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Antimicrobial Potential of Egg Yolk Ovoinhibitor, a Multidomain Kazal-like Inhibitor of Chicken Egg

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ABSTRACT: Chicken egg ovoinhibitor is a multidomain Kazal-type serine protease inhibitor with unknown function. Comparison of expression between different tissues indicated that ovoinhibitor is highly expressed in the magnum and liver followed by the uterus, which secrete egg white, egg yolk, and eggshell precursors, respectively. The results also revealed that ovoinhibitor expression is increased in the liver during sexual maturation followed by a subsequent decrease in mature hens. Ovoinhibitor was purified from the egg yolk plasma from nonfertilized eggs using two consecutive affinity chromatographies and gel filtration. Purified egg yolk ovoinhibitor was shown to inhibit trypsin and subtilisin. It was shown that purified egg yolk ovoinhibitor exhibited antimicrobial activities against *Bacillus thuringiensis*. The results suggest that this anti-protease plays a significant role in antibacterial egg defense against *Bacillus* spp., preventing contamination of table eggs (nonfertilized eggs) and protecting the chick embryo (fertilized eggs).

KEYWORDS: chicken egg, anti-protease, expression, sexual maturation, antibacterial activity

INTRODUCTION

Kazal-type inhibitors are canonical serine proteinase inhibitors that interact with their cognate enzymes through their reactive site.[1] These inhibitors are widely distributed in all kingdoms of life and have various physiological functions. The reactive site of Kazal-type inhibitors is extremely variable, but this domain is structurally conserved. A Kazal domain is characterized by the presence of six well-conserved cysteine residues forming three intradomain disulfide bridges. Several of these inhibitors are believed to be involved in innate immune response.[2−4] The specificity of their antimicrobial activity would rely on their ability to inhibit microbial proteases, which are essential to pathogens to invade host tissues. Such inhibitors have been identified in the metazoan Hydra that display potent in vitro bactericidal activity against *Staphylococcus aureus*.[4] In crustaceans, Kazal-type serine proteinase inhibitors in the hemolymph are suggested to function as regulators of host-defense reactions.[2]

Many Kazal-like inhibitors have been identified in egg. Among them there are two well-known inhibitors, ovoinhibitor and ovomucoid, which are major egg proteins.[5] Additionally in egg, numerous (one to nine) Kazal-like domains have been recently identified in Flik protein, SPARC, trypsin inhibitor ClTI-1, complement component C6, agrin, follistatin, and follistatin-related protein 1.[6−12] The biological activities of these various proteins have not yet been explored.

In this study, we focused on ovoinhibitor, which has been identified in all egg compartments including egg white, egg yolk, vitelline membranes, and eggshell. This anti-protease possesses several Kazal-like domains that display potent anti-protease activity.[5,13]

We first analyzed the expression of ovoinhibitor in various tissues of laying hens by quantitative RT-PCR. Egg yolk ovoinhibitor is expressed by the liver and is secreted into the blood to reach the follicle, whereas the ovoinhibitor found in the other egg compartments is expressed by the oviduct. In fact, it has been shown that egg yolk ovoinhibitor, also named vitelloinhibitor, is derived from a plasmatic precursor form.[14] This precursor form was previously named α2-proteinase inhibitor and consists of two components related to ovoinhibitor that contain different post-translational modifications.[14] As the egg yolk ovoinhibitor gene is an estrogen-responsive gene[15] that is secreted by the liver to be further incorporated in growing follicles, we analyzed its expression in livers of laying and prelaying hens to better appreciate the temporal expression of this anti-protease during sexual maturation.

In a second approach, we developed a new strategy of purification of this anti-protease from freshly laid eggs (unfertilized) to further explore its functional activities and more particularly its antimicrobial potential. Ovoinhibitor is commonly purified from egg white ovomucoid preparation using gel filtration and ion-exchange chromatography.[16,17] Some have suggested that preparations of ovoinhibitor from egg white are contaminated with egg white lysozyme, which displays potent antibacterial activity against Gram-negative and Gram-positive bacteria.[18] Because ovoinhibitor is also present in egg yolk, which contains less lysozyme than egg white,

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we developed a process of purification of ovoinhibitor from egg yolk plasma using two affinity chromatographies followed by gel filtration. The antimicrobial activity of pure ovoinhibitor was then assessed against several bacterial strains secreting proteases including *Pseudomonas aeruginosa*, *S. aureus*, and *Bacillus* spp.

