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Purification and Characterization of Avian β-Defensin 11 (AvBD11): an Antimicrobial Peptide of the Hen Egg.

Running title: **Avian β-defensin 11, an egg antimicrobial peptide**

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19 Abstract: Natural antimicrobial peptides are present in different compartments (eggshell, egg
20 white, vitelline membranes) of the hen egg and are expected to be involved in the protection
21 of the embryo during its development and to contribute to production of pathogen-free eggs.
22 In the present study, we used vitelline membranes from hen (*Gallus gallus*) eggs, as a source
23 of avian β-defensin 11 (AvBD11). A purification scheme using affinity chromatography and
24 reverse phase chromatography was developed. Purified AvBD11 was analysed by a
25 combination of mass spectrometry approaches to characterize its primary sequence and
26 structure. A monoisotopic molecular specie at $[M+H]^+$ = 9,271.56 Daltons was obtained and
27 its N and C-terminal sequences were determined. We also examined post-translational
28 modifications and identified the presence of 6 internal disulfide bonds. AvBD11 was found to
29 exhibit antimicrobial activity towards both Gram-positive and Gram-negative bacteria.

INTRODUCTION

The avian egg is a unique and original biological system, which is formed according to a well-defined spatial and temporal sequence as it passes along the reproductive tract of the hen. The segments of the oviduct involved in the egg formation are the infundibulum, the magnum, the isthmus and the uterus. Each segment expresses and secretes specific molecules that become successively incorporated into: the vitelline membranes in the infundibulum, the egg white in the magnum, the eggshell membranes in the isthmus and the eggshell in the uterus. Thus, the hen deposits into the egg, which can be considered as a closed chamber, all the nutrients and protective systems that are necessary to support the development of an embryo during 21 days of incubation. Recently, transcriptomic and proteomic approaches have identified almost 1000 putative proteins and peptides in the various compartments of eggs (6). Amongst these are a number of polypeptides that are likely to resist microbial contamination of the eggs, some of which belong to the family of avian β-defensins (AvBDs).

A total of 14 avian β-defensin genes have been identified through *in silico* studies. Because most of them were simultaneously described by two research groups, some confusion was generated due to different nomenclatures (9, 10, 18, 31). In a collaborative venture, we proposed a novel nomenclature that adopted the numbering system used by Xiao *et al*. (31) and replaced the term "gallinacin", used formerly by Lehrer'group, with "avian β-defensin" (AvBD) (19). AvBDs are small cationic non-glycosylated peptides (1-9 kDa). Some of them were successfully tested for their antimicrobial activity such as chicken AvBDs 1, 2, 7 and 9, turkey AvBD 1 and 2, ostrich AvBDs 1, 2, 7 and 8, and penguin AvBD103b (3, 30). In mammals, β-defensins are molecules of innate and adaptive immunity, with a broad spectrum of antimicrobial activity (1). β-defensin molecules possess six highly conserved cysteines with the following consensus sequence motif: X_n-C-X_{2-4}-G-X_{1-2}-C-X_{3-5}-C-X_{9-10}-C-X_{5-6}-CC-X_n

where C is a cysteine, G a glycine and X any amino acid. The six cysteines in β-defensins are linked to form disulfide bridges in a 1–5, 2–4 and 3–6 pairing pattern (5).

Little is known regarding the tertiary structure of β-defensins in avian species. Only the three-dimensional structure of the king penguin avian β-defensin 103b, (AvBD103b or Spheniscin-2) has been solved recently. The tertiary structure of AvBD103b is very similar to those of mammalian β-defensins, including the common array of disulfide bonds and the presence of a three stranded β-sheet as the main secondary structure element (15, 26). AvBD103b was isolated from the stomach contents of the king penguin and exhibits a wide spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts and fungi (29). The high potency of AvBD103b against bacteria was correlated with its extreme cationicity, but also with the presence of a hydrophobic patch, which is not present in mammalian β-defensins (15).

AvBDs are expressed in several tissues including genital tract where they are thought to play a role in the antimicrobial defense of the hen genital tract. This protection is essential for embryonic development and also ensures that the *Gallus gallus* table egg is free of pathogens. mRNA of AvBD-1, -2, -3, -4, -5, -8, -9, -10, -11 and -12 have been shown to be expressed with moderate levels, in the infundibulum, the magnum, the isthmus, the uterus and the vagina of the laying-hen oviduct (20). A recent study reported that primary epithelial cell cultures of laying hen oviduct, constitutively expressed most AvBDS, at moderate to high levels, with the exception of AvBD2 and AvBD-6, -7 and -8. All these studies showed the mRNA expression of a large panel of AvBDs in the different regions of the laying hen oviduct, suggesting that they might together exhibit a very large spectrum of antimicrobial activity. Surprisingly, in spite of mRNA expression of AvBD-1, -2, -3, -4, -5, -8, -9, -10, -11 and -12 in the different segments of the oviduct, only three AvBDs have been identified in the hen egg by proteomic approaches: AvBD11 in the eggshell, the egg white and the vitelline

membranes; AvBD10 in the eggshell and gallin, related to AvBD family (8), in the egg white (21-23).

AvBD11 corresponds to a molecule initially identified as vitelline membrane outer layer protein 2 or VMO-2 (21-23), one of the four major proteins in vitelline membranes along with ovomucin, lysozyme and VMO-1 (11, 27). VMO-2 was first described as a molecule of 53 amino acids, a molecular mass of 6,000 Daltons, high cysteine content, four disulfide bonds and LPRDTSRXVGYHGYXIRSKV as N-amino-terminal sequence (12). In 2004, an *in silico* approach, based on the chicken genome, predicted that AvBD11 possessed 80 amino acid residues, with RDTSRCVGYHGYCIRSKVCP as the N-terminus and an expected monoisotopic molecular mass of 9,078.56 Daltons for the mature peptide (31).

