Journal of
Proteomics & Bioinformatics

Kannan et al., J Proteomics Bioinform 2013, 6:2
DOI: 10.4172/jpb.1000258

Research Article Open Access

# Identification and Structural Characterization of Avian Beta-Defensin 2 Peptides from Pheasant and Quail

**L Kannan[1,2], R Liyanage[3], JO Lay Jr.[3], B Packialakshmi[1,2], NB Anthony[2] and NC Rath[1]***

[1]USDA/ARS, Poultry Science Center, University of Arkansas, Fayetteville, AR 72701, USA
[2]Cell and Molecular Biology Program, Poultry Science Department, University of Arkansas, Fayetteville, AR 72701, USA
[3]Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, USA

**Abstract**

Pheasant and quail orthologs of Avian β-Defensin 2 (AvBD2) were identified by screening heterophil extracts using Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS), comparative profiling, chemical, and bioinformatic characterizations. In heterophil extracts of each pheasant and quail, we observed a single high intensity mass peak, corresponding to *m/z* 4114.8 and 4163.8, respectively which upon reduction and alkylation were shifted by a mass difference of 348 Da indicative of the modification of 3 internal disulfide bonds that exist in these peptides. The unmodified peptides, *m/z* 4114 and 4163, and their Carbamidomethylated (CAM) derivatives were purified by reverse phase HPLC. Using the purified peptides we determined their partial sequences by trypsin digestion followed by MALDI-TOF-MS, MALDI LIFT-TOF/TOF, and Edman degradation which showed their unambiguous homologies with other AvBD2. Combining all the results and aligning the sequence stretches with mature AvBD2 from other avian species, we deduced the amino acid sequences of pheasant and quail orthologs as, LFCKRGSCHFGRCPSHLIKVGSCFGFRSCCKWPWNA and LFCRRGTCHFGNCPSDQIKVGNCFGFRSCCRWPWDA, respectively. These sequence information were further confirmed by MALDI *in-source decay* (ISD) analyses. Both pheasant and quail AvBD2 peptides showed significant identities (>80%) with other known AvBD2 sequences.

**Keywords:** Avian beta-defensin 2; Peptide; Amino acids; MALDI-TOF-MS; Pheasant; Quail

**Abbreviations**: ACN: Acetonitrile; AvBD: Avian Beta-Defensin; CAM: Carbamidomethylation; 1,5 DAN: 1,5 Diaminonaphthalene; DHB: 2,5 Dihydroxybenzoicacid; DTT: Dithiothreitol; FITC: Fluorescein Isothiocyanate; IAA: Iodoacetamide; ISD: *In Source Decay*; MALDI-MS: Matrix-Assisted Laser Desorption Ionization Mass Spectrometry; *m/z:* Mass/Charge; PI: Propidiumiodide; RP-HPLC: Reverse Phase-High Pressure Liquid Chromatography; TOF: Time-of-Flight

## Introduction

Mass Spectrometry (MS) has become an essential tool for discovery, identification, and characterization of proteins and peptides present in low concentrations in complex pools of biomolecules such as cell and tissue extracts [1-5]. Identifying tissue specific abundance of mature peptides in their biological niches can be useful to understand their physiological roles. Previously using direct MALDI-TOF-MS; we profiled crude heterophil, macrophage, and bone marrow extracts of chicken and turkey, and identified the occurrence of β-defensins and thymosin β4 in their mature forms [6-8]. These findings suggest the possibility to identify bioactive peptides and their homologues in different species in complex mixtures. Derache et al. [9], similarly identified several chicken beta-defensins in avian bone marrow extracts using MALDI-TOF-MS.

The defensins are cationic antimicrobial peptides which are evolutionarily conserved and occur as beta-defensin isoforms in birds [10-13]. The Avian Beta-Defensin 2 (AvBD2) predominantly occurs in heterophils and epithelial tissues [13,14]. The MALDI-TOF-MS profiles of both chicken and turkey heterophils showed the occurrence of AvBD2 as a single high intensity spectral peak [7]. Thus, we asked whether similar profiling would detect otherAvBD2 orthologs in species where the functional genes have not been annotated. Accordingly, the objective of the current study was to identify and structurally characterize the AvBD2 peptides of pheasant and quail. Using comparative pattern profiling along with MALDI Peptide Mass Finger printing (PMF), MALDI LIFT-TOF/TOF fragmentation, MALDI-ISD fragmentation, and N-terminal peptide sequencing, we present the identification and amino acid sequences of pheasant and quail AvBD2.

## Materials and Methods

### Chemicals and reagents

One Step Polymorphprep™ gradient (Accurate Chemicals Co, Westbury, NY), K-EDTA Vacutainer tubes (BD Bioscience, Franklin, NJ), C18 ZipTip micropipette tips (Millipore, Billerica, MA), peptide and protein calibration standards (*m/z* 500-16000, Bruker Daltonics, Bremen, Germany), and Bio wide Pore $C_{18}$ reverse phase HPLC column (Supelco, St. Louis, MO) were purchased from their respective vendors. All other reagents including Fluorescein Isothiocyanate (FITC), Propidium Iodide (PI), 1, 4-Dithiothreitol (DTT), 2-Iodoacetamide

***Corresponding author:** Narayan C. Rath, USDA/ARS, Poultry Science Center, University of Arkansas, Fayetteville, AR 72701, USA, Tel: 479-575-6189; Fax: 479-575-4202; E-mail: narayan.rath@ars.usda.gov

**Received** January 15, 2013; **Accepted** February 14, 2013; **Published** February 18, 2013

**Citation:** Kannan L, Liyanage R, Lay JO Jr., Packialakshmi B, Anthony NB, et al. (2013) Identification and Structural Characterization of Avian Beta-Defensin 2 Peptides from Pheasant and Quail. J Proteomics Bioinform 6: 031-037. doi:10.4172/jpb.1000258

(IAA), 2, 5-Dihydroxybenzoic acid (DHB), 1,5-Diaminonaphthalene (DAN), were purchased from Sigma Aldrich (St. Louis, MO).