MATERIALS AND METHODS

Tissue Specificity. Tissues (eight samples for each organ) of the oviduct (infundibulum, white isthmus, magnum, and uterus) and other organs (liver, kidney, and duodenum) were harvested from 41-week-old hens. Additionally, livers of 13-, 14-, 15-, and 16-week-old prelaying hens and 41-week-old laying hens were collected. Total RNA was extracted from frozen tissues using the commercial kit Nucleospin RNA II (Macherey-Nagel, Düren, Germany) for the infundibulum, magnum, white isthmus, kidney, and duodenum and the RNA NOW method (Biogentex, Ozyme, Saint-Quentin-en-Yvelines, France) followed by a treatment with DNase I (Applied Biosystems, Courtaboeuf, France) for the liver. RNA concentrations were obtained by measuring absorbance at 260 nm, and the quality of RNA was controlled using the Bioanalyzer Agilent 2100 (Agilent Technologies, Massy, France). Five micrograms of total RNA was reverse-transcribed using the Superscript II kit (Invitrogen, Cergy Pontoise, France) and random hexamers (GE Healthcare, Uppsala, Sweden). Primers for ovoinhibitor were 5′-TAAGGATGGCAGGACTTTGG-3′ (forward) and 5′-GAGTTTGCCACCAGTGGTTT-3′ (backward) at 0.4 μM in UptiTherm buffer 1× (Interchim, Montluçon, France). Quantitative RT-PCR was used to evaluate tissue expression of ovoinhibitor in infundibulum, white isthmus, magnum, uterus, liver, kidney, and duodenum and in livers of hens at 13, 14, 15, 16, and 41 weeks of age. cDNA was amplified in real time using the qPCR Master mix plus for Sybr Green I (Eurogentec, Seraing, Belgium) with a LightCycler 480 apparatus (Roche Diagnostics, Meylan, France). A melting curve program was carried out from 65 to 95 °C in 1 min for each individual sample amplified with Sybr Green. Each run included triplicates of control cDNA corresponding to a pool of cDNA from all tissues. The control cDNA was diluted from 1:6.25 to 1:102,400, and relative arbitrary quantities were defined. The threshold cycle (CT), defined as the cycle at which fluorescence rises 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, and relative amounts of unknown samples were deduced from this curve. The PCR efficiency was calculated. To account for variations due to mRNA extraction and reverse transcription reaction, ovoinhibitor mRNA levels were normalized by two different methods. Levels of 18S RNA in each sample were first measured using a predeveloped TaqMan assay reagent (Applied Biosystems). Additionally, TATA box binding mRNA levels were determined using Sybr Green reaction and the following primers: 5′-GCGTTTTGCTGCTGTTATTATGAG-3′ (forward) and 5′-TCCTTGCTGCCAGTCTGGAC-3′ (backward). The ratio value was calculated for each sample as ovoinhibitor/18 S rRNA or TBP RNA. The log of the ratio was used for statistical analysis using StatView software (SAS Institute Inc., version 5). A one-way analysis of variance was performed to detect statistically significant differences in ovoinhibitor expression in various tissues and in livers during sexual maturation of hens.