In the present work, we confirm the identification of mature AvBD11 peptide in the hen egg and describe its purification from vitelline membranes. Using bottom-up and top-down proteomic approaches, its primary structure has been redefined, as well as confirming the N- and C-terminal sequences of the mature peptide and its post-translational modifications. Additionally, the antimicrobial activity of AvBD11 has been characterized against different bacterial strains and compared to that of the king penguin avian β-defensin, AvBD103b.

MATERIALS AND METHODS

Polyclonal anti-AvBD11 antibodies preparation. N- and C-terminal extremities (RDTSRCVGYHGYC and GLCPLKRWTCCKEI, respectively) of AvBD11 were synthesized and conjugated with keyhole limpet hemocyanin (KLH) (Genosphere biotechnologies, Paris, France). Sera against AvBD11 were prepared as follows. Two rabbits were immunized four times at three weeks of intervals by intramuscular injection of 500 µg of synthesized extremities of AvBD11 bounded to KLH, emulsified in 50% complete Freund adjuvant for the first injection and in 50% incomplete Freund adjuvant for the next three injections. Rabbits were euthanized three weeks after the last injection. The blood was allowed to clot at room temperature for 2 h, stored overnight at 4°C and then centrifuged at 2,000 *g* to remove blood cells. Sera were collected and stored at -20°C.

Vitelline membrane preparation. Vitelline membranes were obtained from unfertilized Isabrown eggs. Following separation of egg white from the yolk, the vitelline membrane was punctured to evacuate the yolk. After several successive washings in distilled water, membranes were dried, lyophilized and frozen in nitrogen liquid to be crushed very finely. Vitelline membrane proteins were solubilized in 50 mM Tris-HCl, 0.5 M NaCl pH 7.4. After a first sonication and a gentle overnight agitation at 4°C, one additional sonication was performed. Samples were then centrifuged 10 min at 10,000 *g*, at 4°C. Supernatant was collected and the protein concentration was determined using the Protein Dc Assay (Biorad, Marnes-la-Coquette, France), and with bovine serum albumin (BSA) (Interchim, Montluçon, France) as standard, according to manufacturer's instructions.

Heparin-sepharose chromatography. Heparin-sepharose chromatography was performed using the batch method, following manufacturer's instructions. Briefly, solubilized proteins of

vitelline membrane were incubated with heparin-sepharose beads in 50 mM Tris, 150 mM NaCl, pH 7.4, overnight at 4°C, under constant shaking. Beads were washed extensively with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, until the absorbance at 280 nm reached zero, and loaded onto a polypropylene column (Qiagen, Courtaboeuf, France). Elution of bound proteins was achieved using 50 mM Tris-HCl, 1 M NaCl, pH 7.4. The protein concentration of unretained (unbound) and eluted (bound) fractions of vitelline membranes was determined using Protein Dc Assay as described above.

Gradient SDS-PAGE. Equal amounts of total proteins (total vitelline membrane proteins, eluted and unretained fractions from heparin-sepharose) were denatured in sodium dodecyl sulphate (SDS) sample buffer 5X (0.31 M Tris-HCl pH 6.8, 20% SDS, 25% glycerol, 0.01% bromophenol blue, 25% 2-β-mercaptoethanol) and boiled for 5 min. Proteins were separated on one-dimensional 4–20% gradient SDS-PAGE (SDS-PolyAcrylamide Gel Electrophoresis) GeBaGel, according to manufacturer's instructions (GeBaGel, BioScience Innovations, Interchim, Montluçon, France), and gels were stained with Coomassie Brilliant Blue R250.

Western blotting. Proteins were transferred to nitrocellulose membrane after migration on 4–20% GeBaGel. Nitrocellulose membranes were blocked for 1 hour in 5% Blotto (Biorad, Saint-Quentin, France, in phosphate-buffered saline-PBS), and incubated overnight, at 4°C, with Anti-AvBD11 antiserum (1/1000) in PBS, 2% Blotto. After further washes in PBS, 0.1% Tween 20, membranes were incubated 90 minutes, at room temperature, with anti-rabbit IgG secondary antibody coupled to Alexa 680 (Molecular Probes, Eugene, OR, USA), that was diluted at 1/2500 in PBS, 2% Blotto. Immunoreactive bands were detected using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

In-gel digestion and protein identification by nanoLC-Ion trap mass spectrometry. Protein bands were excised from the gel, rinsed with water and acetonitrile, reduced with dithiothreitol, alkylated with iodoacetamide and then incubated overnight at 37°C in 25 mM NH_4HCO_3 with 12.5 ng/µL trypsin (Sequencing Grade, Roche, Paris) as described by Shevchenko *et al.* (28). The tryptic digestion fragments were extracted, dried, and reconstituted with 0.1% formic acid before sequencing by nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). The Ettan MDLC system (GE Healthcare, Germany) was used for desalting and separation of tryptic peptides prior to online MS and MS/MS analyses. Ten µL of digested sample were injected and automatically desalted and preconcentrated using a Zorbax 300-SB C_{18} trap column, 300 µm [inside diameter] x 5 mm (Agilent Technologies, Germany). Peptide separations were conducted on a Zorbax 300-SB C_{18} column, 75 µm [inside diameter] x 150 mm (Agilent Technologies, Germany). Buffer A consisted of water with 0.1% formic acid while 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 over 60 min. Eluted peptides were online analysed with the LTQ Linear Ion Trap Mass Spectrometer (Thermo Electron, US). 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 centroïd mode. For CID spectra (MS2), 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). In order to identify the proteins, the peptide and fragment masses obtained were matched automatically against a locally maintained copy of the nr NCBI database (downloaded 2 February 2009). MS/MS ion searches were performed using MASCOT Daemon and search engine (Matrix Science, UK) against the Chordata section (7873120 sequences). Enzyme specificity was set to trypsin with

2 missed cleavages using carbamidomethylcysteine and methionine oxidation 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 < 0.05 were considered positively identified with one peptide when the presence of 5 consecutive fragment ions was confirmed.