### Isolation of heterophils

Heterophils were isolated from K-EDTA anti-coagulated peripheral blood of pheasant (*Phasianus colchicus*) and quail (*Coturnix coturnix*) using One Step Polymorphprep™ gradient centrifugation [6,7,15]. 3 ml of blood was layered on 2 ml Polymorphprep medium and spun at 500 g for 30 min leading to the enrichment of heterophils in the medium between monocytes at the proximal aspect of the gradient and red blood cells at the bottom. The Polymorphprep media containing granulocytes were mixed with an equal volume of 0.5 N RPMI-1640 and centrifuged per manufacturer's instruction, and washed three times successively to pellet the cells. The heterophil enrichment was assessed by staining the cells with FITC, which stain the cytoplasmic granules [15] (Supplementary figure S1). The bone marrow was collected from snapped tibia by centrifugation at 200 g for 2 min and kept frozen at -20°C until extraction.

### Sample preparation for MALDI

Approximately, $1\times10^6$ heterophils or ~100 mg of bone marrow from pheasant (*Phasianus colchicus*) or quail (*Coturnix coturnix*) were homogenized in 1 mL of 70% methanol containing 0.2% acetic acid followed by precipitation overnight at 4°C then centrifuged at 21,000 g for 10 min to obtain the supernatant [7]. For preliminary screening, the crude supernatants were mixed with an equal volume of 1 M 2,5-DHB prepared in 90% methanol containing 0.1% formic acid, and spotted on to a MTP 384 ground steel MALDI target plate. Mass spectra were acquired on Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics GMBH, Bremen, Germany) operated in the positive-ion reflectron mode. For Carbamidomethylation (CAM), the methanol acetic acid extracted samples were first dried using a SpeedVac concentrator then reconstituted with 100 mM $NH_4HCO_3$, reduced using 10 mM 1,4-dithiothreitol (DTT) for 1 h at 55°C followed by alkylation with 20 mM 2-iodoacetamide in the dark for 25 min at 37°C [7]. The high intensity MALDI-TOF-MS spectral peaks showing expected CAM modification induced mass shifts of 348 Da, were provisionally considered as their respective beta-defensins pending subsequent characterizations.

### Reverse phase HPLC purification AvBD2

Both, non-reduced/alkylated intact and reduced alkylated (R/A) peptides were purified by reverse phase HPLC from bone marrow extracts according to an earlier described procedure [7]. Briefly, the methanol extracts of bone marrow were evaporated using SpeedVac concentrator/evaporator to an approximate 1/20$^{th}$ volume then centrifuged at 21,000 g to remove insoluble materials and the clear supernatant was used for HPLC purification using a C18 reverse phase column (4.6×250 mm, 5 µm). An Agilent HPLC system (Hewlett 1100; Hewlett Packard, Palo Alto, CA) was used along with a Bruker Electrospray Ionization (ESI) quadrupole ion trap mass spectrometer as the mass analyzer (Bruker Daltonics GMBH, Bremen, Germany) to identify the peptide fractions of interest. The HPLC fractions, consistent with ESI-MS multiple charge ion distribution matching to the mass of the presumed AvBD2, was collected and verified further for purity by MALDI-TOF-MS. The respective peptide fractions were then used for N-terminal sequencing, trypsin digestion, MALDI peptide mass fingerprinting, and MALDI *in-source decay* (ISD) analyses. The accurate mono isotopic protonated masses of intact oxidized and Carbamidomethylated (CAM) peptides of both pheasant and quail were also determined by MALDI-TOF-MS using combinations of external and internal calibration procedures.

### N-terminal sequencing

The N-terminal sequence analyses of the purified intact, non-reduced/alkylated peptides were performed using an ABI Procise® 1P000145 protein sequencer (Invitrogen, USA).

### Trypsin digestion and MALDI-TOF-MS peptide mass finger printing (PMF) of AvBD2

For trypsin digestion, the purified CAM AvBD2 peptides from each species of birds were dried, reconstituted in 25 mM $NH_4HCO_3$, and digested with 100 ng of trypsin for 16 hours at 37°C. The tryptic digests were desalted using Zip Tip per manufacturer's suggestion and 2 µl of eluted peptides were premixed with HCCA, and spotted on MALDI target plate to obtain Peptide Mass Finger Prints (PMF). Subsequent MS/MS sequencing of the tryptic peptides were done by MALDI LIFT-TOF/TOF [16]. All MALDI LIFT-TOF/TOF experiments were performed on an Ultraflex II MALDI TOF/TOF instrument (Bruker Daltonics GMBH, Bremen, Germany). The tryptic PMF of both chicken and turkey AvBD2 generated were used as references.