Purification of Egg Yolk Ovoinhibitor. A pool of 10 egg yolks collected from nonfertilized freshly laid eggs (table eggs) was diluted to 1:10 in ultrapure deionized water and acidified to pH 5 with HCl according to the method of Ahn et al.[19] The resulting egg yolk was then centrifuged at 10000*g* for 60 min at 4 °C. The supernatant was considered as the hydrosoluble fraction of the egg yolk (plasma) and was used for all experiments. Purification of ovoinhibitor was performed using a three-step procedure. Heparin-Sepharose beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were first equilibrated with 50 mM Tris and 50 mM NaCl, pH 7.4, and incubated with egg yolk hydrosoluble fraction overnight at 4 °C. This step allowed the removal of some major egg yolk proteins. Ovoinhibitor was recovered in the unbound fraction to heparin. Trypsin-Sepharose beads were obtained after coupling bovine trypsin (Sigma-Aldrich, Saint-Quentin-Fallavier, France) to CNBr-activated Sepharose according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB). They were first equilibrated in 50 mM Tris and 50 mM NaCl, pH 7.4, and incubated with the unbound fraction to heparin-Sepharose chromatography overnight at 4 °C. Trypsin-Sepharose beads were washed in 50 mM Tris and 150 mM NaCl, pH 7.4, and elution was performed using 100 mM glycine and 0.5 M NaCl, pH 2. Eluted samples were immediately neutralized with 1 M Tris and concentrated. Ovoinhibitor contained in eluted concentrated samples was further purified by gel filtration (Sephacryl S-100 High Resolution, Hi-prep 16/60, GE Healthcare) and concentrated. The protein concentration was determined using a Protein Dc Assay (Bio-Rad, Marnes-la-Coquette, France), using bovine serum albumin (Sigma-Aldrich) as the standard. Samples were then analyzed by SDS-PAGE under denaturing but nonreducing conditions using a 12.5% acrylamide−bisacrylamide gel, followed by Coomassie blue staining.

In-Gel Digestion and Identification by Nano LC-Q-TOF Mass Spectrometry. The SDS-PAGE band corresponding to 50−60 kDa was cut from the gel after Coomassie blue staining and further rinsed with water and acetonitrile. It was then reduced with dithiothreitol, alkylated with iodoacetamide, and incubated overnight at 37 °C in 25 mM NH_4HCO_3 with 12.5 ng/μL trypsin (sequencing grade, Roche, Paris, France) as described by Shevchenko et al.[20] The tryptic fragments were extracted, dried, reconstituted with 0.1% formic acid, sonicated for 10 min, and sequenced by nanoscale capillary liquid chromatography−tandem mass spectrometry (LC-MS/MS) using a linear ion trap mass spectrometer.

The Ettan MDLC controlled by UNICORN software (GE Healthcare, Germany) was used for desalting and separation of tryptic peptides prior to online MS and MS/MS analyses. Ten microliters of digested sample was injected using microliter-pickup mode. Each sample was automatically desalted and preconcentrated using a Zorbax 300-SB C_{18} trap column, 300 μm i.d. × 5 mm (Agilent Technologies, Germany). Peptide separations were conducted on a Zorbax 300-SB C_{18} column, 75 μm i.d. × 150 mm (Agilent Technologies). Buffer A consisted of water with 0.1% formic acid, whereas buffer B was 84% acetonitrile with 0.1% formic acid. Separation was performed at a flow rate of 350 nL/min by applying a gradient of 15−55% B for 30 min. Eluted peptides were online analyzed with an LTQ Linear Ion Trap mass spectrometer (Thermo Electron) using a Thermo Electron dynamic nanospray probe interface. Ionization was performed (1.8−2.1 kV) with liquid junction and noncoated fused-silica nano ESI 25 μm i.d. emitters (New Objective, Woburn, MA). The ion transfer capillary was set to 200 °C. Each scan cycle consisted of one full-scan mass spectrum (m/z 500−2000) collected in enhanced mode followed by three MS/MS events in centroid mode (Qz = 0.25, activation time = 40 ms). For CID spectra (MS^2), the isolation width was 2 m/z units and the normalized collision energy was 40%. Dynamic exclusion was actived during 30 s with a repeat count of 1. Raw data files were converted to mzXML with Bioworks 3.3.1 software (Thermo Fischer Scientific, San Jose, CA). To identify the proteins, the peptide and fragment masses obtained were matched automatically against a locally maintained copy of the nonredundant nr NCBI database (downloaded January 10, 2010). MS/MS ion searches were performed using MASCOT Daemon and a search engine (Matrix Science, U.K.) against the *Chordata* section (10299319 sequences). Enzyme specificity was set to trypsin with two missed cleavages using carbamidomethylcysteine (+57 Da), methionine oxidation (+16 Da), and propionamide cysteine (+71) as variable modifications. The tolerance of the ions was set to 1.4 Da for parent and 1.0 Da for fragment ion matches. Proteins detected with a *P* value of <0.05 were considered to be