Reverse-Phase HPLC. Proteins present in heparin-sepharose eluted fractions, were further separated by preparative X-Bridge® C_{18} (Waters) reverse-phase high-performance liquid chromatography (HPLC). A linear 5-65% acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) was used at a constant flow rate of 0.90 mL/min, during 85 minutes. The content of each collected peak was analyzed by MALDI-TOF Mass Spectrometry.

MALDI-TOF Mass Spectrometry.

All mass spectra were generated on a M@LDI LR (Waters, Micromass, Ltd., Manchester, United Kingdom) MALDI-TOF mass spectrometer, operating in positive linear mode. Mass spectra were recorded in the m/z range of 2,000 to 20,000, acquiring 10 shots per spectrum at a laser firing rate of 10 Hz. Data processing was performed using MassLynx 4.0 software. The background of the spectrum result for each sample well was subtracted using a polynomial order of 10% below the curve and smoothed with the minimum peak width at half height set to 15 channels. Smoothing was performed twice using the Savitzky Golay algorithm. All the spectra were processed using the same parameters. For reverse-phase HPLC eluted-peptide mass fingerprint analysis, 1 µL of each fraction and the matrix (1:1, v/v) were loaded on the target by using the dried droplet method. The matrix used was sinapinic acid at 20 mg/mL dissolved in 50% acetonitrile-50% H_2O-0.1% TFA.

In-solution digestion and identification by nanoLC-Q-TOF mass spectrometry.

Molecular specie purified by reverse-phase HPLC was identified after tryptic in-solution digestion. Ten µL of purified peptides were mixed in 10 µL of 50 mM NH_4HCO_3, then reduced with 5 mM dithiothreitol, alkylated with 12.5 mM iodoacetamide, and incubated overnight at 37°C with 0.5 µg of bovine trypsin (sequencing grade; Roche, Paris, France) and 1 µL of ProteasMax™ Surfactant (Promega, Charbonnière-les-Bains, France). The tryptic digests were incubated 1 h at 60°C, acidified with 10 µL of 5% trifluoroacetic acid and then sonicated for 10 min. Nanoscale capillary liquid chromatography-tandem MS (nano-LC–MSMS) analysis of the digested peptides was performed using a CapLC system coupled to a hybrid Quadrupole-Time of Flight (Q-TOF) Ultima Global mass spectrometer (Waters, Manchester, United Kingdom). Samples were desalted and concentrated using an on-line precolumn (Monolithic Trap column, 200 µm [inside diameter] by 5 mm; LC Packings, Dionex, Voisins-le-Bretonneux, France). Peptide separations were conducted on two on-line analytic columns (Pepswift Monolithic Nanocolumn, 100 µm [inside diameter] by 50 mm, LC Packings, Dionex, Voisins-le-Bretonneux, France); and Nanocolumn Strategy Silice, 75 µm [inside diameter] by 500 mm, Interchim, Montluçon, France) running with a 200 nl/min flow. The gradient profile consisted of a gradient from 100% A (0.1% formic acid-2% acetonitrile-98% H_2O, vol/vol) to 10% B (0.1% formic acid-20% H_2O-80% acetonitrile, vol/vol) in 1 min and 15% to 60% B in 120 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 four most intense peptide ions detected. Only doubly and triply charged ions were allowed to be selected as precursors over an m/z range of 400 to 1,300. Data were processed using ProteinLynx Global server 2.2 (Waters) and the peak list was exported in pkl format file. In order to identify the proteins, the precursor and fragments masses obtained were matched automatically against a locally maintained copy of the nr NCBI database (download 2 July 2009) in the chordata section (9244709 sequences), using the MS-MS ion search option

in the MASCOT v 2.2.04 software program (Matrix Science, United Kingdom). Enzyme specificity was set to semitrypsin with four missed cleavages, using carbamidomethylcysteine and methionine oxidation as variable modifications. The tolerance of the ions was set to 0.1 Da for both parent and fragment ion matches. All hits with *P* values of < 0.05 were manually verified to confirm the presence of five consecutive fragment ions.

Structural characterization of intact AvBD11 by NanoESI-Q-TOF mass spectrometry

Five µL of HPLC fraction was diluted with 10 µL of a mixture of formic acid 1%-acetonitrile (1:1) and analyzed by MS and MS-MS on a nanoelectrospray ionization (nano-ESI)–Q-TOF Ultima Global mass spectrometer (Waters, Manchester, United Kingdom). The sample was loaded into nanoelectrospray capillaries (Proxeon, Odense, Denmark). Argon was used as the collision gas. Data acquisition and analysis were performed with reflector W mode for MS and with V reflector mode for MS-MS using MassLynx version 4.0 software (Waters, Manchester, United Kingdom). Multicharged precursor ion with m/z values of 1325.5 (7+) was selected for the fragmentation of intact molecular specie.

Bacterial strains. Six different strains were used to test the antimicrobial activity: *Staphylococcus aureus* ATCC 29740, *Listeria monocytogenes* strain EGD, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Salmonella enterica* serovar Enteritidis LA5 and *Salmonella enterica* serovar Thyphimurium ATCC 14028. *Salmonella enterica* serovar Enteritidis LA5 is a wild-type strain (nalidixic acid resistant at 20 µg/mL) isolated from natural chicken infections. *Salmonella enterica* serovar Enteritidis ATCC 13076, and *Salmonella enterica* serovar Thyphimurium ATCC 14028 were purchased from the Centre de Ressources Biologiques de l'Institut Pasteur (CRBIP, Institut Pasteur, Paris, France). *Staphylococcus aureus* ATCC 29740 was kindly provided by Dr Pascal

Rainard (INRA, UR1282, Nouzilly, France), *Listeria monocytogenes* strain EGD and *Salmonella enterica* serovar Enteritidis LA5 were kindly provided by Dr Philippe Velge (INRA, UR1282, Nouzilly, France).