### MALDI-ISD analysis

Both, non-reduced and reduced/alkylated purified AvBD2 peptides were subjected to MALDI *in source decay* fragmentation [17-20]. The use of ISD fragments in MALDI mass spectra as a means of sequencing is a 'top down' approach [19]. MALDI-ISD facilitates $c_n$ (N terminus) and $z+2_n$ (C-terminus) type fragmentation ions with sequence specific information for peptides with and without disulfide bonds. A saturated solution of 1,5-Diaminonaphthalene (DAN) MALDI matrix prepared in 50% ACN/ 0.1% formic acid was employed at the sample to matrix ratio of 1:1 for ISD studies [18], of both purified intact and CAM AvBD2 peptides. ISD spectra were acquired using a Bruker reflex III MALDI-TOF mass spectrometer.

### Data analysis and sequence assembly

All raw MS data were processed using Bruker BioTools 3.1 software. Mature AvBD2 peptide sequences of chicken, turkey, ostrich, mallard duck, Peking duck, and zebra finches from literature [7,9,14,21-28] were aligned using PRALINE multiple protein sequence alignment program (http://www.ibi.vu.nl/programs/pralinewww/) with the N-terminal

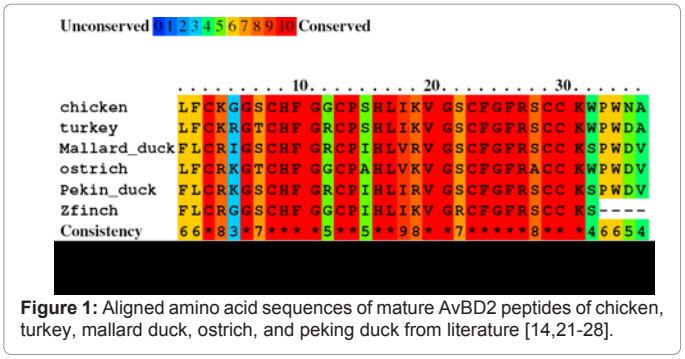


**Figure 1:** Aligned amino acid sequences of mature AvBD2 peptides of chicken, turkey, mallard duck, ostrich, and peking duck from literature [14,21-28].

amino acid at position 1 and a total of 36 amino acids for most known AvBD2 (Figure 1). The tryptic peptide fragments of chicken and turkey AvBD2 were used as primary references for most comparisons. Respective AvBD2 sequence stretches of pheasant and quail were first aligned using common fragment information obtained from MALDI-TOF-MS, MALDI-LIFT-TOF/TOF fragmentation of tryptic peptides, and N-terminus sequence data of intact peptides obtained by Edman's degradation. The proposed mutations in amino acid sequences were based on conservative substitutions using universal genetic code and comparative bioinformatics of known AvBD2 sequences [14,21-28]. The obtained sequences were then confirmed by MALDI-ISD.

## Results and Discussion

Figures 2a and 2c represent the respective MALDI-TOF-MS profiles of heterophil extracts from pheasant and quail respectively. In both species we observed high intensity spectral peaks (indicated by arrows) similar to the profiles seen with chicken and turkey heterophils (Figures S2a and S3a). Our previous studies with chicken and turkey