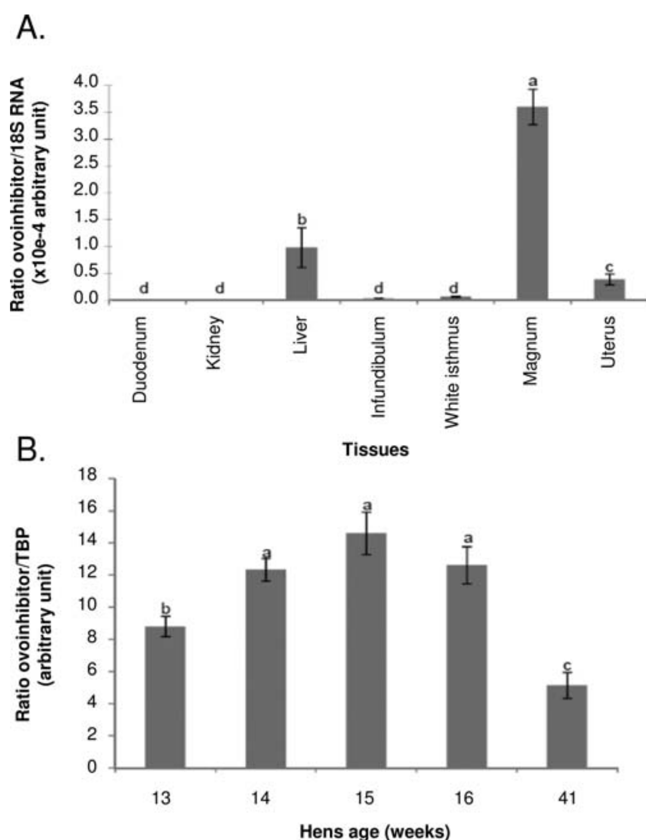

Figure 1. Tissue expression of ovoinhibitor in chicken females: (A) tissue distribution of ovoinhibitor; (B) temporal expression of ovoinhibitor in liver during sexual maturation. TBP, Tata binding protein. Means that do not share a common letter are significantly different ($P < 0.05$; $n = 8$).

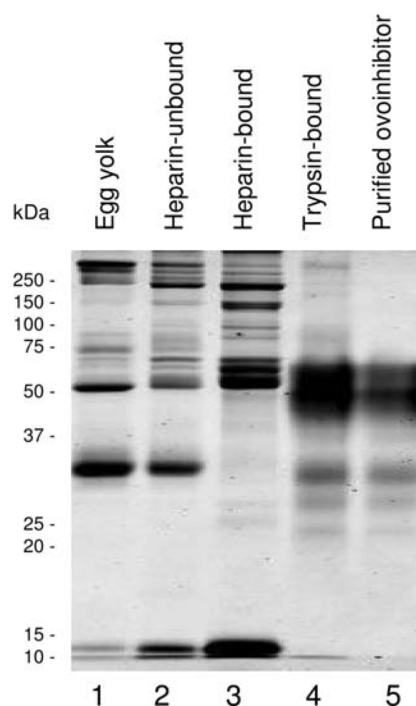

Figure 2. Purification of ovoinhibitor from egg yolk. Lanes: 1, egg yolk; 2, egg yolk fraction that does not bind to heparin-Sepharose (unbound fraction); 3, egg yolk fraction that does bind to heparin-Sepharose (bound fraction); 4, fraction eluted from trypsin-Sepharose; 5, egg yolk ovoinhibitor after gel filtration and concentration. Protein samples (5 μg) were analyzed by SDS-PAGE under nonreducing conditions after Coomassie blue staining.

positively identified with one peptide when the presence of five consecutive fragment ions was confirmed.