Antimicrobial activity tests. Antibacterial activities of AvBD11 were measured by radial diffusion assay according to the method described by Lehrer *et al.* (17). Bacteria incubated overnight were diluted in Trypticase Soy Broth (TSB) or Brain Heart Infusion (BHI) at an absorbance of 0.02 and were incubated 2.5 to 4 hours depending on the bacterial strain at 37°C to obtain a mid-logarithmic-phase culture. Bacterial concentrations were determined by plating serial ten-fold dilutions of bacterial suspension on Trypticase Soy Agar (TSA) plates and by counting colony forming units (cfu) after 24 hours incubation at 37°C. A volume containing bacteria at 1.10^7 cfu was centrifuged at 900 *g* for 5 min at 4°C and bacteria were washed once with cold 10 mM sodium phosphate buffer (pH 7.4), resuspended in small volume of cold sodium phosphate buffer and mixed with 25 mL of previously autoclaved, warm (42°C) 10 mM phosphate buffer, containing 0.03% TSB medium, 1% (w/v) low-endosmosis-type agarose (Sigma-Aldrich, Saint Quentin Fallavier, France), and 0.02% Tween 20. The agarose solution containing bacteria was poured into a Petri dish to form a 1 mm deep uniform layer. A 2.5-mm-diameter gel punch was used to make 36 evenly spaced wells. In each well, 5 µL of peptide dilutions or control solutions were added. MSI-94 (a linear amphipathic magainin variant displaying a broad antimicrobial spectrum) and AvBD103b (spheniscin-2) were used as positive controls. MSI-94 was a kind gift from Dr Philippe Bulet (CNRS UMR 5525, Archamps, France). AvBD103b was a synthetic peptide with its 3 disulfides bonds intact, purchased from Genepep (Montpellier, France). Peptides were allowed to diffuse in the bacteria-containing gel while incubating the plates for 3 hours at 37°C. The gel was then overlaid with 25 mL of agar consisting of a double-strength (6%

[w/v]) solution of TSB containing 1% (w/v) agarose. After an overnight incubation at 37°C, the diameter of the clearing zone surrounding each well was measured. For each bacterial strain, three identical independent measurements of antibacterial activity were performed. The minimum inhibitory concentration (MIC) of each peptide was determined as described by Lehrer *et al*. (17). The best-fit straight-line was determined using linear regression with Excel Microsoft 2003 software (Office Microsoft software). The MIC was calculated by finding the intersection of the line with the x axis, indicating the lowest peptide concentration at which no clear zone was obtained.

RESULTS

Identification and purification of AvBD11 from vitelline membranes.

We explored AvBD11 purification from vitelline membrane, since this protein was described as a major constituent. Following extraction, heparin-sepharose chromatography was used as the first step of the purification scheme; proteins that bind to this resin were eluted at high salt concentration and were analysed by SDS-PAGE. The fraction containing proteins with affinity to heparin-sepharose exhibits a wide range of proteins (10 to 100 kDa), with a major band of 10 kDa, corresponding to AvBD11's molecular weight. Western blotting with specific anti-AvBD11 antibodies confirmed its presence and revealed an enrichment of AvBD11 in the fraction eluted from heparin-sepharose. The corresponding band (about 10 kDa) was excised from the SDS-PAGE gel and its peptide sequence analyzed by nanoLC-Ion Trap. Analysis of the data revealed 3 peptides matching with the AvBD11 sequence which was predicted from the *Gallus gallus* genome, and 3 peptides corresponding to *Gallus gallus* lyzosyme (Table 1). In the aim to separate AvBD11 from lysozyme and additional proteins, this fraction obtained using heparin-sepharose chromatography was further purified by RP-HPLC using a X-Bridge® C-18 column (Figure 1). Collected fractions were subjected to tryptic digestion, and the resulting peptides were identified using nanoLC-Q-TOF. Pure AvBD11 without any contaminant was identified in the RP-HPLC fraction collected at 35 minutes (Figure 1, Table 2): Avian β-defensin 11 [*Gallus gallus* (gi|49169808)] was identified using NCBInr database, with a Mascot protein score of 514 and a protein sequence coverage of 53.8% (Table 2).

Structural characterization of AvBD11 using top-down proteomic approach

A top-down mass spectrometry strategy was applied to purified AvBD11 to determine its primary structure at the N- and C-termini and to characterize post-translational modifications.

An aliquot of intact AvBD11, purified by the chromatographic procedures described above, was subjected to MS and MS/MS analysis using nanoESI-Q-TOF without any prior reduction or alkylation steps. The multicharged ions of the mature AvBD11 were measured with a mass accuracy of 100 ppm affording m/z of 1,159.83; 1,325.36 and 1,546.09 respectively for the (+8), (+7) and (+6) charge states. Through the use of high resolution obtained with a Q-TOF mass spectrometer in W reflectron mode, the monoisotopic mass $[M+H]^+$ was calculated and determined at 9,271.56 ± 0.12 Daltons (Figure 2). In the MS-MS spectrum of the seven-fold charged precursor ion m/z 1,325, the N-terminal sequence LPRDTSR of mature AvBD11 was determined by six fragment b ions, b_2 to b_7, and the C-terminal sequence KEI was confirmed by three fragment y ions (y_1 to y_3) (Figure 3). Altogether these data suggest that the complete sequence of mature AvBD11 peptide consists of 82 amino acids with the following sequence: LPRDTSRCVGYHGYCIRSKVCPKPFAAFGTCSWRQKTCCVDTTSDFHTCQDKGGHVS PKIRCLEEQLGLCPLKRWTCCKEI. The observed N-terminal sequence LPRDTSR of mature AvBD11 is two amino acids longer than that predicted by *in silico* studies (RDTSR) (31), and corresponds exactly to that predicted by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP). In fact, the N-terminal sequence LPRDTSR is identical to that determined by Kido *et al.* (1992) for VMO-2. Based on its predicted sequence, the theoretical monocharged monoisotopic mass of mature AvBD11 was calculated to be 9,283.37 Daltons, differing from that determined experimentally (9,271.56 Da) by 12 Daltons, which corresponds to the post-translational modifications resulting from six disulfide bonds formed from the 12 cysteine residues of AvBD11.