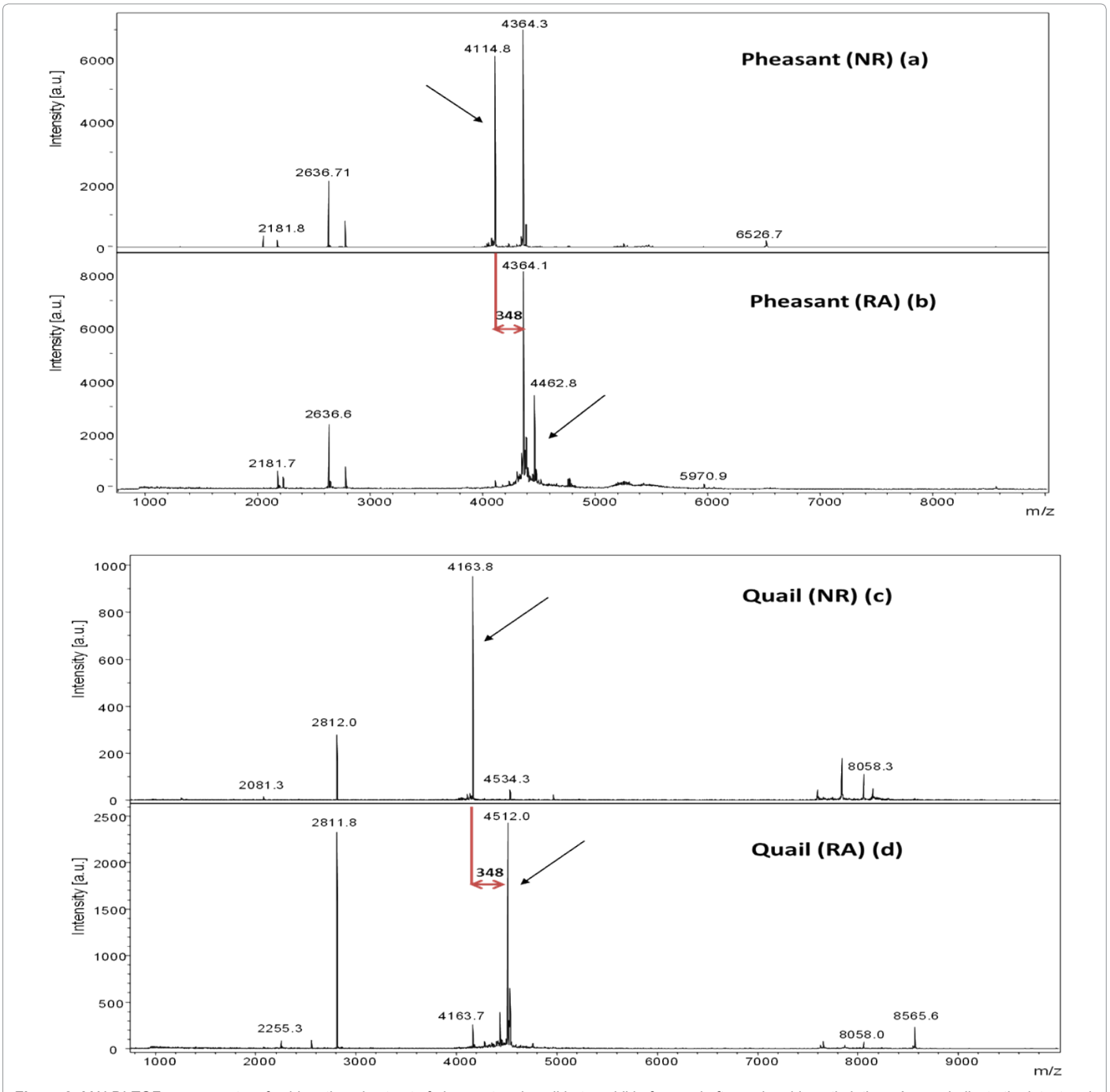


**Figure 2:** MALDI-TOF mass spectra of acid methanol extract of pheasant and quail heterophil before and after carbamidomethylation. Arrows indicate the intact and 348 Da modified of AvBD peaks. NR=Non reduced, RA=Reduced/alkylated.

heterophils showed that these high intensity peaks were corresponding to their respective mature AvBD2 peptides [7,21,24]. Hence, we surmised that, at least, one of the 2 high intensity peaks of pheasant and the one of quail heterophil extract may be their respective AvBD2 peptides. After carbamidomethylation, the peaks at *m/z* 4114.8 of pheasant and *m/z* 4163.8 of quail showed mass shifts of 348 Da each, resulting in peaks *m/z* 4462.8 and 4512, respectively (Figures 2b and 2d). The observed mass difference of 348 Da were due to modifications of 3 internal disulfide bonds present in naturally occurring non-reduced/oxidized forms of AvBD because of the addition of 6 carbamidomethy groups (58 Da each), resulting from reduction and alkylation with iodoacetamide. By contrast, there were other peaks such as at *m/z* 2636 and 4364.1 in pheasant, and *m/z* 2812, and 4534 in quail which remained unchanged suggesting the absence of free cysteine in those peptides (Figure 2). For peptide purification, the bone marrow extracts of pheasant and quail were used with or without CAM modification. Figures S4a and S5a (supplementary) show total ion current chromatogram for unmodified peptides *m/z* 4114.8 (pheasant) and quail *m/z* 4163.8 (quail) respectively. Figures S4b and S5b show the Extracted Ion Current (EIC) chromatogram and figures S4c and S5c show the corresponding ESI-MS multiply charged spectra in supplementary file. The accurate mono isotopic protonated masses for both intact oxidized and CAM AvBD peptides were determined to be *m/z* 4111.8 ± 0.2 and *m/z* 4460.1 ± 0.2 for pheasant, and *m/z* 4160.8 ± 0.2, and *m/z* 4509 ± 0.2 for quail, respectively.

The purified *m/z* 4114.8 (pheasant) and *m/z* 4163.8 (quail), modified by CAM, were subjected to trypsin digestion. Peptide Mass Fingerprint (PMF) of the tryptic digests obtained by MALDI-TOF MS, are shown in figures 3a and 3b. Each tryptic digest showed a single high intensity spectral peak corresponding to *m/z* 929.4 for pheasant and *m/z* 956.5 for quail. This pattern was very similar to MALDI tryptic PMF of chicken and turkey AvBD2 [7]. MALDI LIFT-TOF/TOF fragmentation of the peak at *m/z* 929.4 displayed 'b' and 'y' ions which were consistent with the sequence 'VGSCFGFR' (Figure 4a), with a significant hit for gallinacin-2 peptide of chicken with a MASCOT MS/MS score of 60 (p=0.0024). This stretch of amino acids corresponds exactly to the positions 20-27 of the mature AvBD2 peptides from both chicken

