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Characterization and functional regulation of bioactive peptides in avian macrophages and heterophils

Lakshmi Kannan
University of Arkansas

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**CHARACTERIZATION AND FUNCTIONAL REGULATION OF BIOACTIVE
PEPTIDES IN AVIAN MACROPHAGES AND HETEROPHILS**

**CHARACTERIZATION AND FUNCTIONAL REGULATION OF BIOACTIVE
PEPTIDES IN AVIAN MACROPHAGES AND HETEROPHILS**

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

LAKSHMI KANNAN
University of Madras
Bachelor of Science in Chemistry, 2002
University of Madras
Master of Science in Biotechnology, 2004

December 2009
University of Arkansas

ABSTRACT

Oligopeptides and low molecular weight polypeptides play central roles as effectors and signal transducers acting as hormones, neurotransmitters, growth factors, toxins, and antimicrobial factors that are important for the survival of the organism. Owing to the ubiquitous involvement of peptides in many key regulatory processes, we have been interested to identify native peptides in different cells and tissues and understand their functions. To conduct our studies, we used avian macrophages and heterophils as models of specialized cells which constitute central components of innate immunity. These studies involved (a) qualitative identification and characterization of the peptides associated with high intensity mass spectral peaks in macrophage and heterophil and (b) the quantitative changes in those peptides under immunomodulating effects of toll-like receptor (TLR) activators. The work presented here describes the identification of thymosin beta 4 (T β 4), an actin binding peptide, in macrophages and its modulation under TLR activation. This dissertation also includes identification of mature avian beta defensin 2 (AvBD2), an antimicrobial peptide in heterophils of 4 different avian species (chicken, turkey, pheasant and quail) and its modulation in chicken heterophils under similar conditions.

This dissertation is approved for recommendation
to the Graduate Council

Dissertation Director:

Dr. Narayan C. Rath

Dissertation Committee:

Dr. Annie M. Donoghue

Dr. Gisela F. Erf

Dr. David Mack Ivey

Dr. Jackson O. Lay Jr.

Dr. Kaiming Ye

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ACKNOWLEDGEMENTS

First, I would like to express my sincere appreciation and respect to my major advisor and mentor Dr. Narayan C. Rath for his dedicated guidance, valuable advice, friendship, endless support and encouragement throughout my program of study and research. I am very thankful and grateful to him for providing me the opportunity to work with him and I will always remember him as one of the greatest people I have ever met.

I would like to extend my profound gratitude to my co-advisors Dr. Jackson O. Lay, Jr and Dr. Gisela F. Erf for their constructive advices and suggestions to my graduate study and research. I also thank Dr. Annie Donoghue, Dr. Kaiming Ye, Dr. David Mack Ivey, Dr. Gisela F. Erf and Dr. Jackson O. Lay, Jr for serving in my Ph.D. dissertation committee.

I extend a special thanks to Dr. Rohana Liyanage for his continuous assistance and ingenious solutions to my research project.

Special thanks are due to my colleagues in USDA/ARS, PPPSRU, Dr. Gerry Huff, Dr. William Huff, Dr. Sonia Tsai, Ms. Dana Bassi, Scott Zornes, Wally McDonner, Larry Altman, David Horlick and Lara Baker for their tremendous support and encouragement during the entire program.

My heartfelt thanks to Ms. Dana Bassi and Dr. Sonia Tsai for their love, care, and technical support throughout my doctoral studies. Much appreciation is also expressed to Ms. Lara Baker and Ms. Carol Frederik for all the help.

I would like to offer my most sincere appreciation to all my course instructors and several individuals who have helped me in many ways during my graduate program.

I would also like to thank Ms. Komal Singh Rasaputra and Mr. Vijay Durairaj for being wonderful lab mates.

I am very thankful to Mr. Ajith K. Puthiyaveetil for his help and support all the time. I extend my deep appreciation to my friendship family, Dr. Bronson Stilwell and Ms. Evelyn Stilwell for their love, encouragement and prayers.

My enormous debt of gratitude can hardly be repaid to my good friend and well wisher Mr. Jithesh V. Veetil, for the care and support he has provided since I have known him for last seven years. Many thanks to him for helpful discussions and suggestions he provided that helped me throughout my Masters and Ph.D Program.

Finally it would not have been possible for me to earn my degree without the unfailing support of my family, my mother Vasantha Kannan, aunt Saraswathi Munirathinam and my brother Prabhakar and his family for their unconditional love, constant encouragement and moral support.

DEDICATION

I dedicate this dissertation to my
mother Ms. Vasantha Kannan
and
aunt Ms. Saraswathi Munirathinam

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ABBREVIATIONS

ACN= acetonitrile

AMP= antimicrobial peptide

AU= arbitrary units

AvBD= avian beta defensin

BD= beta defensin

CAM= carbamidomethylation

CpG-ODN= CpG oligodeoxynucleotide

DHB= 2, 5 dihydroxy benzoic acid

DAN= 1, 5 diaminonaphthalene

DTT= dithiothreitol

ESI = electrospray ionization

FA= formic acid

FGN= flagellin

FITC= fluorescein isothiocyanate

IAA= 2- iodoacetamide

IL-1 β = interleukin 1beta

IL-6= interleukin 6

iNOS= nitric oxide synthase

ISD= in source decay

kDa= kilo Dalton

LDH= lactate dehydrogenase

LOX= loxoribine

LPS= lipopolysaccharide

MALDI= matrix assisted laser desorption ionization

MS= mass spectrometry

m/z = mass/charge

PAMP= pathogen associated molecular pattern

PGN= peptidoglycan

PI= propidium iodide

PMF= peptide mass fingerprinting

Poly (I:C)= poly (inosinic:cytidilic acid)

PRR= pattern recognition receptor

PSD= post source decay

PVDF= polyvinylidene fluoride

Q-PCR= quantitative PCR

RP-HPLC= reverse phase-high pressure liquid chromatography

RT-PCR= reverse transcription-polymerase chain reaction

T β 4= thymosin beta 4

TIC= total ion chromatogram

TFA= trifluoroacetic acid

TLR= toll-like receptor

TOF= time of flight

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LIST OF PEER-REVIEWED PAPERS

CHAPTER 2: Published

Kannan L, Rath NC, Liyanage R, Lay JO Jr. Identification and characterization of thymosin beta-4 in chicken macrophages using whole cell MALDI-TOF. *Ann N Y Acad Sci.* 1112:425-34, 2007.

CHAPTER 3: Published

Kannan L, Rath NC, Liyanage R, Lay JO Jr. Direct screening identifies mature beta-defensin 2 in avian heterophils. *Poult Sci.* 88(2):372-9, 2009.

CHAPTER 4: Submitted

Kannan L, Rath NC, Liyanage R, Anthony NB, Lay JO Jr. A direct MALDI MS study to identify avian beta defensin 2(AvBD2) from pheasant and quail heterophils. (*J Proteome Res*)

CHAPTER 5: To be submitted

Kannan L, Rath NC, Liyanage R, Lay JO Jr. Effect of Toll-like receptor activation on production of Thymosin beta 4 by chicken macrophages. (*J Leukoc Biol*)

CHAPTER 6: Published

Kannan L, Liyanage R, Lay JO Jr, Rath NC. Evaluation of beta defensin 2 production by chicken heterophils using direct MALDI mass spectrometry. *Mol Immunol.* 46(15):3151-6, 2009.

INTRODUCTION

Cellular identities are often determined by a repertoire of proteins and peptides which make up their structural components and govern their specialized functions. Therefore, despite genomic unity amongst different cell and tissue types of an organism they emanate proteomic diversities. In recent years there has been major emphasis in proteomics to study and understand the individual proteins, their modifications, and functions in relation to development, differentiation, metabolism, and disease. While significant developments have taken place in the analysis of proteins, particularly using 2D gel electrophoresis, similar developments for the analysis of low molecular weight peptides are lagging behind. It is because peptides are less amenable to 2D analysis and conventional proteomic procedures. Oligopeptides and low molecular weight polypeptides play central roles as effectors and signal transducers acting as hormones, neurotransmitters, growth factors, toxins, and antimicrobial factors that are important for the survival of the organism. Owing to the ubiquitous involvement of peptides in many key regulatory processes, we have been interested to identify native peptides in different cells and tissues and understand their functions. We have used avian heterophils and macrophages as models of specialized cells which constitute central components of innate immunity to conduct our studies. These cells share many common features such as they respond to similar microbial stimuli, exhibit chemotaxis, engulf microbial pathogens, and produce many identical cytokines and metabolites to maintain immune homeostasis. Yet, these cells also differ in many ways with respect to their morphology, physiology, longevity, priming adaptive immunity, and also in the pattern of their response to inflammatory stimuli. To understand the similarities and differences in the

peptide profiles of these cells, we used matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry which has proved to be a formidable tool to analyze biomolecules. Using whole cells or their extracts, the studies in this dissertation involves both qualitative and quantitative characterization of molecules associated with high intensity mass spectral peaks in macrophage and heterophils. Thus, the study in this dissertation is divided into two categories with specific objectives in each. The categories are: (A) qualitative identification and characterization of the peptides associated with high intensity mass spectral peaks in macrophage and heterophil and (B) study the quantitative changes in those peptides under immunomodulating effects of toll-like receptor (TLR) agonists which activate these cells. The work presented here describes identification of thymosin beta 4 (T β 4), an actin binding peptide in macrophages and its modulation by toll-like receptor activation, and the identification of mature avian beta defensin 2 (AvBD2), an antimicrobial peptide in heterophils of 4 different avian species and its modulation in chicken heterophils under similar conditions.

Based on the two categories, the dissertation is divided into six chapters including the literature review. Chapters 2-4 submit to the identification and characterization of differentially associated peptides such as thymosin beta 4 and avian beta defensin 2 in avian macrophages and heterophils respectively, and the chapters 5 and 6 include the regulation of these peptides by toll-like receptor activation.