Antibacterial activity of AvBD11.

Antimicrobial activities of purified AvBD11, AvBD103b and MSI-94 were evaluated using a radial diffusion assay against six different pathogens: *Staphylococcus aureus* ATCC 29740 (*S.*

329 *aureus*), *Listeria monocytogenes* strain EGD (*L. monocytogenes*), *Escherichia coli* ATCC
330 25922 (*E. coli*), *Salmonella enterica* serovar Enteritidis ATCC 13076 (*S*. Enteritidis ATCC
331 13076), *Salmonella enterica* serovar Enteritidis LA5 (*S*. Enteritidis LA5) and *Salmonella*
332 *enterica* serovar Typhimurium ATCC 14028 (*S*. Typhimurium). Clearing units (U) were
333 calculated by the diameter in mm of the clear zone surrounding the well minus the diameter in
334 mm of the well. They were further plotted as a function of $\log_{10}$ (peptide concentration) for
335 each bacterial strain, as illustrated on Figure 4 for AvBD11 against *L. monocytogenes*. The
336 various MICs obtained from each peptide are shown in µM in Table 3. Antimicrobial peptides
337 MSI-94 and AvBD103b were used as controls, and similar MICs were observed for AvBD11
338 against *S.* Enteritidis ATCC 13076 and *S.* Typhimurium. AvBD11 antibacterial activity
339 towards *S. aureus*, was three and five times lower than MSI-94 and AvBD103b controls.
340 However, for *E. coli*, results showed that AvBD11 and AvBD103b displayed a particularly
341 low MIC of 50 and 90 nM, respectively, compared to 370 nM obtained for MSI-94. AvBD11
342 and AvBD103b displayed also a higher antibacterial effect against *L. monocytogenes* than
343 MSI-94. While AvBD11 was two times less effective than AvBD103b against *S*. Enteritidis
344 LA5, their MICs were similar against *S*. Enteritidis ATCC 13076.

DISCUSSION

AvBD11 has been previously identified by proteomic approaches in different compartments of the hen egg: the eggshell, the egg white and the vitelline membranes (21-23). In this work, we have developed a fast, simple and reliable method to purify AvBD11 from vitelline membranes. Mature AvBD11 peptide was purified from vitelline membranes, by a combination of heparin-sepharose chromatography and by C_{18} RP-HPLC. We obtained around 750 µg of AvBD11 / 250 mg of solubilized vitelline membranes (extracted from 30 eggs). Characterization of mature AvBD11 was achieved using a hybrid strategy combining "bottom-up" and "top-down" proteomic approaches, as previously described for AvBD1, AvBD2 and AvBD7 (3). The use of complementary mass spectrometry techniques allowed us to obtain structural information concerning the mature AvBD11 peptide sequence and its post-translational modifications. The sequence of mature AvBD11 was shown to be composed of 82 amino acids. The amino-terminal sequence, identified as LPRDTSR, corroborates the previous determination by Kido *et al.* (12). Top-down proteomic approaches put in evidence the formation of six disulfide bonds from the 12 cysteine residues. We attempted to carry out complementary mass spectrometry analyses, but failed to determine the position of the disulfide bonds. These results are partly explained by the high resistance of AvBD11 to tryptic digestion and to heat denaturation. The strong stability of mature AvBD11 is compatible with the prediction of six internal disulfide bonds. The entire AvBD11 sequence has no homology with any other β-defensin. To our knowledge, AvBD11 is the first β-defensin containing a double β-defensin motif with 6 disulfide bonds to be purified and characterized. Its C-terminal sequence possesses 27% identity to the corresponding region of human β-defensin 3 (Swiss-Prot P81534), which seems to include the biologically active part of the molecule (14). Solving the three-dimensional structure by two-dimensional NMR and molecular modeling is needed in order to determine the pairing pattern of the six disulfide

bridges and to understand the structure-function relationship of AvBD11, as it has been performed for AvBD103b (15).

Avian β-defensins interact electrostatically with negatively charged components of bacterial membranes, such as lipopolysaccharides, lipoteichoic acid and anionic phospholipids. Depending on their amino acid composition, AvBDs exhibit more or less antimicrobial activity against a specific bacteria strain (30). In our study, we found that both AvBD11 and AvBD103b exhibited potent antimicrobial activity against the six bacterial strains tested. *S.* Enteritidis LA5 corresponds to a field isolated strain and because of the high incidence of *S.* Enteritidis contamination of hen eggs, any antimicrobial peptide that is effective against this particular strain is of interest.