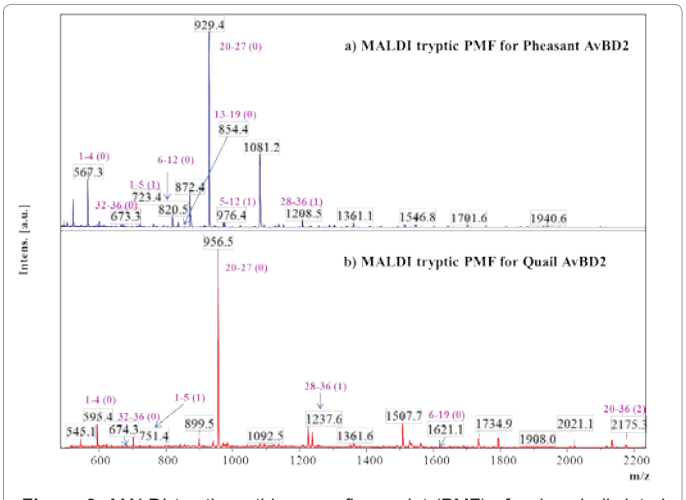


**Figure 3:** MALDI trypticpeptide mass fingerprint (PMF) of reduced alkylated (R/A) AvBD peptides of pheasant (upper panel, a) and quail (bottom panel b). (0) no miscleavage and (1) single miscleavage.

| MH+ | Sequence range | Partial | Sequence |
|---|---|---|---|
| 567.3 | 1-4 | 0 | LFCK 3: CAM (C) |
| 723.4 | 1-5 | 1 | LFCKR 3: CAM (C) |
| 976.4 | 5-12 | 1 | RGSCHFGR 4: CAM (C) |
| 820.5 | 6-12 | 0 | GSCHFGR 3: CAM (C) |
| 854.4 | 13-19 | 0 | CPSHLIK 1: CAM(C) |
| 929.4 | 20 - 27 | 0 | VGSCFGFR 4: CAM (C) |
| 1208.5 | 28 - 36 | 1 | SCCKWPWNA 2: CAM (C) 3: CAM(C) |
| 673.3 | 32 - 36 | 0 | WPWNA |

**Table 1:** MALDI-TOF-MSb observed tryptic peptide ions from pheasantAvBD2 digest.

and turkey (Figure 1). Besides *m/z* 929.4, there were mass peaks at *m/z* 567.3, 673.3, and 1208.5, similar to tryptic fragment masses of chicken AvBD2, corresponding to amino acid sequences 'LFCK'(positions 1-4), 'WPWNA'(positions 32-36), and 'SCCKWPWNA'(28-36), respectively (Table 1, Figure 4a). The presence of amino acids 'Asn-Ala (NA)' at the C-terminus of the intact pheasant AvBD2, was evident from two sets of multiply charged ESI-MS spectra (Figure S4c), most likely, generated by the consecutive loss of both the amino acid residues 'Asn-Ala (NA)'. Such C-terminus fragmentation was also observed for chicken AvBD2 in LC-ESI mass spectrum because of *in source* fragmentation of the intact peptides due to voltage induced heating at the skimmer [7]. Two other pheasant tryptic peptides, *m/z* 723.1 and 854.4, also matched with turkey AvBD2 PMF corresponding to 'LFCKR', positions 1-5, and 'CPSHLIK', positions 13-19, respectively (Table 1, figure S1 and 3a). MALDI LIFT-TOF/TOF spectra for peaks at *m/z* 567 and 723 were also suggestive of the possible sequence as, 'LFCK' and 'LFCKR', respectively though the mass spectra would not distinguish 'Leu (L)' from 'Ile (I)' due to their identical masses. However, the N-terminal sequence information of intact pheasant AvBD2 peptides, obtained by Edman degradation, confirmed the identities of the first 7 residues as 'LF_KRGS' with the missing 3rd residue as 'Cys (C)'thus, confirming the amino acid identities at positions 1-5. NCBI database search combining the PMF (MS) and LIFT-TOF/TOF(MS/MS) spectra, resulted in a very significant hit for the chicken AvBD2 with a MASCOT score of 117 (p=3.3e$^{-6}$). Since six amino acids 'CHFG[G/R]C' (positions 8-13), with either 'Gly (G) or Arg (R) substitution [G/R] in the 12th position, are conserved in all AvBD2 peptides of chicken, turkey, duck, ostrich, and zebra finch [24,26-28] (Figure 1), we reasoned that the 8-13 sequence tag is most probably, similar in the pheasant. Placing 'Arg (R)'at position 12 matched the MALDI tryptic fragment ion *m/z* 820.5 corresponding to 'GSCHFGR' position 6-12 (Figure 3 and Table 1). Assembling all of the above information of different stretches of amino acids, the pheasant AvBD2 peptide sequence was deduced to be 'LFCKRGSCHFGRCPSHLIKVGSCFGFRSCCKWPWNA'. This sequence was consistent with the accurate mono-isotopic mass of oxidized and CAM pheasant peptide. The predicted sequence information was further verified by MALDI-ISD fragmentation using both intact oxidized and reduced/alkylated (CAM) pheasant AvBD2 peptides, observing the expected $c_n$ and $z+2_n$ fragment ions 7 through 29 (Figure S6 and Table S1).