Chapter 1: Peptidomic Perspective of Avian Biology: A Literature Review

Chapter 2: Identification and Characterization of Thymosin Beta 4 in Chicken

Macrophages using Whole Cell MALDI-TOF (Ann. N. Y. Acad. Sci. 1112: 424-434, 2007).

Chapter 3: Direct Screening Identifies Mature Beta Defensin 2 in Avian Heterophils (Poult. Sci. 88(2):372-9, 2009).

Chapter 4: A Direct MALDI MS Study to Identify Avian Beta Defensin 2 (AvBD2) from Pheasant and Quail Heterophils (Submitted to Journal of Proteome Research).

Chapter 5: Effect of Toll-Like Receptor Activation on Production of Thymosin Beta 4 by Chicken Macrophages (In internal review).

Chapter 6: Evaluation of Beta Defensin 2 Production by Chicken Heterophils using Direct MALDI Mass Spectrometry (Mol Immunol. 46(15):3151-6, 2009).

CHAPTER 1

PEPTIDOMIC PERSPECTIVE OF AVIAN BIOLOGY: A LITERATURE REVIEW

PEPTIDOMIC PERSPECTIVE OF AVIAN BIOLOGY: A LITERATURE REVIEW

INTRODUCTION

Peptides are strings of amino acids covalently linked through secondary amide or peptide bonds which include dipeptides, oligopeptides to as long as 100 amino acid stretch polypeptides that can be loosely classified as low molecular weight proteins (Norbert and Hans-Dieter, 2003a; Petra and Harald, 2007). The oligopeptides and polypeptides play many central roles in maintenance of homeostasis regulating many metabolic and physiological functions essential for the survival of organism (Norbert and Hans-Dieter, 2003b). For instance, tripeptide glutathione takes part in intermediary metabolism and acts as an antioxidant (Martin and Teismann, 2009). Ubiquitin which regulates many cellular processes such as protein sorting, inflammation and proteosomal degradation is a widely occurring 8.5 kDa polypeptide (Ramage et al., 1994). Many oligopeptides and polypeptides serve as signaling and growth factors acting as hormones (ghrelin, urotensin, obestatin), and neurotransmitters (opioid peptides, vasopressin, angiotensins, bradykinins). These peptides function in autocrine, endocrine, or paracrine fashions (Konig, 1996). Others such as toxins and antimicrobial peptides are designed to function as defense factors protecting the organism. Most of these polypeptides are produced or stored in specific cells and tissues and are released upon physiological exigencies. The instantaneous actions of peptides are presumably suited through their low molecular mass that allows their easy access into cell environments to participate in physiological mechanisms. The peptides are classified as ribosomal, non-ribosomal, and peptide fragments, depending on how they are produced (Petra and Harald, 2007). Most peptides

in higher organisms that play important role as effector molecules and signal transducers are ribosomal produced by the translation of mRNA. Similar to proteins, they also undergo posttranslational modifications such as phosphorylation, acylation, amidation, glycosylation, sulfonation and disulfide bond formation (Baggerman et al., 2004). By contrast, non-ribosomal peptides are synthesized by modular enzyme complexes called *nonribosomal peptide synthetases* and are commonly found in unicellular organisms, plants, and fungi (Schwarzer et al., 2003). Peptides are also generated by proteolysis of larger proteins during physiological or pathological processes. For example, neutrophil invasion, and metastatic cancer cell migration induce tissue proteolysis generating protein fragments (Geho et al., 2005; Romero et al., 1991). Also aberrant quantities of peptides can be produced in the course of disease processes such as in Alzheimer's (Nilsson et al., 2001) or neoplastic transformation that are likely to be found in body fluids which have been exploited as diagnostic markers for specific diseases (Schrader and Selle, 2006).

In addition to the native occurrence of many biologically active peptides in cells and tissues that function as hormones and neurotransmitters, bioactive peptides can also derived artificially from food/biological sources by proteolysis of larger proteins. These polypeptides have received much attention because of their specific biological activities and pharmacological properties as antioxidative, antimicrobial, anticancer or immunomodulatory efficacies (Fields et al., 2009; John Howl, 2009; Mine, 2007; Norbert and Hans-Dieter, 2003a). However, most cell/tissue-associated peptides are naturally occurring and are synthesized in the cell in the form of large prepro forms, which are then cleaved and modified, by selective action of peptidases to give biologically active products in their mature forms (Fricker et al., 2006; Hook, 1998). Owing to ubiquitous

involvement of peptides in many key regulatory processes, we have been working with the hypothesis that different cells and tissues exhibit their own repertoire of low molecular proteins/peptides that contribute to their specialized functions. Hence, identifying and characterizing these native mature peptides will not only provide insight into their functional physiology involving development, differentiation, metabolism, and their disease related changes but also to prospect for their bioactivities in relation to their clinical applications. Compared with mammalian species, the analysis of low molecular weight (LMW) proteins or bioactive peptides in avian cells and tissues are lagging behind. To date, very few peptides, less than 100 amino acids, have been identified in avian species most of which are largely limited to chicken. Many of these peptides belong to neuropeptide, neuroendocrine signaling pathways such as vasotocin, gonadotropin or corticotrophin releasing hormones in the brain (Takahashi et al., 1992; Troskie et al., 1997), galanin (Norberg et al., 1991), gastrin releasing peptide (McDonald et al., 1980) (Campbell et al., 1990), neuromedin-U-25 (Domin et al., 1992), neurokinin A (Conlon et al., 1988), motilin (De Clercq et al., 1996), Substance-P (Conlon et al., 1988) that have been isolated from chicken intestine. Others include host defense related antimicrobial peptides such as beta defensins and fowlicidins present in chicken myeloid, epithelial cells, and liver (Harmon, 1998; Harwig et al., 1994; Lynn et al., 2004; Xiao et al., 2006; Xiao et al., 2004). Also mineral homeostasis maintaining calcitonin and parathyroid hormones (Homma et al., 1986; Khosla et al., 1988), play central roles in avian skeletal biology. Despite identification of few such peptides there is paucity of information about the identities and functions of many other polypeptides that occur naturally in the cells of avian species. Thus, characterization of native peptides and their

changes has potential to provide better understanding under a variety of physiological and pathological conditions.

PROTEOMICS IN POST-GENOMIC ERA

Among the avian species, chicken is the first livestock animal to enter the post genomic era and has reached the status of model organism, since the publication of chicken genome sequence and the development of high-throughput tools for functional genomics (Burt, 2004; Cogburn et al., 2007; Dodgson, 2007). Functional genomics refers to the study of function and regulation of gene and gene products on a global level. Although genomic technologies such as DNA microarrays are available to study gene products in the form of mRNAs, analyzing gene expression on the transcript level and extrapolating this data to the protein or peptide level is not sufficient. That is because of the differential mRNA translation, alternative splicing or post-translational modifications of proteins/peptides. Such changes that are not apparent from the DNA sequence can be determined only by proteomic and peptidomic methodologies and thus verification of a gene product by these methods is an important step in “annotating the genome” (Cogburn et al., 2007).

Proteomics, a new discipline in functional genomics involves study of proteins in cells, tissues, or body fluids. Thus, since the sequencing of the genome, there has been increasing number of reports on proteomic studies to understand the molecular basis of chicken’s normo- and pathophysiology. Some reports include protein profiling in the facial development of chicken embryo (Mangum et al., 2005), skeletal muscles (Doherty et al., 2004), eye lens (Wilmarth et al., 2004), chicken germ line stem cells (GSCs) (Han

et al., 2009), whole organ bursa of Fabricius (McCarthy et al., 2006) and the analysis of chicken serum proteome (Huang et al., 2006). Also, different parts of chicken egg such as egg yolk plasma, vitelline membrane, calcified eggshell layer and egg white have been well characterized by proteomic analysis (Mann, 2007; Mann, 2008; Mann et al., 2006; Mann and Mann, 2008). Though proteome research in the chicken is still rudimentary stage, similar studies at the peptidomic level are under developed. Therefore, like proteins, it is necessary to understand the native peptides of cells and tissues in health and disease that also contribute to the fundamentals of chicken genome's functional capacity.

SCOPE OF PEPTIDOMICS

The term peptidomics refers to the comprehensive qualitative and quantitative analysis of LMW proteins/peptides present in the biological system, thus covering the gap between proteomics and metabolomics (Clynen et al., 2001; Schulz-Knappe et al., 2005; Soloviev and Finch, 2006). Although there has been a rapid development in proteome research over the last few decades, "peptidomics" in general, is under explored. The development of gel electrophoresis techniques for separation of proteins has been of great importance especially to the development of protein-derived drugs. However, the gel based systems have been less efficient for recovering components with molecular weights below 10 kDa, because of low resolution (Baggerman et al., 2004). Hence, research on naturally occurring low molecular weight polypeptides has not progressed sufficiently. Also, in this context it is easy to understand why conventional proteomics has focused on proteins rather than on small and mature forms of peptides. Since there are many peptides that are abundant in biological fluids, cells and tissues yet unknown,

there is a strong interest and need for rapid and less laborious approaches to identify and quantify native peptides.

Traditionally identification of peptides mostly involved separation techniques such as affinity chromatography, size exclusion chromatography, and multiple high performance liquid chromatography (HPLC) fractionation steps followed by detection via immunoassay techniques, and elucidation of primary structure by Edman sequencing. However, all of these methods for identification are considered laborious. The advent of genome projects, advancement in proteomic tools such as mass spectrometry (MS), improved separation techniques such as nanoscale HPLC, and bioinformatics has paved way for peptidomics to analyze LMW proteins/peptides in complex mixtures. Therefore, the current trend in peptidomics is largely based on MS, enabling simultaneous mass and sequence determination of peptide (Petra and Harald, 2007). A mass spectrometry is an analytical technique used to determine the molecular mass of a sample based on the mass-to-charge (m/z) ratio of charged ions. Different kinds of mass spectrometers exist and the two main technologies used for peptidomic research (both qualitative and quantitative analysis) include matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and the electrospray ionization MS coupled with reversed phase HPLC (LC-ESI-MS) (Baggerman et al., 2004; Clynen et al., 2003; deHoffman, 2003). One of the major challenges for analysis of peptides in crude mixtures such as biological fluids is the interference by lipids, ions, and other small molecules. Some of these aspects can be surmounted using fractionation procedures such as liquid chromatography, capillary electrophoresis techniques (Guerrera and Kleiner, 2005).