In this study, AvBD11 and AvBD103b possess an elevated cationic net charge of (+9) and (+10), respectively. And even if AvBD103b was more cationic than AvBD11, similar levels of antibacterial activity were found for AvBD11 and AvBD103b towards *L. monocytogenes*, *S.* Enteritidis, *S.* Thyphimurium and *E. coli*. We only noted that AvBD103b possessed better antibacterial activity than AvBD11 against *S. aureus*. Derache *et al.* (3) compared antibacterial activity of AvBD2 (+4), AvBD1 (+8) and AvBD7 (+6). In accordance with our finding, they couldn't establish a direct link between the net charge of the peptides and their antibacterial potency. In our study, identical antibacterial assay and positive control (MSI-94), as for Derache *et al.*, were used to characterize AvBD11 and AvBD103b. Therefore, MICs obtained for AvBD-1, -7, -11 and AvBD103b could be compared. Even if AvBD11 and AvBD103b were positively charged at a higher level than AvBD-1 and -7, they did not exhibit better antimicrobial activities towards *Salmonella* strains and *S. aureus*; but they were more efficient against *E. coli* (3). These comparisons taken together reinforce the fact that no direct relationship could be established between the net charge of these AvBD and their antimicrobial potency against Gram-positive and -negative bacteria. It also suggests that

AvBD efficiency not only depends on its amino acid composition but also certainly on their positioning. However, it is noteworthy that the relationship between the high positive net charge (+10) of AvBD103b and its potent antimicrobial activity is evident when tested in a salt-rich environment (15).

The innate immune system is known to play a major role in protecting the female reproductive tract against microbes. Previous studies have shown the presence of a large panel of AvBDs in the different regions of the hen oviduct, presumably exhibiting a large spectrum of antimicrobial activities in order to protect the future embryo against pathogenic microorganisms. In humans, during late pregnancy and delivery, human β-defensin 3 (hBD3), which possesses homology with AvBD11, was expressed at key sites of the uterus, and would provide enhanced protection from infection for the uterus and/or fetus (13). In hens, the onset of egg-laying activity takes place at approximately 5 months of age. AvBD-1, -2 and -3 mRNA are not expressed in the immature oviduct of younger hens or in the mature oviduct of non-laying hens, but their oviduct expression increases when the hens are forming eggs (25, 34). These results suggest modulation of AvBD expression, as a function of egg-laying activity, possibly as a consequence of the dilatation of the oviduct by the presence of the forming egg as shown for osteopontin and ovocalyxin 36 (7, 16). Furthermore, several studies have shown a modulation of β-defensin expression correlated with stimulation by pathogens. In the amnion FL cell line, hBD3 expression increases in response to peptidoglycan and lipopolysaccharide (LPS), which mimic the effect of bacterial infection (2). Modification of AvBD expression has also been demonstrated in the hen oviduct. AvBD-1, -2 and -3 mRNA expression is upregulated when cultured hen vaginal cells were stimulated with LPS or with *S.* Enteritidis (33). In the hen vagina, expression of AvBD-3, -5, -10, -11 and -12 were significantly increased in response to LPS treatment (20). In primary hen isthmus epithelial cells, an infection with wild type *S.* Enteritidis decreased the expression of AvBD-4, -9, -10

and -11, which are constitutively highly expressed and, reversely, increased the normally low expression of AvBD-2, -7 and -8 (4). This mechanism might be present in other regions of the hen oviduct, in order to ensure an appropriate antimicrobial response in case of infection (24). Moreover, in humans, several studies have reported chemotactism of hBD3 for monocytes and macrophages (32), allowing recruitment of these cells into uterine tissues. This suggests that hBD3, produced in the uterine tissues, may have a role in activation of the maternal adaptive immune system in the event of infection (13). In the hen oviduct, AvBD11 might possess similar functions and interact with the adaptive immune system, in addition of their innate molecular antimicrobial activities.

The presence of AvBD11 in different compartments of the egg (eggshell, egg white, vitelline membranes) suggests that it plays a protective role during embryonic development. If there are changes in AvBD11 stability during egg storage, this might explain the decreased protective efficiency of vitelline membrane which is noted during egg storage at 20°C (27). Lysozyme, VMO-1 and VMO-2 (AvBD11) are the three major proteins of the salt soluble fraction of vitelline membranes. During 20 days of egg storage at 5°C or 20°C, the lysozyme content increases in the salt soluble fraction of vitelline membranes, while AvBD11 decreases from 5% (initially observed) to 3% and 1% at 5°C and 20°C, respectively. The authors attributed the deterioration of vitelline membranes during storage to the disappearance of the VMO-1 and AvBD11 proteins. However, an alternative hypothesis is that breakdown of the vitelline membranes was due to denaturation of ovomucin, the major component of vitelline membrane, which is not present in the salt soluble fraction. In any event, the loss of the vitelline membranes and its components is likely to alter the antimicrobial protection in these compartments during storage.

The current study put in evidence the large antibacterial spectrum of AvBD11, a β-defensin with an unusual structure containing two repeated β-defensin consensus motifs

consisting of 6 disulfide bonds. Structural characterization of AvBD11 is expected to lead to superior modelling for the design of synthetic antimicrobial peptides. AvDB11 is present in different compartments of the egg, including the egg white. The availability of antibodies against this peptide will permit development of a quantitative assay aiming to evaluate the variability of AvBD11 in table eggs, as a function of hen physiological stage and/or environment. Finally, this work contributes to a broader understanding of how previously unknown egg components contribute to the numerous biological functions of the egg, including its antimicrobial activities (24). Such studies underline the potential of the table egg as a source of new antimicrobial peptides and its value for human and animal health.

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Figure legends

Figure 1. Reverse-phase HPLC to separate molecules of the vitelline membranes eluted fraction obtained after heparin-sepharose affinity chromatography.

Figure 2. Multicharged ions spectra for intact AvBD11. MS spectra (zoom on multicharged species) obtained with nanoESI-Q-TOF with reflector W mode (resolution R=15000) from intact AvBD11 purified by reverse-phase HPLC. The monoisotopic molecular mass of the mature AvBD11 was observed at m/z 1,159.83; 1,325.36 and 1,546.09 respectively for the (+8), (+7) and (+6) charge states. The monocharged molecular specie $[M+H]^+$ was calculated and determined at 9,271.56 ± 0.12 Daltons.