The MALDI tryptic PMF of quail AvBD2 showed no obvious mass match with either chicken or turkey's, except a superficial pattern similarity. The peak at *m/z* 956.5 (Figure 3b) was dominant in quail similar

to *m/z* 929.4 in pheasant, chicken, and/or turkey, which corresponds to the stretch of amino acid sequences between positions 20-27. Presuming the observed mass difference was related to mutation in some amino acids between positions 20-27, a MALDI LIFT-TOF/TOF fragmentation of the *m/z* 956 peptide yielded $y_1$-$y_5$ ions corresponding to *m/z* 174.8, 321.8, 378.8, and 685.9 (± 0.5), similar to the LIFT-TOF/TOF fragment ions of *m/z* 929 (Figures 4a and 4b). This suggested that the amino acids in positions 23-27 were identical to pheasant, chicken, and turkey. Because the 'CFGFR' stretch of amino acids are conserved in most known AvBD2 peptides (Figure 1, positions 23-27), a *de novo* sequencing of the MALDI LIFT-TOF/TOF data, corresponding to the fragment *m/z* 956, yielded the most probable amino acid sequence of 'VGNCFGFR' for positions 20-27. As suggestive from the chicken, turkey, duck, and ostrich AvBD2 sequences (Figure 1), the 'VG' appears to be highly conserved and the substitution of 'N' to 'S' is possible with single base mutation. Edman sequencing of the intact quail peptide yielded a sequence of 'LF_RRGT_HFGN_PS' at the N-terminus with the missing amino acids indicated in blank, as 'Cys'. The positional information was also supported by the observed peaks at *m/z* 595 and 751 corresponding to the sequence stretches 'LFCR' (positions 1-4) and 'LFCRR' (positions 1-5), respectively (Figure 3b, Table 2) in MALDI tryptic PMF data. Comparing the tryptic PMF profile of quail's with that of pheasant (Figure 3a) and chicken AvBD2 (not shown), the peak at *m/z* 674 appears to be associated with the amino acid positions 32-36 (Table 2). This tryptic fragment corresponds to the C-terminus sequence 'WPWDA' ion which is 1 Da higher than the tryptic peptide fragment *m/z* 673, 'WPWNA', present in both chicken and pheasant AvBD2 (Figure 3a, Tables 1 and 2). An identical C-terminus sequence 'WPWDA' is also present in turkey [21,29] (Figure 1) that may relate to a single point mutation resulting from change of Asp (D) to Asn (N). Similar to the pheasant AvBD2, the C-terminus of the intact quail AvBD2 also showed two sets of multiply charged ESI-MS spectra (Figure S5c), due to consecutive loss of 'Asp-Ala (DA)'. Extrapolating from chicken, turkey, duck, and ostrich AvBD2 sequences (Figure 1) along with pheasant AvBD2 sequence data, the positions 28-30 (SCC) seem to be well conserved, and the amino acids at positions 19 and 31 is either a 'Lys (K)' or 'Arg (R)'. Collectively, the information thus far lead to a tentative sequence for quail AvBD2 as, 'LFCRRGTCHFGNCPS)(XXX)(R/K)(VGNCFGFRSCC)(R/K)WPWDA', with positions 16-18 stretch of amino acids undetermined, shown as 'X', with one of the positions at 19 or 31 being either a 'Lys (K)' or 'Arg (R)' (R/K). The use of 'R' at position 31 following 'SCC' appears consistent with the observed tryptic fragments, *m/z* 1237 (positions 28-36) and *m/z* 2175 (Figure 3b and Table 2), leading to the assignment of amino acids for positions 20-36 as 'VGNCFGFRSCCRWPWDA'. In most known AvBD2 sequences 'R' or 'K' at position 19, appears to be conserved (Figure 1) with 'Ile (I)'occurring as the preceding amino acid. It is also interesting to note that amino acids 'IK', at positions 18,19, seem to be predominant in