Qualitative analysis

Identification and characterization of targeted proteins/peptides involve two types of analysis; (a) peptide mapping or peptide mass fingerprinting which refers to the analysis of intact peptide masses generated by enzyme digestion and (b) or analysis of peptide fragment ions for similar algorithm based *in silico* digestion and or *de novo* sequencing of novel peptides (Twyman, 2004). Also, identification of peptides in cells and tissues using whole cells, tissues or their extract are gaining momentum due to the advancement in direct mass spectrometric profiling by MALDI-TOF-MS. The direct analysis include identification of the mature forms of native peptides in whole cell/tissue/organs which is also accomplished by techniques called MALDI imaging where a spatial distribution of peptides in the tissues is observed (Chaurand et al., 1999). There are several reports on such direct analysis in both invertebrate and vertebrate species which have lead to studies and discoveries of novel neuropeptides and their relation to behavior and physiology (DeKeyser and Li, 2006; Dong et al., 2009; Herring et al., 2007; Hummon et al., 2006; Li et al., 2000; Ma et al., 2009; Yuan and Desiderio, 2005). In addition to MALDI, ESI MS is another powerful technique that can fragment even femtomole amounts of peptides with high efficiency making it an ideal tool for amino acid sequencing (Clynen et al., 2003). Nano LC coupled with ESI-TOF MS, has been used to unravel peptidome of the nervous system of drosophila (Baggerman et al., 2002), the rat brain (Skold et al., 2002), and the human urine samples (Heine et al., 1997). The advancement in peptide discovery is further accomplished using tandem mass spectrometry (MS/MS) such as collision induced dissociation and post source decay, coupled to MALDI or ESI which favors structural and sequence determination of peptides because of its efficiency, accuracy and

sensitivity (Cape et al., 2009). This has led to the finding of several peptides such as insulin-related peptides (de With et al., 1997), small cardioactive peptides (Jimenez et al., 1998), and several neuropeptides (Cape et al., 2009; Ons et al., 2009).

Using such formidable tools, peptide discovery in avian species has been increasing over the years. For instance, the isolation and characterization of antimicrobial peptides from avian heterophils (Evans et al., 1994), chicken liver (Li et al., 2007), and identification of avian beta defensins from king penguin and ostrich (Sugiarto and Yu, 2006; Thouzeau et al., 2003) were accomplished using MS. Myers and Patnoy (2006) developed a new strategy utilizing electrospray-ionization mass spectrometry for the qualitative determination of gonadotropin releasing hormone (GnRH) peptides in several species including chicken I and chicken II GnRH peptide. They also demonstrated by *de novo* sequencing of intact GnRH peptides and by using ESI-MS/MS proposed adequate sequence coverage for these peptides (Myers and Patonay, 2006).

Quantitative analysis

Quantitative peptidomics is an additional dimension to peptide identification and characterization when it is required to know the peptide abundance in the sample. Similar to quantitative proteomics, the peptidomic approaches also involve mass spectrometry although it was realized that MS could not be simply used for quantification because it is not inherently a quantitative technique (Yan and Chen, 2005). However recent advances in stable isotope labeling methods, MS instrumentation, and bioinformatic analysis have facilitated platforms to obtain quantitative information about the peptide components of the cell. Comparing various conditions such as two disease states or the effects of genetic

knockout, the stoichiometry of proteins/peptides in a large complexes or monitoring changes to a proteome/peptidome over time has been accomplished (Brockmann et al., 2009; Che and Fricker, 2005; Che et al., 2006). The quantitative approaches solely based on signal intensities of spectral peaks have been difficult. However, combining sample preparations such as affinity matrices (lectins) C18 matrix based isolation of peptides free of salts and ions and the use of novel algorithm based softwares the detection of quantitative difference detection are becoming feasible (Chernokalskaya, 2006). Besides, the advent of new approaches such as stable isotope labeling of proteins/peptides have facilitated mass spectrometry based quantification and the various approaches are discussed in detail in reviews by different research groups (Bantscheff et al., 2007; Ong and Mann, 2005; Yan and Chen, 2005). Stable isotope labeling of proteins was first introduced by Gygi and his research team in 1999 using isotope coded affinity tags (ICAT) (Gygi et al., 1999). Since then, many groups have adopted the principle of this strategy creating specific mass tags using differential stable isotopes which can be introduced into proteins or peptides metabolically, chemically, or enzymatic labeling such as SILAC, ^{18}O labeling, isobaric tag for relative and absolute quantitation (iTRAQ), isotope coded protein labeling (ICPL) (Fenselau and Yao, 2009; Ong et al., 2002; Schmidt et al., 2005; Shevchenko et al., 1997; Wiese et al., 2007). By measuring the signal intensities of light and heavy isotope tagged peptides in the same spectrum, the quantitative differences can be calculated under different conditions. Metabolic labeling by stable isotope labeling of amino acids in cell culture (SILAC) was introduced by Matthias Mann's group in 2002 has been used in many studies (Ong et al., 2002; Ong and Mann, 2007). By contrast, label-free quantification correlates to the mass spectrometric

signal of intact proteolytic peptides or the number of peptide sequencing events with the relative or absolute protein quantity directly (Asara et al., 2008; Wang et al., 2008). Thus different MS-based approaches have been generated with their own strengths and weaknesses (Moritz and Meyer, 2003) and the most commonly used MS tools for quantification of proteins and peptides are ESI or MALDI. Additionally, protein array based quantification similar to DNA microarray technology, also exists to detect and quantify proteins interacting with individual target proteins however, this method does not majorly rely on MS (Stoevesandt et al., 2009). Thus, most of the MS strategies are promising because they can reveal cell/tissue associated mature and functional peptides on both qualitative and quantitative basis.

AVIAN MACROPHAGES AND HETEROPHILS

Native bioactive peptides are more often than not, are produced by cells and tissues that play active roles in homeostasis such as neural, endocrine, and immune cells. While significant developments haven taken place in the analysis of neural and endocrine peptides, similar studies with immune cells are less explored. Multicellular organisms have evolved various defense mechanisms for protection against microbial invasions. Phagocytes such as the macrophages and neutrophils are principal cells responsible for innate immunity (Scott and Frank, 2009). As the first line of defense against pathogen invasion, these cells have the ability to be mobilized for migration to the site of inflammation and counter pathogens or foreign components by producing factors to prevent their spread. In the process, these cells engulf pathogens. The macrophages also ingest apoptotic neutrophils thereby removing inflammation (Kennedy, 2009). In birds,

the heterophils (neutrophil equivalent) and macrophages are major phagocytes (Juul-Madsen et al., 2008). These cells play essential roles in all aspects of multicellular life ranging from inflammation and defense against pathogens to wound healing, development and tissue remodeling. They initiate immune response through cell signaling cascade by expression of effector molecules including chemokines, cytokines and other secretory factors such as anti-inflammatory or anti-microbial peptides (Juul-Madsen et al., 2008; Kogut et al., 2005; Kogut et al., 2006; Qureshi, 2003). The immune responses in these cells are predominantly triggered by their 'pattern recognition receptors' (PRR), which are transmembrane or cytoplasmic proteins that identify the microbial pathogens or their derived products known as 'pattern associated molecular patterns' (PAMPs) (Janeway and Medzhitov, 2002). Thus, in recent years, an increasing number of pattern recognition receptors (PRRs), has been described to participate in innate recognition of microbes, through PAMPs evoking the production of effector molecules. Different kinds of PRRs such as Toll-like receptors (Sabroe et al., 2003), NOD-like receptors (Inohara and Nunez, 2003), glucan receptors and mannose receptors exist (Apostolopoulos and McKenzie, 2001), specific to cell types. Both TLRs and NOD receptors induce signal cascade mechanisms triggering the expression of pro inflammatory and co-stimulatory molecules and are thus involved in a variety of functions in regulation of inflammatory and apoptotic responses. Other receptors such as glucan and mannose PRRs triggers endocytosis and phagocytosis of the microbe and do not rely on signaling mechanisms. At present, TLRs have been found in many species ranging from insects to mammals (Sabroe et al., 2003). In birds, so far chicken is the only species where 10 TLRs have been reported to be expressed in a wide range of tissues

(Boyd et al., 2007; Temperley et al., 2008) whereas, NOD-like, mannose and glucan receptors are not well characterized in chickens unlike mammalian genome (Juul-Madsen et al., 2008).

To understand the function of these cells, it is necessary to know the proteins and peptides involved, which are the active and functional product of most genes. Over the years, there has been considerable research at protein level in these cells as evident from the reports of different research teams that has contributed to the establishment of functional profile of the neutrophil and macrophage proteome in mammals (Gadgil et al., 2003; Kang et al., 2009; Lominadze et al., 2006; Piubelli et al., 2002). However, to date we know of no reports on proteomic studies related to avian immune cells although an immunoproteomics program was developed at Mississippi State University (MSU) College of Veterinary Medicine by Dr. Burgess and his research group in January 2002 (Burgess, 2004). While significant developments have taken place in the analysis of macrophage or neutrophil proteome, similar analysis at peptidome level is in nascent stage on global scale including mammals. In general, knowledge on the database of peptides in immunology is very limited except for certain peptides such as thymopoietin I and II in the induction of early T cell differentiation (Goldstein, 1974; Sunshine et al., 1978), thymosin α 1 in T cell differentiation (Bach et al., 1979; Deber et al., 1980) and antimicrobial peptides in host defense mechanisms. In birds, antimicrobial peptides such as avian beta defensins and fowlicidins are the widespread immune related LMW native peptides identified mostly in heterophils of chickens including few in other species of birds (Lynn et al., 2007; Sugiarto and Yu, 2006; Thouzeau et al., 2003; van Dijk et al., 2008; Xiao et al., 2004).