Inhibition Assays. Subtilisin A type VIII, elastase from porcine pancreas type IV, trypsin from bovine pancreas TPCK treated, α-chymotrypsin from bovine pancreas type II, *N*-succinyl-ala-ala-ala-*p*-nitroanilide, *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide, and *N*-(*p*-tosyl)-gly pro-arg *p*-nitroanilide were all purchased from Sigma-Aldrich. The inhibitory activity of purified egg yolk ovoinhibitor was assayed in 0.1 M Tris-HCl and 0.1 M NaCl, pH 8. The reaction mixture consisted of 0.3 mM *N*-succinyl-ala-ala-ala-*p*-nitroanilide, *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide, and *N*-(*p*-tosyl)-gly pro-arg *p*-nitroanilide for elastase (17 nM), chymotrypsin (2 nM), subtilisin (1 nM), and trypsin (2 nM), respectively. Proteinases were independently incubated with increasing concentrations of purified ovoinhibitor (1−200 nM) in the buffer for 30 min at 37 °C. The remaining activity of proteases was evaluated after addition of their respective substrates by measuring the absorbance at 410 nm during 15 min at 37 °C. Each reaction was performed in triplicate using a microplate reader (Tecan, Infinite M200, Tecan France S.A.S., Lyon, France).

Antimicrobial Assays. *P. aeruginosa* PAO1 and *S. aureus* CIP 103 811 were provided by S. Attucci (Inserm U618, Proteases et vectorisation pulmonaires, Tours, France). *Bacillus cereus* ATCC 6464, *Bacillus subtilis* ATCC 6633, and *Bacillus thuringiensis* LMSA 3.06.004 were from the American Type Culture Collection (ATCC) and from the "Souchothèque de Bretagne" culture collection (Université de Brest, Plouzané, France), respectively. Bacteria were grown to mid-logarithmic phase in 9 mL of brain−heart infusion (BHI) broth (AES Chemunex, Bruz, France). They were then washed twice in modified Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride (buffer A, Sigma-Aldrich) and centrifuged for 10 min at 10000*g*. Antimicrobial tests were conducted in 96-well low-binding plates (Corning 3355, Sigma-Aldrich) in a 150 μL final volume. Bacteria (3×10^5 cfu/150 μL) were incubated with various concentrations of ovoinhibitor (0.47, 0.94, and 1.88 μM) diluted in buffer A for 3 h at room temperature. Twenty microliters of the mix bacteria−ovoinhibitor was then sampled and serially diluted. The different dilutions were plated onto tryptic soy agar (TSA) (Biomérieux, Craponne, France) and incubated overnight at 30 °C to count colonies and determine bacterial concentrations.

RESULTS

Tissue Distribution and Sequential Expression of Ovoinhibitor in Hens. The expression of ovoinhibitor gene was analyzed by quantitative RT-PCR in different tissues involved in egg formation (liver for the yolk; infundibulum for vitelline membranes; magnum for the egg white; white isthmus for shell membranes), in the kidney and in the duodenum. Levels of ovoinhibitor expression were normalized by 18S RNA to control for possible differences in RNA extraction and reverse transcription efficiencies between samples. The relative normalized ovoinhibitor expression was higher in the magnum compared with all other tissues tested (Figure 1A; $P < 0.001$). In the liver, the expression was 27% of that expressed in the magnum and was significantly higher than that detected in all other tissues (Figure 1A; $P < 0.05$). Ovoinhibitor expression was also revealed in the uterus but at a lower magnitude (Figure 1A).

Table 1. Characteristics of Ovoinhibitor Peptides Identified by Mass Spectrometry

protein accession, description	sequence coverage	precursor mass observed	precursor mass theoretical	peptide score	peptide sequence
gi|71895337, ovoinhibitor precursor [*Gallus gallus*]	30.1	1078.2586	1078.5151	28.03	TLNLVSMAAC
		1715.8192	1715.8487	50.33	HVMIDCSPYLQVVR
		1762.6205	1762.7832	82.07	QEIPEIDCDQYPTR
		1869.0589	1868.919	51.17	LEIGSVDCSKYPSTVSK
		1891.1715	1890.8782	50.55	QEIPEIDCDQYPTRK
		2078.9304	2078.915	70.36	CRQEIPEIDCDQYPTR
		2197.5155	2197.0685	56.17	LEIGSVDCSKYPSTVSKDGR
		2205.6597	2207.0099	28.89	CRQEIPEIDCDQYPTRK
		2628.4579	2628.1407	60.21	VSPICTMEYVPHCGSDGVTYSNR
		2709.6046	2708.2786	41.82	LHDGECKLEIGSVDCSKYPSTVSK
		2776.8539	2777.2207	68.38	NLKPVCGTDGSTYSNECGICLYNR
		3083.018	3083.3172	102.5	ILSPVCGTDGFTYDNECGICAHNAEQR

