embryonic development.

We analyzed the mechanism by which hen egg pro-MMP-2 could be activated. In our trials, chicken MMP-2 proform purified from egg was not activated by APMA, a mercuric derivative commonly used to activate metalloprotease precursors. These results are consistent with previous studies: pro-MMP-2 from egg yolk and egg white is copurified with TIMP-2, and the pro-MMP-2/TIMP-2 complex is much more resistant to activation by APMA than pro-MMP-2 free of TIMP-2 (*20, 25*). Some serine proteases also have been described to be activators

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of mouse and human pro-MMP-2 (*26, 43*). In this context, we analyzed the activation of chicken pro-MMP-2 by serine proteases that have been shown to process human pro-MMP-2, human cathepsin G, and human neutrophil elastase (*26*) and by two other serine proteases with different enzymatic specificities (chymotrypsin and trypsin). Purified chicken pro-MMP-2 from yolk or egg white was partially activated by the proteases in our conditions and was also degraded into smaller fragments as described previously for human MMP-2 from rheumatoid synovial cells (*44*). The activation of pro-MMP-2 by cathepsin G was previously shown to depend on the presence of cell-anchored MT-MMP (*26*). In our model using purified pro-MMP-2 and serine proteases, we detected an activated form, suggesting that the processing of pro-MMP-2 by soluble proteases in egg might not necessarily require MT-MMPs. However, because both egg white and yolk contain numerous serine protease inhibitors (*9, 45*), the physiological activation of pro-MMP-2 in egg white and yolk is unlikely to be due to serine proteases. This prediction is partly corroborated by the fact that the gelatinolytic activity of elastase, trypsin, and chymotrypsin tends to be lower after incubation with egg white or yolk (**Figure 4B**, lanes 8−10 and 13−15 vs lanes 2−4, which correspond to elastase, trypsin and chymotrypsin alone), which suggests that these serine proteases are inactivated by endogenous antiproteases contained in egg yolk and white. Some authors have suggested that the removal of the propeptide is not essential for MMP-2 activity. In fact, some members of the SIBLING (small, integrin-binding ligand, N-linked glycoproteins) family bind to certain MMPs, inducing structural changes that release the activity (*46*). That mechanism might occur in the egg as ovocleidin-116, a SIBLING protein initially described as eggshell-specific (*47*), and has been detected in egg yolk, although not in egg white (*9, 45*).

To investigate the physiologic pro-MMP-2 activation in eggs, we studied the effect of storage of unfertilized and fertilized eggs at 37.8 °C, the temperature required for proper embryonic development. By zymography, we could not detect any 62 kDa form corresponding to the activated MMP-2 in egg white or yolk but only the precursor (**Figure 5A**, zymograms). The activity of this pro-MMP-2 was stable in yolk during egg storage. In contrast, egg white pro-MMP-2 lost its gelatinolytic activity in a time-dependent manner during the storage of fertilized or unfertilized eggs at 37.8 °C. This decrease was not observed when whole unfertilized eggs were stored at 4 or 20 °C or when egg whites were initially separated from yolk at day 0 and independently stored in a tube during the same storage time at 37.8 °C. These results emphasize the fact that the regulation of this activity in egg white is specific to the intact egg and to the temperature. The activity of pro-MMP-2 in egg yolk from unfertilized eggs was not affected by the temperature or the time of storage; therefore, we did not carry out an analysis of egg yolks from fertilized eggs. Because many changes occur in egg yolk during embryonic development, it would therefore be interesting to study as to how this specific activity is regulated in yolk during the incubation of fertilized eggs. The mechanism by which pro-MMP-2 is regulated in egg whites of unfertilized and fertilized eggs could not be elucidated in this work, but our results suggest that the pro-MMP-2 activity of shelled egg white is controlled by environmental and physicochemical changes occurring during incubation at 37.8 °C.

Chicken MMP-2 shares 84% sequence identity with human MMP-2 or mouse MMP-2 (*48*) but also *Xenopus* or rainbow trout. This high percentage of sequence identity of MMP-2 suggests that this metalloprotease has a role that is strongly conserved among the different animal species. As reported in the literature, it would be mainly involved in tissue remodelling required in many physiological processes such as angiogenesis and embryogenesis. Pro-MMP-2 expression by chicken embryo has been largely reviewed: during chicken embryonic development, the role of chicken MMP-2 has been found to be essential for the epithelial-mesenchymal transformation of the embryo (*14*) and angiogenesis (*49, 50*) but also for neural crest cell migration (*38, 51*). However, the presence of pro-MMP-2 in egg, originating from oviduct tissues, had not been reported before. The physiological role of egg pro-MMP-2 is unclear. Its fast inactivation in egg white during incubation and the consumption of egg white within the first 10 days combined with putative functions reported in the literature suggest that this protease has a local effect in early embryonic development. Interestingly, the first 10 days of chicken development correspond to morphogenesis, while egg white is gradually consumed up to day 16 (*52*). With regard to the possible antimicrobial activity of egg white MMP-2, there is little information about its counterparts in the other species except that MMP-2 could regulate indirectly the production of the antimicrobial peptide parasin I in catfish skin mucosa (*53*). Concerning other metalloproteases, to our knowledge, only MMP-7 (also called matrilysin) could have a role in antimicrobial defense, by activating antibacterial peptides such as defensins (*54*). The development of techniques allowing for a specific silencing of such genes in the magnum would be a promising advance to assess the role of egg white proteins in embryonic development.

ABBREVIATIONS USED

ACKNOWLEDGMENT

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