CURRENT AND FUTURE PROSPECTS

Over a period of time, the chicken has proved to be an outstanding model for biomedical research, especially in the field of immunology which can be exemplified by the invention of attenuated vaccines, the discovery of B cells and interferon, the first successful vaccines against a cancer, and more (Burgess, 2004). With the recent publication of the chicken genome sequence and development of functional genomics complemented with proteomics and bioinformatics, biomedical research in chicken has gained a whole new dimension. However, biologically active peptides in chicken and related species are still under explored. The advancements in proteomic technologies and bioinformatics also increase the likelihood of the identification, sequencing, and biological characterization of novel peptides that are hitherto unknown. Identification of endogenous bioactive peptides would not only be valuable for therapeutic purposes but also would help in improving poultry production, health, and welfare. For example, antimicrobial peptides can be useful for protection against pathogens, increasing food safety. Thus, the future for prospecting endogenous bioactive peptides is bright.

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CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF THYMOSIN BETA 4 IN CHICKEN MACROPHAGES USING WHOLE CELL MALDI-TOF

IDENTIFICATION AND CHARACTERIZATION OF THYMOSIN BETA 4 IN CHICKEN MACROPHAGES USING WHOLE CELL MALDI-TOF

Lakshmi Kannan^{1,2,3}, Rohana Liyanage⁴, Jackson O. Lay, Jr⁴, Narayan C. Rath¹

¹PPPSRU/Agricultural Research Service/USDA, ²Cell and Molecular Biology Program, ³Department of Poultry Science, ⁴Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701

Address of correspondence:

Narayan C. Rath, Ph.D.
Agricultural Research Service, USDA,
Poultry Science Center, University of Arkansas,
Fayetteville, AR 72701
Email: narayan.rath@ars.usda.gov
Tel: 479-575-6189
FAX: 479-575-4202

Published: Annals of New York Academy of Sciences, 1112:425-34, 2009

Manuscript Style: Full-length Research Article

Abbreviations: AU= arbitrary units; BD= below detection; DHB=2, 5 dihydroxy benzoic acid; ESI-MS = electrospray ionization mass spectrometry; LPS= lipopolysaccharide; MALDI-TOF-MS = Matrix assisted laser desorption ionization time-of-flight mass spectrometry; m/z = mass/charge; PGN= peptidoglycan; PMF = peptide mass fingerprinting; RP-HPLC= reverse phase-high pressure liquid chromatography; T β 4= thymosin beta 4; TIC= total ion chromatogram.

ABSTRACT

The aim of the study was to determine chicken monocyte- and granulocyte-associated peptides and proteins using “whole cell” matrix assisted laser desorption/ionization time-of-flight mass spectrometry and to characterize the peptides based on their abundance. The mass spectra showed a prominent peak at m/z 4963 in monocytes/macrophages but not in the granulocytes. Subsequent purification and characterization of the m/z 4963 peptide from an avian macrophage cell line HTC, revealed it to be thymosin β 4 (T β 4), an actin modulating peptide. HTC cells when treated with bacterial lipopolysaccharide and peptidoglycan to determine the modulation of T β 4 gene expression or its secretion, showed no changes.

Key words: *chicken macrophage thymosin beta 4, mass spectrometry*

INTRODUCTION

Mononuclear phagocytes and granulocytes are two major effectors of innate immunity that defend against microbes, mediate inflammatory response, and contribute to the tissue repair process. Despite an overlap of their functions that are mediated through many common cytokines, growth factors, and signal transduction agents, it is likely that these cells produce an abundance of different signatory proteins and peptides that may be of importance to their function and physiology. Thus, we have been interested in identifying protein and peptide profiles of these cells based on their occurrence and relative abundance. Mass spectrometry has emerged as a formidable tool not only to characterize biomolecules but has also been used to identify different prokaryotic cells by virtue of their molecular profiles. Matrix assisted laser desorption ionization/time of

flight mass spectrometry (MALDI-TOF MS) has been used for this purpose [1, 2]. However, the potential of this technique to study the molecular profiles of eukaryotic cells has not been much explored. To determine the applicability of this technique to differentiate between cell types we performed “whole cell MALDI” [2] on mononuclear cells and granulocytes of chicken peripheral blood. This report deals with the identification of thymosin beta 4 (T β 4) as an abundant peptide present in avian monocytes/macrophages and its regulation by bacterial lipopolysaccharides (LPS) and peptidoglycans (PGN) which modify macrophage functions.

MATERIALS AND METHODS

Whole cell MALDI of mononuclear cells, granulocytes, and macrophages

All animal procedures followed Institutional Animal Care and Use Committee guidelines. Potassium-EDTA anti-coagulated blood was collected from 3 week-old broiler chickens by cardiac puncture and subjected to density gradient centrifugation on a PolymorphoprepTM medium (Accurate Chemical Company, Westbury, NY) to isolate both mononuclear cells and granulocytes as described earlier for avian blood[3]. After two successive washings and centrifugation, the cell pellets were resuspended in RPMI 1640, to a concentration of 1×10^6 cells/ ml. Two μ l of each cell suspension was mixed with an equal volume of one molar 2,5-dihydroxybenzoic acid (DHB) in 90% methanol containing 0.1% formic acid and spotted onto a Bruker MTP 384 stainless steel MALDI target. MALDI-TOF MS analysis was performed in the mass range of 1-20 kDa using a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik GMBH, Bremen, Germany) in positive ion mode. Spectral distributions and relative intensities were the

primary criteria of selecting peaks for further investigation. Using the mononuclear cell MALDI spectrum as reference we compared it with two avian macrophage cell lines HTC [4] and HD11 [5]. All these cells gave a prominent peak at m/z 4963. Subsequent experiments including the purification and the characterization of the peptide responsible for the peak at m/z 4963 and its regulation was performed using the HTC macrophage cells grown in culture.

Reverse phase HPLC Purification and ESI Mass Spectrometry

HTC cells were extracted with 70% ethanol containing 0.2% acetic acid at concentrations of 1×10^7 cells/ml and centrifuged at $21,000 \times g$ for 10 min to obtain the supernatant. The supernatant was dried using a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, NY) and reconstituted to one tenth of its original volume with distilled water. It was then subjected to reverse phase high pressure liquid chromatography (RP-HPLC) using a Hewlett Packard (Palo Alto, CA) 1100 series HPLC instrument and monitored using a single wavelength UV detector and a Bruker Esquire 2000 quadrupole ion trap electrospray ionization mass spectrometer (ESI MS) (Billerica, MA). A Bio wide Pore C_8 reverse phase column ($4.6 \text{ mm} \times 15 \text{ cm}$, $5 \mu\text{m}$) (Supelco, St. Louis, MO) was used with a solvent flow rate of 0.7 ml/min using a 0.1% formic acid/acetonitrile gradient of 0-50% over 40 min. The ESI MS was operated in positive ion mode with the nebulizing pressure (N_2) at 2.1×10^5 Pa (30 Psi), and a drying gas temperature at 300°C with flow of 12 ml/min. The mass spectrometer was optimized at m/z 1000 with low skimmer voltage. After optimizing the HPLC separation by monitoring the effluent simultaneously at 214 nm and by ESI MS, appropriate fractions

with m/z 4963 were collected, pooled, and evaporated to dryness. This fraction was tested by MALDI TOF MS to confirm the presence and the purity of the compound.

Peptide mass fingerprinting

The HPLC fraction obtained corresponding to m/z 4963 was dissolved in 25 mM ammonium bicarbonate solution and an aliquot was subjected to trypsin digestion at 37°C for 22 h using sequencing grade recombinant trypsin (Promega, WI). The trypsin digest was then desalted and concentrated using Ziptip C₁₈ pipette tips (Millipore, Bedford, MA) according to the manufacturers suggested procedure. Two μ l of the eluted sample was mixed with an equal volume of saturated α -cyano-4-hydroxycinnamic acid in 34% acetonitrile containing 0.1% formic acid, spotted onto a stainless steel MALDI target, and analyzed by MALDI-TOF MS as described above. The peptide mass fingerprint was subjected to a NCBI data base search for identification using the MASCOT search engine (<http://www.matrixscience.com>). The search was performed using a compiled list of the 30 most abundant monoisotopic peaks in the mass spectrum of the tryptic digest.

Effects of immunomodulators on T β 4 gene expression

Two immunostimulatory agents, *Salmonella typhimurium* lipopolysaccharide (LPS) and *Streptococcal pyrogenes* peptidoglycan (PGN-PS) (Becton Dickinson, Franklin Lakes, NJ) were used to activate HTC cells and determine their effects on T β 4 gene expression and its secretion into the culture medium. HTC cells were grown in RPMI-1640 medium containing 10% fetal bovine serum for 12 h at a concentration of 2×10^6 cells/ml/well in 24 well cell culture plates. Duplicate cultures were then treated with

either phosphate buffered saline (PBS) (control) or 1 μ g LPS or 10 μ g PG-PS in PBS for an additional 12 h. At the end of incubation, the cells were centrifuged at 250 \times g and the supernatant was used to determine T β 4 concentration by a competitive enzyme immunoassay using a rabbit anti-human T β 4 antiserum (Abcam, Cambridge, MA) and human T β 4 (ProSpec-Tany TechnoGene Ltd, Israel) as the coating antigen and the standard [6, 7]. The nitrite and the IL-6 concentrations in the culture supernatant was determined using Griess reagent [8] and a B9 hybridoma bioassay[9] as described earlier [3, 4].