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MRTARQFVQVALALCCFADIAFGIEVNCSLYASGIGKDGTSWVACPRNLKPVCGTDGSTY
SNECGICLYNREHGANVEKEYDGECRPKHVMIDCSPYLQVVRDGNTMVACPRILKPVCGS
DSFTYDNECGICAYNAEHHTNISKLHDGECKLEIGSVDCSKYPSTVSKDGRTLVACPRIL
SPVCGTDGFTYDNECGICAHNAEQRTHVSKKHDGKCRQEIPEIDCDQYPTRKTTGGKLLV
RCPRILLPVCGTDGFTYDNECGICAHNAQHGTEVKKSHDGRCKERSTPLDCTQYLSNTQN
GEAITACPFILQEVCGTDGVTYSNDCSLCAHNIELGTSVAKKHDGRCREEVPELDCSKYK
TSTLKDGRQVVACTMIYDPVCATNGVTYASECTLCAHNLEQRTNLGKRKNGRCEEDITKE
HCREFQKVSPICTMEYVPHCGSDGVTYSNRCFFCNAYVQSNRTLNLVSMAAC
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Figure 3. Mass spectrometry coverage of ovoinhibitor sequence (IOV7_CHICK, P10184). Peptides identified by mass spectrometry are shaded in gray, glycosylation sites are shown in bold, and the signal peptide is underlined.

The expression of ovoinhibitor was analyzed in the liver of prelaying and laying hens during sexual maturation. As shown in Figure 1B, the relative normalized ovoinhibitor expression increased gradually from 13 weeks of age to reach its maximum of expression at 15 weeks of age. A significant decrease in expression was observed in livers of 41-week-old hens ($P < 0.0001$) to return to a level that is significantly lower than initially measured in livers of 13-week-old pullets ($P < 0.0005$).

Purification of Egg Yolk Ovoinhibitor Using Affinity Chromatography. SDS-PAGE analysis of various purification steps is presented in Figure.2. Purified ovoinhibitor consists of a major heterogeneous band (Figure 2, lane 5) with an apparent molecular weight ranging from 50 to 65 kDa, in accordance with its theorical molecular weight (49.4 kDa). As shown in Figure 2, lane 3, heparin-Sepharose chromatography allowed the removal of numerous proteins from the unbound fraction (Figure 2, lane 2). In fact, we have shown by mass spectrometry analysis that heparin-Sepharose allowed the binding of apovitellenin, similar to avidin, apolipoprotein B, and vitellogenins II and III (data not shown). Ovoinhibitor was identified by mass spectrometry with 12 unique peptides (Table 1) corresponding to 30.8% sequence coverage of the mature form (without the signal peptide) (Figure 3).

Anti-protease Activity of Purified Ovoinhibitor. The inhibitory activity of purified ovoinhibitor was assayed toward four proteases, trypsin, subtilisin, chymotrypsin, and pancreatic elastase. The results indicate that the purified ovoinhibitor displayed activities against trypsin and subtilisin (Figure 4). Ovoinhibitor exhibited a weak activity toward chymotrypsin, and no activity could be detected against pancreatic elastase in our conditions (data not shown).