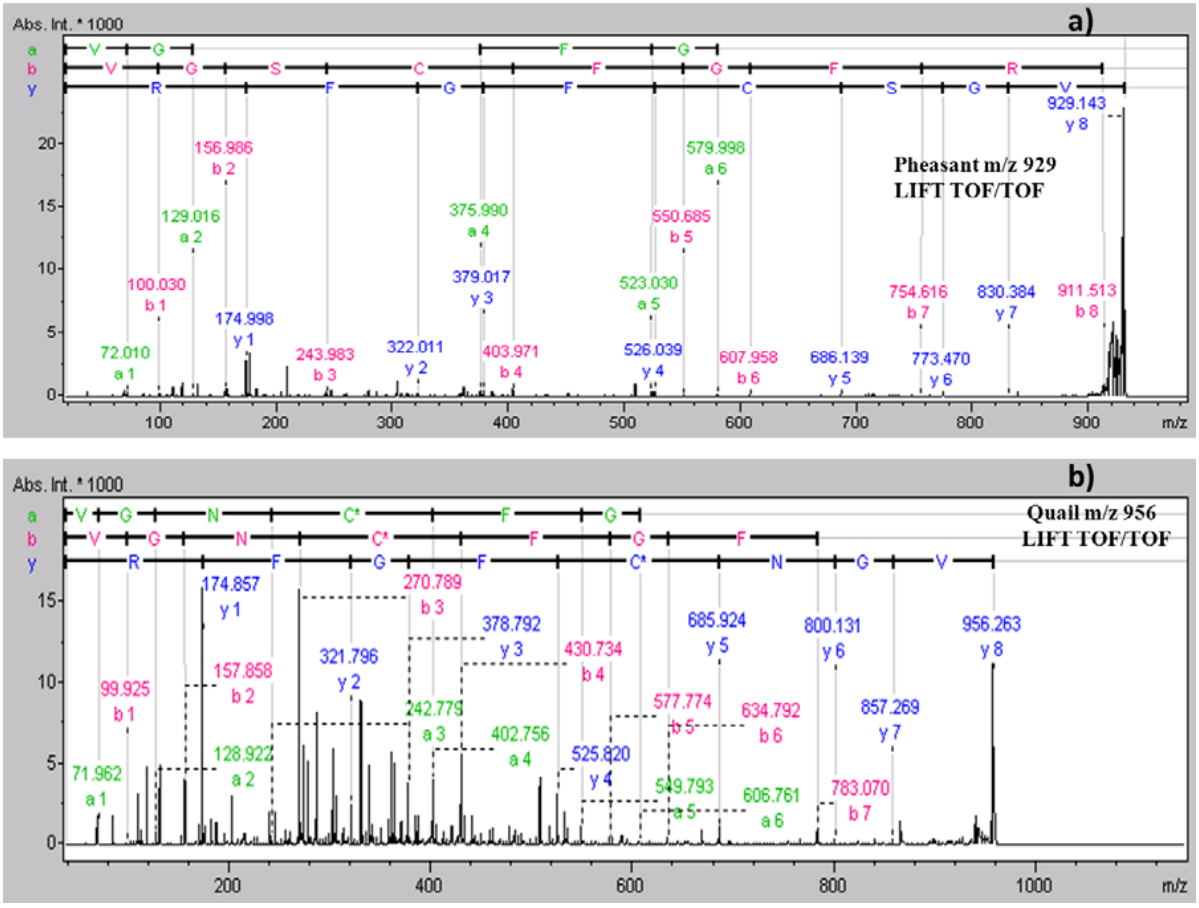


**Figure 4:** MALDI LIFT-TOF/TOF spectra of pheasant *m/z* 929 quail *m/z* 956of tryptic peptides showing a, b, and y ions.

| MH+ | Sequence range | Partial | Sequence |
|---|---|---|---|
| 595.4 | 1-4 | 0 | LFCR 3: CAM (C) |
| 751.5 | 1-5 | 1 | LFCRR 3: CAM (C) |
| 1777.1 | 5-19 | 1 | RGTCHFGNCPSDQIK 4: CAM (C) 9: CAM(C) |
| 1621.1 | 6-19 | 0 | GTCHFGNCPSDQIK 3: CAM (C) 8: CAM(C) |
| 956.7 | 20 - 27 | 0 | VGNCFGFR 4: CAM (C) |
| 1237.8 | 28 - 36 | 1 | SCCRWPWDA 2: CAM (C) 3: CAM (C) |
| 2175.3 | 20 - 36 | 2 | VGNCFGFRSCCRWPWDA 4: CAM (C) 10: CAM (C) 11: CAM(C) |
| 674.3 | 32 - 36 | 0 | WPWDA |

**Table 2:** MALDI-TOF-MS observed tryptic peptide ions from quail AvBD2 digest.

most known AvBD2 sequences. In case of quail peptide, 'IK' at positions 18-19 also, satisfy the intact protonated ion mass with either for 'Asp-Gln (DQ)' or 'Glu-Asn (EN)' placed in positions 16,17. No other permutation including substitution of Lys (K) by Arg (R) at position 19 was able to satisfy the observed protonated monoisotopic mass of 4160.8 ± 0.2 (oxidized) and *m/z* 4509 ± 0.2 (CAM). The amino acid substitutions with a single base mutation, using an universal genetic code permitting the mutations of the amino acids 'HL' in most known AvBD2, therefore, would be 'His (H)' with 'Asp (D)' and' Leu (L)' with 'Gln (Q)'at positions 16,17, respectively. Hence, the most logical combination of amino acid sequence for positions 16-19 appears to be 'DQIK'. Assembling all the above information, the sequence for mature quail AvBD2 'LFCRRGTCHFGNCPSDQIKVGNCFGFRSCCRWPWDA' is derived. Positional mutations are consistent with the observed MALDI PMF peaks at *m/z* 1621, corresponding to the sequence stretch of 'GTCHFGNCPSDQIK' (positions 6-19) and *m/z* 1777 corresponding to the sequence stretch of 'RGTCHFGNCPSDQIK'(positions 5-19) (Table 2). MALDI-ISD data of the CAM and intact oxidized quail AvBD2, shown in table S1 and figure S7, confirm this amino acid sequence observing the $c_n$ and $z_{n+2}$ fragment ions from 7 through 29.A multiple sequence alignment of both, pheasant and quail AvBD2 peptides show significant identities (>80%) with other mature AvBD2 sequences (Figure S8).

## Conclusion

In conclusion, our results with pheasant and quail AvBD2 shows that by using mass spectrometry approach and comparative bioinformatics it is possible to prospect and characterize functional peptides in their biological niches. Significant molecular homologies of AvBD2 among different avian species may allow developing reagents that can be broadly useful to monitor avian health, immunity, and welfare, and better understand their role in physiological processes other than their microbiocidal effects.

### Acknowledgements

We thank Martha Scharlau, Dept of Chemistry for N-terminal sequencing and Scott Zornes, and Sonia Tsai for help.