After removing the medium, the cells were washed once with sterile PBS and DNA free total cellular RNA was purified using RNAeasy and On-column DNA digestion kits (Qiagen Corp, Chatsworth, CA). RNA was reverse transcribed to cDNA using a Retroscript kit (Ambion, Austin, TX). Two microgram RNA equivalent of cDNA was amplified using a “hot start” Taq Polymerase Multiplex PCR kit (Qiagen Corp, Chatsworth, CA) in a 200 gradient Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) for 30 cycles. The conditions included denaturation at 94°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 1 min using primers for T β 4, interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) along with β -actin as the reference gene [10]. The primers were designed with the Primer 3 software program [11] from the coding sequences of chicken T β 4 (Genbank Accession # NM_001001315) [12], IL-6 (Genbank accession # AJ309540) [13], and iNOS (Genbank accession # U46504 [14, 15]. The primer sequences for T β 4: forward: 5'GCCGAGATCGAGAAATTTGA3'; reverse: 5'GAAGGCAATGCTTGTGGAAT 3'. The coding sequences of chicken IL-6 precursor and iNOS were, forward: 5'CTCCTCGCCAATCTGAAGTC3'; reverse:

5'TCATAGAGACGCTGCTGCCAG3'; forward: 5'AGGCCAAACATCCTGGAGGTC3'; reverse 5'TCATAGAGACGCTGCTGCCAG3' respectively. The PCR products were analyzed by 2% agarose gel electrophoresis using a low range DNA ladder as standard (Fermentas, Hanover, MD). The gels were stained using ethidium bromide and photographed. The expression of genes for IL-6 and iNOS, along with the accumulation of IL-6 and nitrite in the conditioned media were used as positive controls as the indicators of macrophage activation.

RESULTS

Whole cell MALDI of mononuclear cells, granulocytes, and macrophages

The whole cell MALDI TOF mass spectra of mononuclear cells and the HTC cells are shown in Figs. 1 and 2. There were several small peaks associated with the mononuclear cell population along with a prominent peak at m/z 4963. The macrophage cell lines HTC and HD11 showed profiles similar to mononuclear cells with a prominent spectral peak at m/z 4963 (Fig. 2). The granulocytes, on the other hand, showed a prominent peak corresponding to m/z 3915 but not 4963 (data not shown).

Reverse phase HPLC Purification of 4963 Da peptide

The reverse phase-HPLC chromatogram of acid-alcohol extract of HTC cells is shown in Fig. 3a. Based on the analysis of HPLC fraction, a peptide corresponding to m/z 4963 was observed to elute at about 10.8 min. The HPLC/ESI-MS spectrum of this peptide is shown in Fig 3b. The peaks at m/z 829, 994, 1242 and 1655 correspond to the

+6, +5, +4 and +3 charged peptide ions, respectively (Fig. 3b). An oxidized form of the peptide with m/z 4979 was a minor peak in the chromatogram.

Peptide mass fingerprinting

Attempts to determine the N-terminal sequence of the peptide with m/z 4963 by Edman degradation failed, suggesting that it may be N-terminally blocked. MASCOT database searches using the 30 most abundant monoisotopic tryptic fragments along with a single set of search parameters (4 missed cleavages, 100 ppm mass error, and N-terminus variable acetyl modification in the protein level) resulted in a statistically significant hit corresponding to T β 4 in *Gallus gallus*. The search also indicated that T β 4 contains methionine at the 6th position that can be oxidized to thymosin β 4 sulfoxide, which accounts for the peak observed at m/z 4979 (Fig. 2).

Expression of T β 4 gene

The agarose gel electrophoresis profile of PCR products showed no change in the expression of T β 4 in response to either LPS or PGN-PS. However, an increased expression of both IL-6 and iNOS genes were evident (Fig. 4). Enzyme immunoassay showed no detectable levels of T β 4 in the culture medium of either control or treated cells but the concentrations of IL-6 and nitrite increased by both LPS and PGN treatments (Table 1). The absence of any significant peak corresponding to T β 4 was also confirmed by MALDI-TOF MS using the Ziptip purified culture supernatant (data not shown).

DISCUSSION AND CONCLUSION

The foregoing results demonstrate that whole cell MALDI-TOF has the potential to provide insight into cellular characteristics and their possible functions based on the relative abundance of their signatory proteins and peptides. The results show that the peptide corresponding to m/z 4963 is indeed thymosin β 4, an evolutionarily conserved peptide that regulates actins polymerization [16, 17]. Its presence in chicken tissues was shown using hybridization of a rat T β 4 probe with chicken blood DNA [18]. However, the complete structure of the chicken peptide was not known until 2004 when Dathe and Brand-Saberi [12] predicted its sequence from the T β 4 EST nucleotide sequence. The reported peptide sequence did not suggest N-terminal modification and it also contained an additional lysine at the C terminus [12] resulting in an average mass of 5181 Da. However, our results show that the chicken T β 4 has a molecular mass of 4963 Da, identical to that of human peptide [19, 20]. While, the N-terminal peptide modification may not be apparent from nucleotide sequences, the presence of a C-terminal lysine in the chicken sequence remains puzzling.

The relative abundance of T β 4 in macrophages and the tissues rich in macrophages and lymphocytes such as the spleen, bone marrow, and lungs, [18, 21, 22] suggests that it may possibly have other physiological significance aside from its G-actin sequestering property. It has been shown that monocyte/macrophages produce T β 4, and suggested that its oxidized form, thymosin β 4 sulfoxide, has better anti-inflammatory efficacy compared with T β 4 itself [23]. Whereas intracellular up-regulation of T β 4 has been linked to cancerous and metastatic transformation of cells [24, 25] it has also been shown to promote angiogenesis, wound healing, mediate tissue remodeling, subvert

inflammation, and prevent apoptosis when administered extracellularly [26-29]. Other lines of evidence are suggestive of T β 4 having anti-microbial activity against several gram positive and gram negative bacteria [30]. These extra cellular actions of T β 4 are consistent with the functions of macrophages that not only have antimicrobial activities but are also involved in the resolution of inflammation by promoting tissue healing and angiogenesis [31]. A high level of occurrence of T β 4 in macrophages is likely to help these functions. However, the secretion of T β 4 has been controversial due to an apparent absence of a signal sequence [18, 32]. Several studies have shown the occurrence of T β 4 in wound fluids, [33] suggesting that it can not only be secreted but also actively internalized. To examine whether activation of macrophages results in T β 4 secretion and alters the expression of its gene, the HTC cells were treated with bacterial LPS and peptidoglycan. Although these treatments changed the expression of genes encoding IL-6 and iNOS along with their corresponding products, IL-6 and nitrite, there was neither any effect in the expression of T β 4 mRNA nor its concentration in the condition medium. Thus, it is likely that the T β 4 in the wound fluids may be derived from dying or dead cells or is secreted under different conditions and by other cell types as has been demonstrated by Bock-Marquette *et al* [34]. Dead macrophages and similar other cells such as the platelets, in the inflamed areas, may also provide T β 4 to initiate extracellular functions such as wound healing. In conclusion, although the physiological significance of T β 4 in macrophages is speculated, the mechanism and conditions under which it is expressed and regulated need to be determined.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Mr. David Horlick for his technical assistance.

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Table 1: Effects of lipopolysaccharide (LPS) and peptidoglycan on nitrite, IL-6, and T β 4 concentrations of HTC cell culture supernatant.

| Treatment | Nitrite $\mu\text{g/ml}$ | Interleukin-6 Units/ml | Thymosin β4 |
|------------------------------|--|-----------------------------------|-------------------------------------|
| PBS (Control) | 0.14 ± 0.06 | 0.03 ± 0.003 | BD |
| LPS (1 $\mu\text{g/ml}$) | 1.66 ± 0.03 | 1.752 ± 0.02 | BD |
| PG-PS (10 $\mu\text{g/ml}$) | 1.48 ± 0.03 | 1.618 ± 0.04 | BD |

BD= below detection

FIGURE LEGENDS

Figure 1: Whole cell MALDI-TOF mass spectrum of chicken peripheral blood mononuclear cells isolated by density gradient separation showing m/z 4963 as a major peak.

Figure 2: Whole cell MALDI-TOF mass spectrum of HTC cells showing m/z 4963 as a major peak.

Figure 3: (a) RP-HPLC trace of HTC cell extract showing the elution of 4963 Da and 4979 Da peptides. (b) Electrospray mass spectrum from the HPLC fraction collected at 10.8 min.

Figure 4: An agarose gel electrophoresis profile of RT-PCR amplicons of T β 4, IL-6, iNOS, and β -actin from HTC cells treated with either control medium (1), 1 μ g LPS (2), or 10 μ g PG-PS (3) for 12 h.

Figure 1.

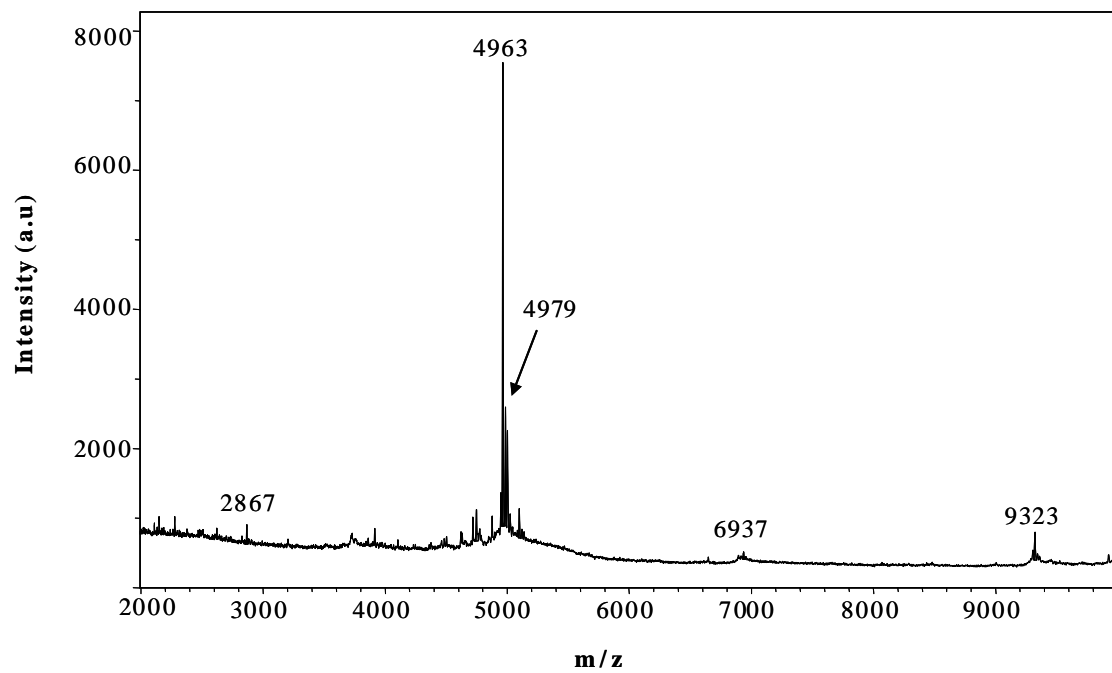


Figure 2.