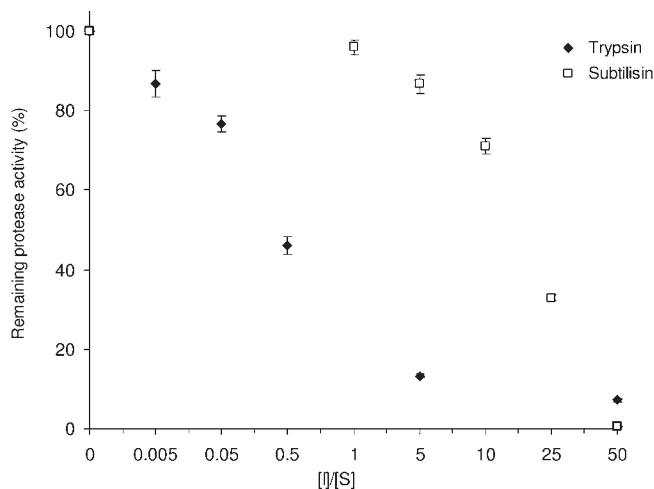

Figure 4. Protease inhibition assay of purified egg yolk ovoinhibitor. The inhibitor was incubated with subtilisin and trypsin, 0.1 M Tris-HCl, 0.1 M NaCl, pH 8, at 37 °C for 30 min before the addition of their respective substrate (see Material and Methods). Substrate hydrolysis was monitored during 15 min. Results are expressed as a percentage of inhibition related to the control (substrate + protease). The molar ratios of ovoinhibitor [I]/trypsin or subtilisin [E] are indicated on the *x*-axis.

Antimicrobial Assays. The antibacterial activity of purified ovoinhibitor was investigated against *S. aureus* CIP 103 811, *P. aeruginosa* PAO1, *B. cereus* ATCC 6464, *B. subtilis* ATCC 6633, and *B. thuringiensis* LMSA 3.06.004. It was found that the purified ovoinhibitor was able to inhibit *B. thuringiensis* (Table 2).

DISCUSSION

The hens export in the egg a large range of nutrients but also protective systems against microbial invasion to ensure the extrauterine development of the embryo. These characteristics are at the origin of the high nutritional value of eggs for human and of its long storage ability at room temperature without alterations. At least 40 different protease inhibitors have been identified in chicken egg[12] including two well-known and ubiquitous Kazal-like proteins, ovoinhibitor and ovomucoid.

Table 2. Antimicrobial Activity of Purified Ovoinhibitor

bacterial strain	antimicrobial activity (MIC, μM)
Staphylococcus aureus CIP 103 811	nd[a]
Pseudomonas aeruginosa PAO1	nd
Bacillus cereus ATCC 6464	nd
Bacillus subtilis ATCC 6633	nd
Bacillus thuringiensis LMSA3.06.004	3.8

[a] nd, not detected.

The physiological roles of these inhibitors in egg remain unexplored. Some of them, including ovoinhibitor, are likely to contribute to regulation of proteolytic degradation of egg yolk proteins during embryonic development.[14] Alternatively, there is increasing evidence in the literature that Kazal-type inhibitors might play a role in innate immunity to inhibit proteases that are secreted by invading pathogens. In this study, we investigated the antimicrobial potential of egg yolk ovoinhibitor, which contains seven Kazal-like domains capable of inhibiting various proteases with different enzymatic specificities. We first showed that the expression of ovoinhibitor is specific to tissues which participate in the formation of the chicken egg, particularly in the magnum and liver (Figure 1A), which secrete egg white proteins and egg yolk proteins, respectively. The transcription rate of the ovoinhibitor gene in liver is stimulated during sexual maturation of pullets (Figure 1). These results are in agreement with the increase of the expression induced in chicks treated with estrogen[15] and the observation that ovoinhibitor expression is highly induced at sexual maturity in geese.[21] The expression of ovoinhibitor coincides with estradiol plasma concentration because the highest concentration of estradiol is reached during the two weeks preceding the onset of egg laying (15 and 16 weeks of age, when the ovoinhibitor expression was highest, Figure 1B) and with a subsequent decrease observed in laying hens (16 and 41 weeks, Figure 1B). From our results, it seems that ovoinhibitor expression in the livers of mature hens is significantly lower than in the livers of 13-week-old pullets (Figure 1B). However, we have shown elsewhere that the expression of ovoinhibitor is not significantly different between 10-week-old pullets and 41-week-old hens in a transcriptomic approach applied to liver (unpublished data). These results indicate that the expression of ovoinhibitor is increased during sexual maturation of pullets and decreases subsequently to reach a level that is similar to that found in immature hens (10 weeks old). It would be interesting to determine whether changes in the ovoinhibitor expression in the liver during sexual maturation coincide with an increase in ovoinhibitor concentrations in blood, in which all yolk precursors are secreted prior to their transfer into the ovary. In fact, an ovoinhibitor analogue has been identified in the serum of laying hens.[14,22]