### References

1. Gucinski AC, Dodds ED, Li W, Wysocki VH (2010) Understanding and exploiting Peptide fragment ion intensities using experimental and informatic approaches. Methods Mol Biol 604: 73-94.
2. Han X, Aslanian A, Yates JR 3rd (2008) Mass spectrometry for proteomics. Curr Opin Chem Biol 12: 483-490.
3. Hummon AB, Amare A, Sweedler JV (2006) Discovering new invertebrate neuropeptides using mass spectrometry. Mass Spectrom Rev 25: 77-98.
4. Tinoco AD, Saghatelian A (2011) Investigating endogenous peptides and peptidases using peptidomics. Biochemistry 50: 7447-7461.
5. Chaurand P, Caprioli RM (2002) Direct profiling and imaging of peptides and proteins from mammalian cells and tissue sections by mass spectrometry. Electrophoresis 23: 3125-3135.
6. Kannan L, Rath NC, Liyanage R, Lay JO Jr (2007) Identification and characterization of thymosin beta-4 in chicken macrophages using whole cell MALDI-TOF. Ann N Y Acad Sci 1112: 425-434.
7. Kannan L, Rath NC, Liyanage R, Lay JO Jr (2009) Direct screening identifies mature beta-defensin 2 in avian heterophils. Poult Sci 88: 372-379.
8. Kannan L, Liyanage R, Lay JO Jr, Rath NC (2009) Evaluation of beta defensin 2 production by chicken heterophils using direct MALDI mass spectrometry. Mol Immunol 46: 3151-3156.
9. Derache C, Labas V, Aucagne V, Meudal H, Landon C, et al. (2009) Primary structure and antibacterial activity of chicken bone marrow-derived beta-defensins. Antimicrob Agents Chemother 53: 4647-4655.
10. Boman HG (1991) Antibacterial peptides: key components needed in immunity. Cell 65: 205-207.
11. Martin E, Ganz T, Lehrer RI (1995) Defensins and other endogenous peptide antibiotics of vertebrates. J Leukoc Biol 58: 128-136.
12. Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. Nat Immunol 6: 551-557.
13. van Dijk A, Veldhuizen EJ, Haagsman HP (2008) Avian defensins. Vet Immunol Immunopathol 124: 1-18.
14. Lynn DJ, Higgs R, Gaines S, Tierney J, James T, et al. (2004) Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. Immunogenetics 56: 170-177.
15. Rath NC, Huff GR, Balog JM, Huff WE (1998) Fluorescein isothiocyanate staining and characterization of avian heterophils. Vet Immunol Immunopathol 64: 83-95.
16. Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, et al. (2003) A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal Bioanal Chem 376: 952-965.
17. Debois D, Bertrand V, Quinton L, De Pauw-Gillet MC, De Pauw E (2010) MALDI-in source decay applied to mass spectrometry imaging: a new tool for protein identification. Anal Chem 82: 4036-4045.
18. Fukuyama Y, Iwamoto S, Tanaka K (2006) Rapid sequencing and disulfide mapping of peptides containing disulfide bonds by using 1,5-diaminonaphthalene as a reductive matrix. J Mass Spectrom 41: 191-201.
19. Hardouin J (2007) Protein sequence information by matrix-assisted laser desorption/ionization in-source decay mass spectrometry. Mass Spectrom Rev 26: 672-682.
20. Quinton L, Demeure K, Dobson R, Gilles N, Gabelica V, et al. (2007) New method for characterizing highly disulfide-bridged peptides in complex mixtures: application to toxin identification from crude venoms. J Proteome Res 6: 3216-3223.
21. Brockus CW, Jackwood MW, Harmon BG (1998) Characterization of beta-defensin prepropeptide mRNA from chicken and turkey bone marrow. Anim Genet 29: 283-289.
22. Evans EW, Beach GG, Wunderlich J, Harmon BG (1994) Isolation of antimicrobial peptides from avian heterophils. J Leukoc Biol 56: 661-665.
23. Xiao Y, Hughes AL, Ando J, Matsuda Y, Cheng JF, et al. (2004) A genome-wide screen identifies a single beta-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics 5: 56.
24. Harwig SS, Swiderek KM, Kokryakov VN, Tan L, Lee TD, et al. (1994) Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. FEBS Lett 342: 281-285.
25. Sugiarto H, Yu PL (2006) Identification of three novel ostricacins: an update

on the phylogenetic perspective of beta-defensins. Int J Antimicrob Agents 27: 229-235.

26. Soman SS, Arathy DS, Sreekumar E (2009) Discovery of Anas platyrhynchos avian beta-defensin 2 (Apl_AvBD2) with antibacterial and chemotactic functions. Mol Immunol 46: 2029-2038.

27. Ma D, Wang R, Liao W, Han Z, Liu S (2009) Identification and characterization of a novel antibacterial peptide, avian beta-defensin 2 from ducks. J Microbiol 47: 610-618.

28. Hellgren O, Ekblom R (2010) Evolution of a cluster of innate immune genes (beta-defensins) along the ancestral lines of chicken and zebra finch. Immunome Res 6: 3.

29. Zhao C, Nguyen T, Liu L, Sacco RE, Brogden KA, et al. (2001) Gallinacin-3, an inducible epithelial beta-defensin in the chicken. Infect Immun 69: 2684-2691.