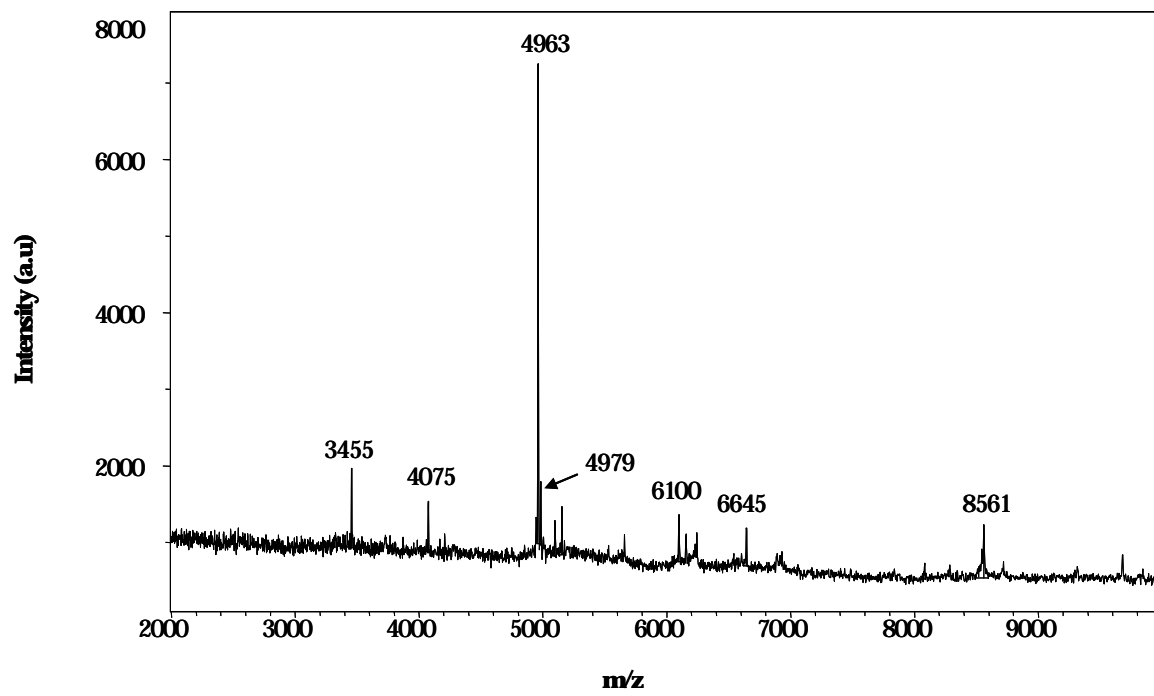


Figure 3.

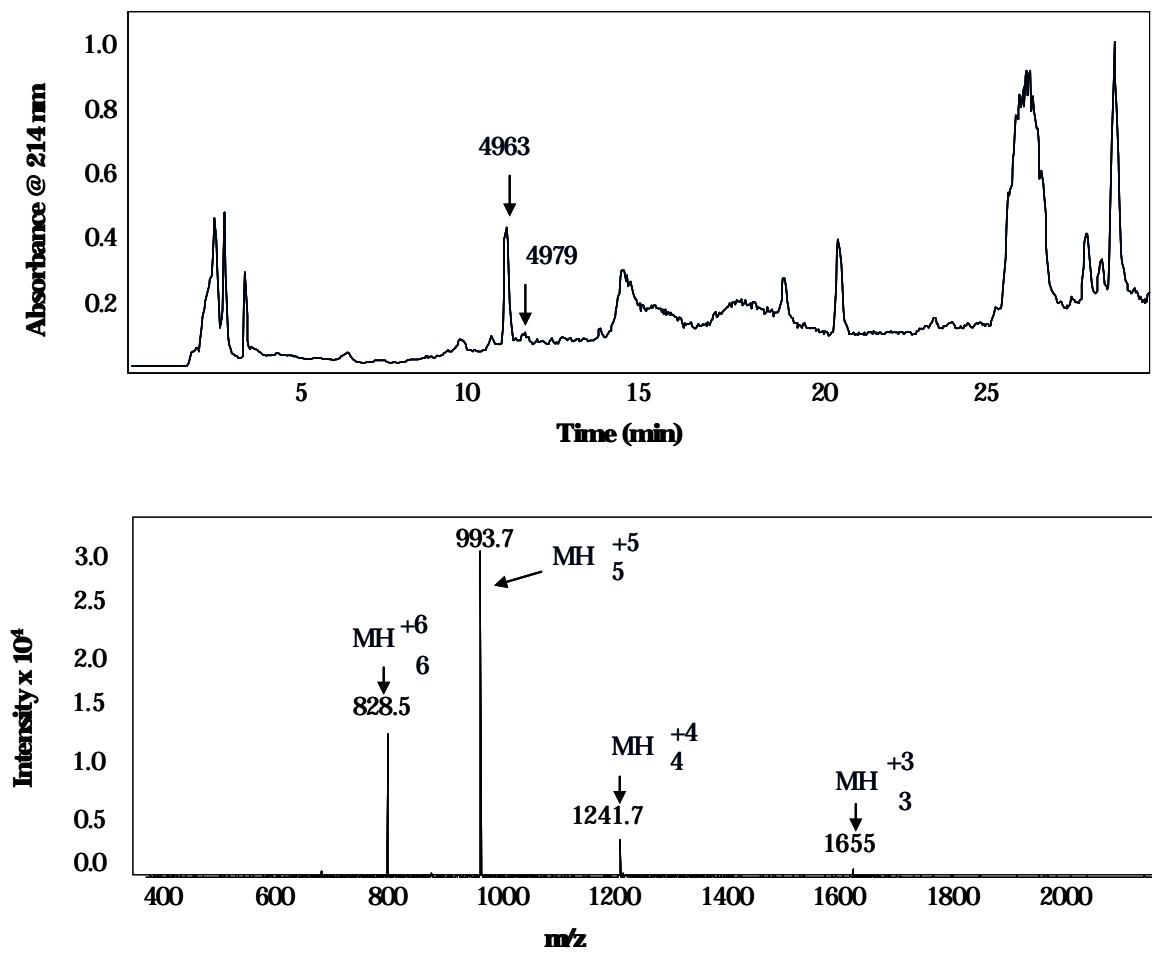
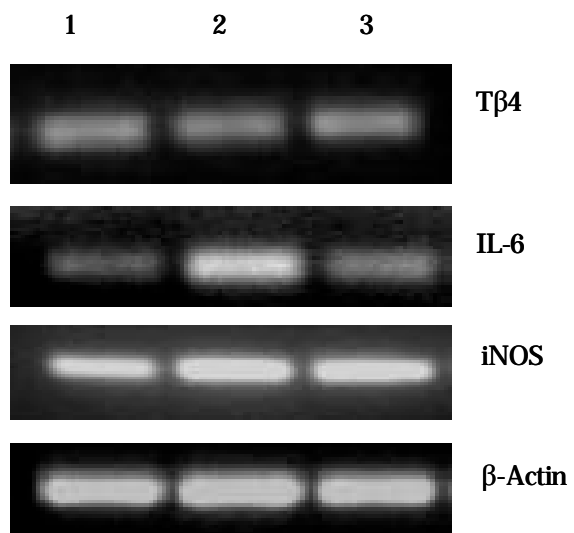


Figure 4.



CHAPTER 3

DIRECT SCREENING AND CHARACTERIZATION OF BETA-DEFENSIN 2 IN AVIAN HETEROPHILS

DIRECT SCREENING AND CHARACTERIZATION OF BETA-DEFENSIN 2 IN AVIAN HETEROPHILS

Lakshmi Kannan^{1,2}, Narayan C. Rath¹, Rohana Liyanage³, and Jackson O. Lay, Jr³

¹USDA, Agricultural Research Service, Poultry Production and Product Safety Research, ²Cell and Molecular Biology Program, ³Department of Poultry Science, ⁴Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701.

Address of correspondence:

Narayan C. Rath, Ph.D.
Agricultural Research Service, USDA,
Poultry Science Center, University of Arkansas,
Fayetteville, AR 72701
Email: narayan.rath@ars.usda.gov
Tel: 479-575-6189
FAX: 479-575-4202

Published: Poultry Science, 88(2):372-9, 2009

Manuscript Style: Full-length Research Article

Section: Molecular, Cellular and Developmental Biology

Abbreviations: AU= arbitrary units; AvBD= avian beta defensin; CAM= carbamidomethylation; DHB=2, 5 dihydroxy benzoic acid, DTT=dithiothreitol; ESI-MS = electrospray ionization mass spectrometry; FITC= Fluorescein isothiocyanate; MALDI-TOF-MS = Matrix assisted laser desorption ionization time-of-flight mass spectrometry; m/z = mass/charge; PMF = peptide mass fingerprinting; PVDF=polyvinylidene fluoride; RP-HPLC= reverse phase-high pressure liquid chromatography; TIC= total ion chromatogram; TFA= trifluoroacetic acid

ABSTRACT

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to screen avian heterophils in the m/z range of 1-20 kDa with an objective to identify the cell associated peptides that may be reflective of their functional physiology. The MALDI-TOF-MS profiles of crude heterophil extract showed a high intensity peak with average mass of m/z 3916.1 for chicken and at m/z 4129.6 for turkey respectively. To identify these peaks, we first purified m/z 3916.1 from chicken bone marrow extract using reverse phase high performance liquid chromatography (RP-HPLC). Edman sequencing and peptide mass fingerprinting exclusively confirmed this peptide as beta-defensin 2 (BD2) or gallinacin-2, a broad range antimicrobial peptide. Uniprot database search followed by the MASCOT sequence query revealed m/z 4129.6 to be the corresponding turkey ortholog of avian beta-defensin 2 (AvBD2), also called turkey heterophil peptide 2 (THP2). Both AvBD2 peptides are 36 amino acids long including a highly conserved region with 6 invariant cysteines forming three disulfide bonds characteristic of defensins. These results demonstrate that screening of the crude extract by MALDI-TOF-MS can identify cell or tissue associated peptides in their functional/mature forms. This study also confirms the existence and the complete mature peptide sequence of the turkey heterophilic BD2 previously proposed based on cDNA analysis.