A new technique of purification was developed to isolate ovoinhibitor from egg yolk plasma from nonfertilized eggs and to further explore its antimicrobial potential. The strategy chosen was based on two affinity chromatographies followed by a gel filtration. We have taken egg yolk plasma as the starting material because ovoinhibitor is mainly recovered in the hydrosoluble fraction of egg yolk.[11] The first step of purification consisted of heparin-Sepharose that was used to remove lipoproteins from ovoinhibitor preparations (Figure 2, lane 3). Then, we used a trypsin-Sepharose to specifically target trypsin anti-proteases contained in this lipoprotein-depleted fraction, considering that ovoinhibitor is a potent trypsin-like anti-protease. Pure ovoinhibitor was finally recovered after gel filtration. The electrophoretic profile of the resulting sample revealed a large band with an apparent molecular weight ranging from 50 to 65 kDa as well as smaller bands around 35 and 32 kDa. After mass spectrometry analysis, it was confirmed that the major band as well as these lower bands corresponded to the ovoinhibitor. The 35 and 32 kDa bands might result from proteolytic degradation of ovoinhibitor during the process of purification (elution performed at pH 2). The apparent heterogeneity of native ovoinhibitor on SDS-PAGE (50−65 kDa) has been previously described[23 14] and can be explained by the presence of three glycosylation sites[24] as indicated in Figure 3.

Ovoinhibitor possesses seven Kazal-type domains that favor inhibition of various serine proteases including trypsin, subtilisin, and, to a lesser extent, chymotrypsin and elastase.[13] Purified egg yolk ovoinhibitor exhibited potent inhibitory activity against trypsin and subtilisin, as previously shown.[14] In contrast, the inhibitory activity of purified ovoinhibitor against chymotrypsin was weak, and no inhibition of pancreatic elastase could be detected (data not shown). Former publications suggest that anti-proteases with inhibitory activity against subtilisin could potentially be bacteriostatic against some *Bacillus* spp. or other serine protease(s) secreting strains.[4,25,26] In fact, protease inhibitors are widely used in several therapeutic strategies to overcome bacterial and viral infections.[27] Most microorganisms secrete proteases that can hydrolyze host proteins to inactivate them or to facilitate their assimilation by microorganisms as nutrients. These microbial proteases can thereby limit the immune response and induce tissue damages that favor pathogen dissemination. However, host organisms possess an arsenal of anti-proteases that regulate and interfere with the deleterious activities of exogenous proteases.[28] Because of the presence of seven different inhibitory sites, ovoinhibitor constitutes a potent antimicrobial candidate. Our results indicate that the purified ovoinhibitor displayed antimicrobial activity against *B. thuringiensis*. These results are consistent with previous data that indicate a similar antimicrobial spectrum for other Kazal-like proteins.[2,26] Thus, hcPcSPI2 from the red swamp crayfish is a three Kazal-like inhibitor with inhibitory activity against sublilisin A and trypsin, which have been shown to exhibit bacteriostatic activity against *B. thuringiensis* and *B. subtilis* to a lesser extent,[26] but none against the other *Bacillus* strains tested. Analysis of the *B. thuringiensis* genome revealed at least seven different protease genes including five serine proteases potentially inhibited by ovoinhibitor, some of them being intracellular, extracellular, or membrane-associated.[29] Analysis of the antimicrobial potential of each Kazal domain produced independently as recombinant molecules would give additional information regarding the enzymatic specificity of the *Bacillus* protease(s) which is (are) actually inhibited by ovoinhibitor. The antibacterial activity of ovoinhibitor against *B. thuringiensis* may also depend on a synergistic action of some or all of its inhibitory domains.

Ovoinhibitor together with cystatin is the second anti-protease from egg that has been described as an antimicrobial agent.[30−33] As many serine protease inhibitors are up-regulated following infection,[34−36] it will be also informative to study how ovoinhibitor expression is regulated in the various chicken female tissues upon bacterial or viral challenges. All of these data will help to increase our appreciation of the role of ovoinhibitor in

innate response of hens and the possible exportation of antimicrobial anti-proteases into eggs to ensure the hygienic quality of table eggs and the protection of developing embryos.

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