Figure 3. C and N terminus vitelline membrane-extracted AvBD11 sequence. Shown is an MS-MS spectrum obtained by collision-induced dissociation with nano-ESI–Q-TOF MS from intact AvBD11 without prior reduction or alkylation. The precursor ion had an *m/z* of 1,325.5 in charge state 7. The N-terminal sequence LPRDTSR, determined by manual interpretation, was essentially obtained by six consecutive b ions (b_2 to b_7) and the C-terminal sequence KEI was confirmed by three y ions (y_1 to y_3). Single letters indicate immonium ions.

Figure 4. AvBD11 Antimicrobial activity against to *Listeria monocytogenes.* (A) Dose-response of AvBD11 and its inhibitory effect on *L. monocytogenes*, reflected by size of the clear zone (Clearing units = the diameter in mm of the clear zone on the plate minus the diameter in mm of the well). (B) The best-fit straight line was determined by linear regression. The MIC of AvBD11 *versus L. monocytogenes* was calculated by finding the intersection of the line with the *x* axis, indicating the lowest peptide concentration at which no clear zone was detected.

597 TABLE 1. Identification by nanoLC-Q-TOF mass spectrometry of heparin binding proteins

598 from egg vitelline membranes, present in the 10 kDa band.

599

Protein accession Gi/NCBI/ Swiss-Prot Trembl accession no.	Protein	Protein MASCOT score	Protein mass	No. of unique peptides	Sequence
gil49169808 NP_001001779 Q6IV20	Avian β-defensin 11 (AvBD11)	703	11642	3	CLEEQLGLCPLK VCPKPFAAFGTCSWR IRCLEEQLGLCPLKR
gil157831653 1KXW_A P00698	Chain A, Analysis Of The Stabilization Of Hen Lysozyme	348	14305	3	NTDGSTDYGILQINSR NLCNIPCSALLSSDITASVNCAK GYSLGDWVCAAKFESNFNTQATNR

600
601

602 TABLE 2. Peptide list obtained from reverse-phase HPLC purified AvBD11, after tryptic
603 reaction, and identified in NCBInr database, using nanoLC-Q-TOF.

m/z	Z	M obs	M theo	Δ mass	Miss cleavage	Pep. score	Sequence	Position	Modif.
484.71	2	967.41	967.43	-0.02	0	20.64	YHGYCIR	33-39	CAM
513.21	2	1024.40	1024.45	-0.06	0	32.90	GYHGYCIR	32-39	CAM
528.23	2	1054.45	1054.47	-0.02	0	65.46	AAFGTCSWR	48-56	CAM
562.75	2	1123.48	1123.52	-0.04	0	44.09	VGYHGYCIR	31-39	CAM
642.76	2	1283.50	1283.55	-0.05	0	57.96	CVGYHGYCIR	30-39	2 CAM
650.29	2	1298.57	1298.59	-0.02	0	86.99	PFAAFGTCSWR	46-56	CAM
671.85	2	1341.68	1341.71	-0.03	1	59.87	EEQLGLCPLKR	87-97	CAM
728.38	2	1454.75	1454.79	-0.04	1	64.13	LEEQLGLCPLKR	86-97	CAM
730.35	2	1458.69	1458.72	-0.03	0	72.70	CLEEQLGLCPLK	85-96	2 CAM
539.27	3	1614.79	1614.82	-0.03	1	98.86	CLEEQLGLCPLKR	85-97	2 CAM
808.40	2	1614.79	1614.82	-0.03	1	85.86	CLEEQLGLCPLKR	85-97	2 CAM
595.27	3	1782.78	1782.83	-0.05	0	79.60	VCPKPFAAFGTCSWR	42-56	2 CAM
658.90	3	1973.69	1973.76	-0.07	0	64.17	TCCVDTTSDFHTCQDK	59-74	3 CAM
1016.37	2	2030.73	2030.78	-0.05	1	22.90	TCCVDTTSDFHTCQDKGG	59-76	2 CAM

604

605 Columns indicates, the ratio mass/charge (m/z) fragmented, the charge (z), the experimental
606 MW of peptide (M obs), the theoretical MW of peptide (M theo), the delta mass (Δmass), the
607 number of missed cleavage, the individual MASCOT score of peptide, the sequence, the
608 position in the full sequence, the observed modification (CAM : carbamidomethylation). All
609 peptides allowed the identification of β-defensin 11 [*Gallus gallus*] (gi|49169808) with a
610 Mascot protein score at 514 and 53.8% for the protein coverage.

TABLE 3. Minimum Inhibitory Concentration (MIC) of purified AvBD11, synthesized king penguin AvBD103b and MSI-94 (magainin variant used as positive control).

Bacterial group	MIC (μM) (95% confidence interval)		
	MSI-94	AvBD11	AvBD103b
Gram positive			
S. aureus ATCC 29740	0.33 (0.19-0.48)	0.90 (0.27-1.7)	0.16 (0.12-0.20)
L. monocytogenes	0.28 (0.13-0.43)	0.18 (0.08-0.27)	0.14 (0.14-0.15)
Gram negative			
S. Enteritidis ATCC 13076	0.31 (0.25-0.35)	0.35 (0.27-0.46)	0.31 (0.15-0.45)
S. Enteritidis LA5	0.15 (0.10-0.21)	0.40 (0.29-0.49)	0.21 (0.20-0.22)
S. Typhimurium ATCC 14028	0.25 (0.11-0.40)	0.32 (0.31-0.32)	0.20 (0.19-0.21)
E. coli ATCC 25922	0.37 (0.23-0.52)	0.05 (0.04-0.05)	0.09 (0.08-0.11)

The MIC was determined by radial diffusion assay for each bacterial strain.