Key words: *heterophils, mass spectrometry, avian beta defensin*

INTRODUCTION

Low molecular weight (MW) proteins (<20 kDa) and peptides regulate many physiological functions as hormones, neurotransmitters, antimicrobials, growth and signaling factors. It is likely that differentiated cells and tissues express many bioactive peptides, which can serve as biomarkers. Identifying and characterizing these peptides in cells and tissues can provide insight into their functional physiology involving development, differentiation, metabolism, and disease related changes. Although there has been a rapid development in peptide research over the last few decades, “peptidomics” is still under explored because small peptides are not well suited for resolution by conventional proteomic techniques such as 2D gel electrophoresis (McNulty and Slemmon, 2004). However, recent advances in mass spectrometry (MS) particularly MALDI and ESI techniques have facilitated direct screening, identification and characterization of small peptides with significant sensitivity and accuracy (Schwartz et al., 2003). MS techniques have been applied at the organismal levels to characterize prokaryotes (Fox, 2006; Lay, 2001), to study physiological and pathological changes at tissue levels (Blomqvist et al., 1999; Chaurand et al., 1999; Nelsestuen et al., 2005), and to characterize biological fluids and cellular secretions (Hida et al., 2005; Li et al., 2000; Romanova et al., 2006; Schrader and Schulz-Knappe, 2001; Thompson et al., 2006; Williams et al., 2006). MS based identification and characterization of tissue associated peptides has been extensively used to study neuropeptides from a variety of organisms (DeKeyser and Li, 2007; Desiderio et al., 2000; Schmidt et al., 2008). Using a whole cell screen, we have previously detected differential profiles of peptides in monocytes and granulocytes and identified thymosin beta4 in chicken monocytes and macrophages

(Kannan et al., 2007; Rath et al., 2007). However, characterizing such peptides and understanding their relevance to specific cell physiologies or use as biomarkers requires extensive study. Also, the homology of peptides and peptide biomarkers between related species needs more study. To explore the detection of biomarker peptides in discrete cells and related species, we screened the crude heterophil extracts of chickens and turkeys by MALDI-TOF-MS. Based on the signal intensities in the spectra from crude extracts we selected 2 peptides (one from each species) for further study. More complete characterization of these potential biomarkers was accomplished by HPLC/MS and Edman degradation to establish their identities. In this case both peptides were identified as AvBD2 from each species. It is noteworthy that the specific AvBD2s reported herein have been confirmed in chicken and from cDNA studies in turkey (Brockus et al., 1998; Evans et al., 1994; Harwig et al., 1994). This report provides experimental evidence in complete agreement with the prior studies on chicken and turkey BD2.

MATERIALS AND METHODS

Chemicals

One Step PolymorphTM medium (Accurate Chemicals Co, Westbury, NY), recombinant trypsin (Promega, Madison, WI), RPMI-1640 medium (Mediatech Inc., Herndon, VA), C18 ZipTip micropipette tips (Millipore, Bedford, MA), and K-EDTA Vacutainer tubes (BD Bioscience, Franklin, NJ) were purchased from the respective companies. All other chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Isolation of Heterophils

All animal procedures were approved by institutional animal use and care committee. Blood was obtained from three week-old broiler chickens and six week-old turkeys by cardiac puncture using K-EDTA Vacutainer tubes prior to killing. Three ml of individual blood samples were layered over 2ml PolymorphprepTM density gradient medium and spun at 500 X g for 40 min at 23°C. The density gradient medium containing granulocytes, between the top mononuclear cell layer and the bottom red blood cells, was treated per manufacturer's protocol to isolate granulocytic heterophils as described previously (Rath et al., 1998). The granulocyte preparations from chickens and turkeys were pooled separately, centrifuged to pellet the cells, washed 3 times with RPMI-1640 medium by successive centrifugation at 380 X g for 8 min each. The purity of cells was determined by staining the cytocentrifuged cells with fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Rath et al., 1998) which showed the cells to be predominantly heterophil granulocytes (Fig 1).

Chicken and Turkey Bone marrow Preparation

The birds were killed by carbon dioxide inhalation and the tibias were removed free of soft tissues and periosteum. The tips of each extremity was cut and the bones were placed in 15 ml centrifuge tubes and spun at 100 X g for 5 min at 23°C to collect the bone marrow. The bone marrows were frozen at -20⁰C until extraction.

MALDI-TOF-MS

The heterophil granulocytes (1×10^6 cells/ml) and the bone marrow (~100 mg/ml) were extracted with 70% methanol containing 0.2% acetic acid for 2 h to overnight, and centrifuged at 21,000 X g for 10 min to obtain the supernatant. Two μ l of supernatant was mixed with an equal volume of 1M 2, 5-dihydroxybenzoic acid (DHB) in 90% methanol containing 0.1% formic acid and spotted onto a Bruker MTP 384 stainless steel MALDI target. MALDI-TOF-MS analysis was performed in the spectral range of m/z 1-20 kDa in positive ion reflectron mode using a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik GMBH, Bremen, Germany). Reproducibility and signal intensity were the primary criteria for the selection of corresponding peaks to purify and characterize the peptides. Average mass values were measured in the MALDI-TOF-MS experiments on crude extracts.

Reverse Phase HPLC-ESI-MS

The chicken bone marrow extract was subjected to RP-HPLC (Hewlett 1100; Hewlett Packard, Palo Alto, CA) separation using SUPELCO C₈ column (15 cm x 4.6 mm, 5 μ m) which is coupled online to a quadrupole ion trap electrospray ionization mass spectrometer (ESI-MS) (Bruker Esquire 2000; Billerica, MA) for MS analysis. The chicken peptide m/z 3916.1 was purified at a solvent flow rate of 0.7 ml /min using a 0.1% formic acid/ acetonitrile gradient of 0-50% over 50 min period. The ESI-MS was operated in positive ion mode with the dry gas temperature of 300°C, drying gas flow of 12 ml/min and nebulizing N₂ pressure of 2.1×10^5 Pa (30 Psi). The mass spectrometer was optimized at m/z 1000 with low skimmer voltage to avoid ion fragmentation and

charge stripping. After optimizing the HPLC separation by monitoring the effluent simultaneously using total ion chromatogram and by ESI-MS, the fractions corresponding to m/z 3916.1 was collected, pooled, and evaporated to dryness using a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, NY). Homogeneities of these fractions were tested by MALDI-TOF-MS to confirm the purity of the peptides.

Automated Protein Sequencing

The RP-HPLC purified m/z 3916.1 peptide was spotted on a PVDF membrane and subjected to an automated Edman degradation using an Applied Biosystems Procise sequencer (Weiterstadt, Germany) to determine the N-terminal sequence.

Peptide mass Fingerprinting using MALDI-TOF-MS

Replicate aliquots of the purified peptide of average mass m/z 3916.1 were reconstituted in 25 mM ammonium bicarbonate and subjected to reduction and alkylation prior to digestion with recombinant trypsin. Peptide aliquots were reduced for 1 hr with 10 mM 1,4- dithiothreitol (DTT) followed by alkylation with 20 mM 2-iodoacetamide in dark for 25 min at 37°C. Excess iodoacetamide was quenched by an addition of DTT. Reduced and alkylated peptides were then digested with trypsin for 22 h at 37°C, desalted, and concentrated using C18 ZipTips as recommended by manufacturer's protocol. Two μ l of the eluted sample was then mixed with an equal volume of saturated α -cyano-4-hydroxycinnamic acid prepared in 34% acetonitrile containing 0.1% formic acid and spotted on to the MALDI target plate for MS analysis. A Bruker reflex III MALDI-TOF mass spectrometer was operated in positive ion reflectron mode and optimized in the m/z

range of 500 Da to 2 kDa. Peptide mass finger print obtained from the MALDI-TOF-MS was subjected to data base search as described below. Monoisotopic mass values were measured and used in the peptide mapping experiments.

Database Search for m/z 3916.1

Using the partial N- terminal sequence of *m/z* 3916.1, a NCBI blast search (<http://www.ncbi.nlm.nih.gov>) and a MASCOT sequence query search (<http://www.matrixscience.com>) were done to establish this peptide's identity. The tryptic peptide mass fingerprint (PMF) MASCOT search confirmed the identification of the peptide. The PMF was performed using a compiled list of 10 most abundant monoisotopic peaks in the mass spectrum. PMF search parameters at NCBI database were set to a peptide tolerance of 100 ppm with fixed carbamidomethyl modification and maximum number of missed cleavages set to one under chordate taxonomy.

Sequence Query for m/z 4129.6

Based on the chicken sequence, the Uniprot database in the ExPASy proteomic server (<http://www.expasy.org/uniprot/P46158>) was used to test for homologous sequences, in this case to determine the identity of the turkey peptide. It was verified by Mascot sequence query search using the reduced average mass 4135.7 (+6 Da for disulfides) and partial Edman sequence of the chicken peptide.

RESULTS

MALDI-TOF-MS Profiles

Table 1 shows a list of twelve most abundant peaks from the MALDI-TOF-MS spectra of chicken and turkey heterophils. Fig. 2 and 3 show the MS profiles of methanol-acetic acid extract of chicken and turkey bone marrow. The most intense peaks in the chicken and turkey bone marrow spectra were found at m/z 3916.1 and m/z 4129.6 respectively similar to their corresponding peaks observed in the MALDI-TOF-MS profile of the heterophils (Fig. 2 and 3, Table 1).