Figure 1

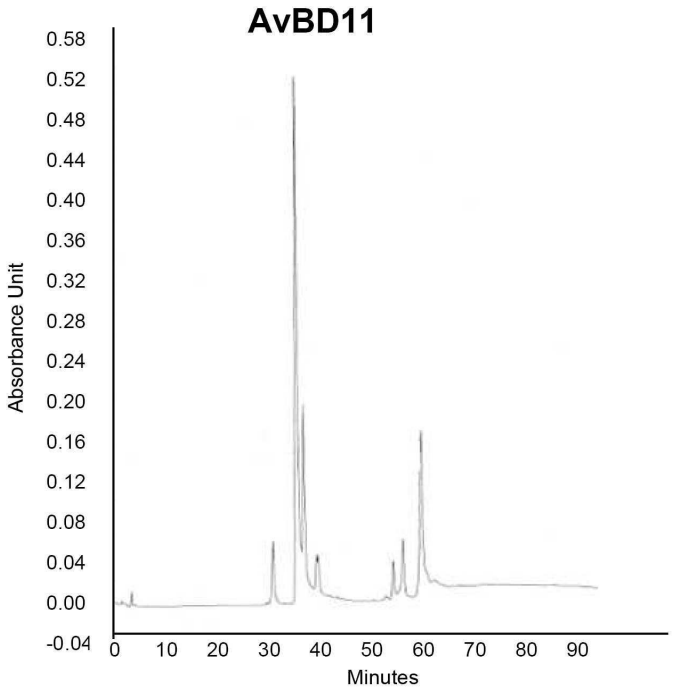

Figure 2

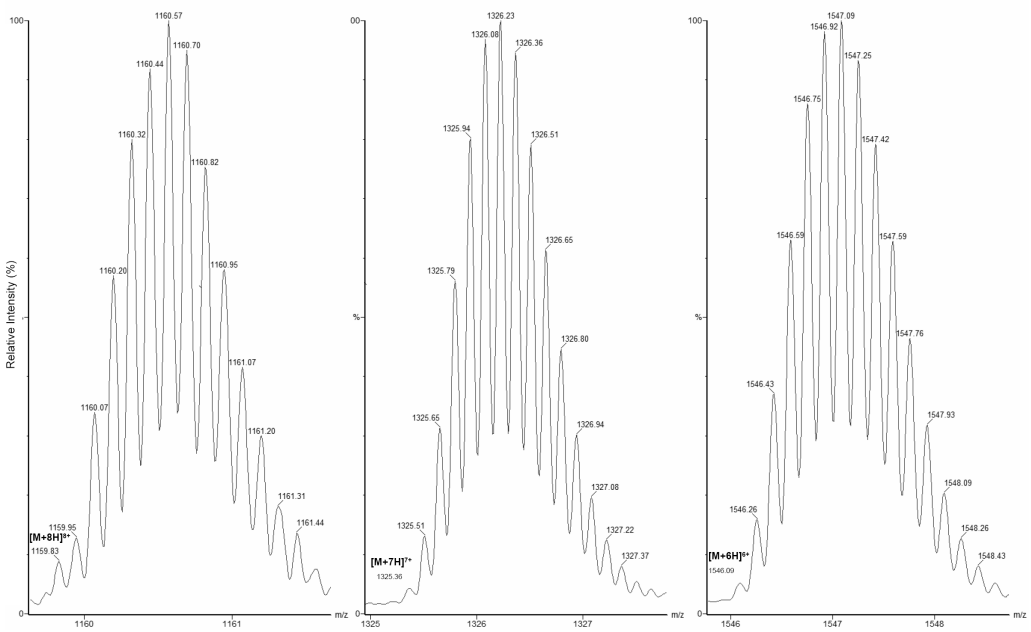

Figure 3

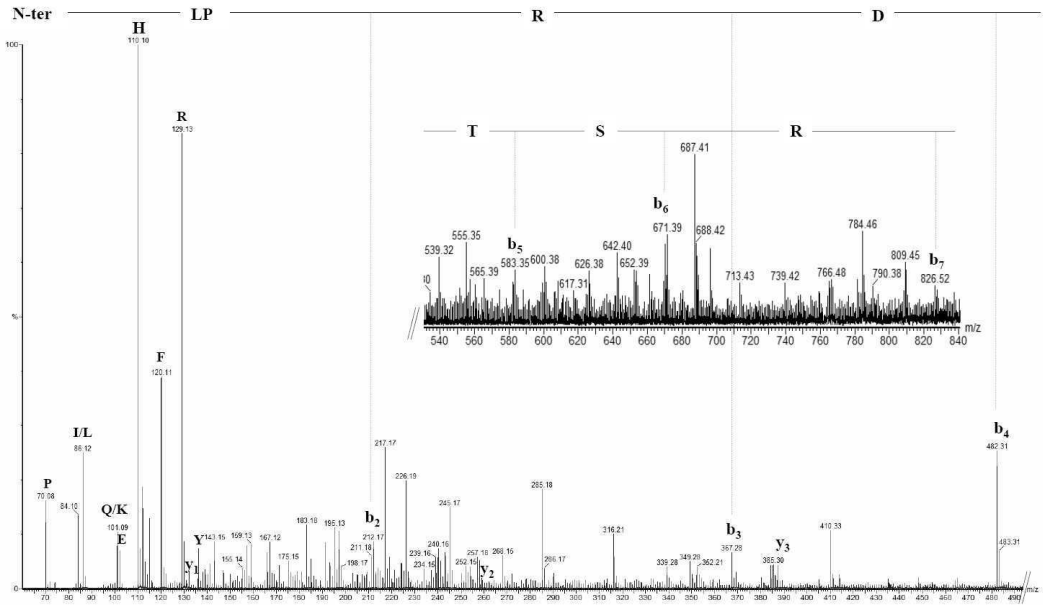

Figure 4