Reverse Phase HPLC Purification of m/z 3916.1 peptide

The total ion chromatogram (TIC) of methanol-acetic acid extract of the chicken bone marrow is shown in Fig. 4. The peptide corresponding to m/z 3916.1 was eluted at about 24.2 min. Corresponding ESI-MS spectrum of the peak, shown in Fig. 5, confirms the occurrence of the peptide in its four multiple-protonated species, m/z 650.9, 784.3, 979.9, and 1305.9, with charges corresponding to +6, +5, +4, and +3 respectively (Fig 5).

Edman Sequencing

Edman degradation of m/z 3916 yielded an N-terminal partial sequence of first 15 amino acids: LF_KGGS_HFGG_PS. The missing amino acid positions in between are conventionally assigned to be unmodified cysteine residues.

Identification of m/z 3916.1 peptide

NCBIInr blast search with the partial sequence LFCKGGSCHFGGCPS produced alignments with *Gallus gallus* beta-defensins 2, 3 and 1. However, the BD2 sequences showed the highest hit with an expected value (E) of 2e-08 compared with BD 3 and 1 that had an E value of 36. The E value refers to the number of matches with equal or better scores that are expected to occur by chance alone. MASCOT sequence query was done using the reduced monoisotopic peptide mass 3919.6 along with the partial N-terminal sequence LFCKGGSCHFGGCPS. The search parameters at Swissprot/NCBIInr database set at peptide tolerance 100 ppm and maximum number of missed cleavages at zero under taxonomy of chordate resulted in a statistically significant hit for *Gallus gallus* BD2.

Identification of 3916 peptide was further established by its tryptic mass fingerprint generated by MALDI-TOF-MS (Fig 6). MASCOT data base search using the tryptic mass fingerprint also resulted in a statistically significant hit for AvBD2 with a high probability MOWSE score of 112 and an E value of 5.9e-06. The peaks from the peptide mass fingerprint, m/z 929.4, 1208.5, 1613.7, 2161.9 (Fig 6), corresponded to the cysteine modified tryptic peptides, VGSCFGFR (position 20-27), SCCKWPWNA (position 28-36), GGSCHFGGCPSHLIK (position 5-19), LFCKGGSCHFGGCPSHLIK (position 1-19) respectively thus, showing a sequence coverage of 100% spanning the mature *Gallus gallus* AvBD2 peptide sequence.

Identification of m/z 4129.6 peptide

The first attempt to identify this peptide was a homology search. The Uniprot database search for chicken AvBD2 homologous peptides identified turkey heterophil peptide 2 with 90% homology. Likewise a MASCOT sequence query using its reduced average mass and partial chicken AvBD2 sequence also resulted in a hit for THP2 with a high probability MOWSE score of 235 and an E value of 3e-18. Because cDNA experiments have also predicted the same sequence for turkey AvBD2 (Brockus et al., 1998), it was evident that this peptide (THP2) was indeed with MW 4129.6. Since the peptide was identified as a homolog, additional experiments were not needed.

DISCUSSION

The study was initiated with the hypothesis that different cells may exhibit their specific profiles of peptides in a manner reflective of their physiology, and also that it might be possible to extrapolate across taxonomy. MALDI-TOF-MS analysis of crude extracts of chicken and turkey heterophils showed different spectral profiles for each population of cells. Using signal intensity as a criterion we selected two high intensity peaks, m/z 3916.1 in chicken and m/z 4129.6 in turkey, to determine their identities. The first was clearly established as chicken AvBD2 and the second was consistent with a known turkey protein, THP2, that was predicted to be AvBD2, an ortholog of chicken AvBD2 or gallinacin-2 (Brockus et al., 1998; Evans et al., 1994; Harwig et al., 1994). AvBD2 is a cationic anti microbial peptide predominantly expressed in heterophils, intestinal and reproductive tissues of the birds (Harwig et al., 1994; Hida et al., 2005; van Dijk et al., 2007; Yoshimura et al., 2006). Since heterophils originate in the bone marrow, it appears

to be a significant source of this peptide. Other than chicken and turkey, AvBD2 orthologs have been found in other species of birds such as penguins, ostriches, and pigeons (Sugiarto and Yu, 2006; Thouzeau et al., 2003; van Dijk et al., 2008). The beta-defensins, in general, have a wide spectrum anti-microbial activity and exhibit evolutionarily high conservancy such as the position specific disulfide bonds (Brogden, 2005; Sugiarto and Yu, 2004; Zasloff, 2002). In chickens approximately 14 different AvBD has been identified (Lynn et al., 2007). The identification of different chicken AvBDs in their pre-protein forms have been made by genome browsing and bioinformatics followed by gene expression studies (Higgs et al., 2005; Lynn et al., 2004; Xiao et al., 2004). Since the exact processing of pro peptide is not understood, the identity of mature peptides that have been deduced by bioinformatics approaches have led to varying masses of the mature form. For example the mature peptide for chicken AvBD2 has been reported to have different masses (Harwig et al., 1994; Higgs et al., 2005; Lynn et al., 2004; Xiao et al., 2004). In our study, the molecular mass of the mature chicken AvBD2 showed to be 3916.1 consistent with the results of Harwig et al. (1994) and the turkey peptide 4129.6 as reported (Evans et al., 1994). A second peptide with m/z 4503 present in the chicken heterophils and bone marrow extracts, also shows to be an exact match with beta-defensin 1 reported by Harwig et al (1994).

In a previous study we compared peripheral blood heterophils, mononuclear cells, and a chicken macrophage line HTC and identified the presence of thymosin b4 in chicken macrophages but not in the granulocytes (Kannan et al., 2007; Rath et al., 2007). Both AvBD1 (m/z 4503) and AvBD2 were absent in the macrophages. Although both macrophages and heterophils are derived from the bone marrow and share many over

lapping innate immune functions, they show distinctive spectral profiles by MALDI-TOF-MS suggesting that these profiles might be tissue or cell specific. While genome based bioinformatics has the advantage of predicting the structure of the whole peptide or proteins, the MALDI based approach can be a tool of discovery and identification of known as well as novel peptides possibly in their functional/mature forms. In this application MALDI-TOF-MS may be a rapid method to probe cell or tissues for physiological changes, and perhaps disease associated changes. A potential downside of direct MALDI approach is that in a typical mixture of peptides, all molecules do not ionize with equal efficiency because of the differences in amino acid composition and some sequences are suppressed by co-occurring peptides. This problem can be easily corrected by peptide fractionation using standard chromatographic approaches before MALDI. However, at this point this limitation is largely theoretical, because most related peptides will have similar spectral behavior. Moreover, the time/cost advantages of the direct analysis of cells or crude extracts suggest that this should be the method of first resort. In conclusion, our study shows that rapid screening of crude extracts by MALDI-TOF-MS can detect important homologous peptides relevant to disease or other peptidomics endpoints, even across species lines.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Ms. Marty Scharlau for peptide sequencing and thank Drs. Yuchun Du and Neil Pumford for their helpful reviews. This work was supported in part by a grant from Arkansas Biosciences Institute.

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Table 1: Average mass and relative intensities of the twelve most abundant peaks associated with the MALDI-TOF of a typical crude chicken and turkey heterophil extract.

The errors for the given masses are ± 100 ppm.

| <u>Chicken Heterophils</u> | | <u>Turkey Heterophils</u> | |
|----------------------------|----------------------|---------------------------|----------------------|
| Peaks (<i>m/z</i>) | Relative Intensities | Peaks (<i>m/z</i>) | Relative Intensities |
| 3916.1 | 1 | 4129.6 | 1 |
| 3844.9 | 0.70 | 2784.1 | 0.91 |
| 3731.2 | 0.53 | 4362.5 | 0.24 |
| 2811.2 | 0.36 | 5516.1 | 0.12 |
| 4503.9 | 0.27 | 8565.0 | 0.12 |
| 2601.6 | 0.18 | 5091.1 | 0.09 |
| 5353.4 | 0.12 | 2588.2 | 0.09 |
| 3531.2 | 0.10 | 2065.2 | 0.08 |
| 8165.7 | 0.09 | 5939.5 | 0.06 |
| 13959.9 | 0.09 | 6026.1 | 0.05 |
| 7646.8 | 0.07 | 6598.1 | 0.05 |
| 6027.1 | 0.05 | 9685.5 | 0.03 |

FIGURE LEGENDS

Figure 1: Chicken peripheral blood heterophils stained with FITC and propidium iodide (X400 magnification)

Figure 2: MALDI-TOF-MS of chicken bone marrow extract shows m/z 3916 (arrow) as a high intensity peak.

Figure 3: MALDI-TOF-MS of turkey bone marrow extract showing several peaks along with m/z 4129.1 (arrow) as the most prominent peak.

Figure 4: Total ion chromatogram of RP-HPLC of chicken bone marrow extract showing the elution of 3916 Da peptide at 24.2 min.

Figure 5: Electrospray ionization mass spectrum of the HPLC fraction collected at 24.2 min showing different protonated species of 3916 Da peptide

Figure 6: Peptide mass fingerprint of m/z 3916 after trypsin digestion. Arrows correspond to MW of tryptic peptides spanning different stretches of amino acids with carbamidomethyl (CAM) modification of the cysteine residues in the positions specified in the parenthesis.

Figure 1.

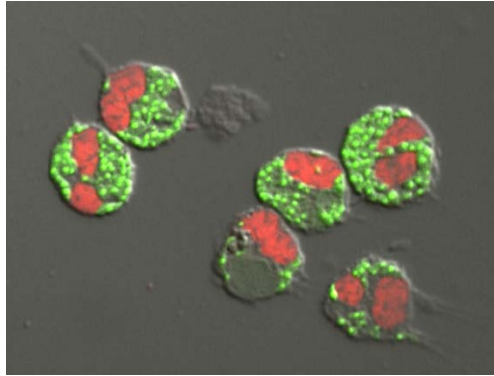


Figure 2.

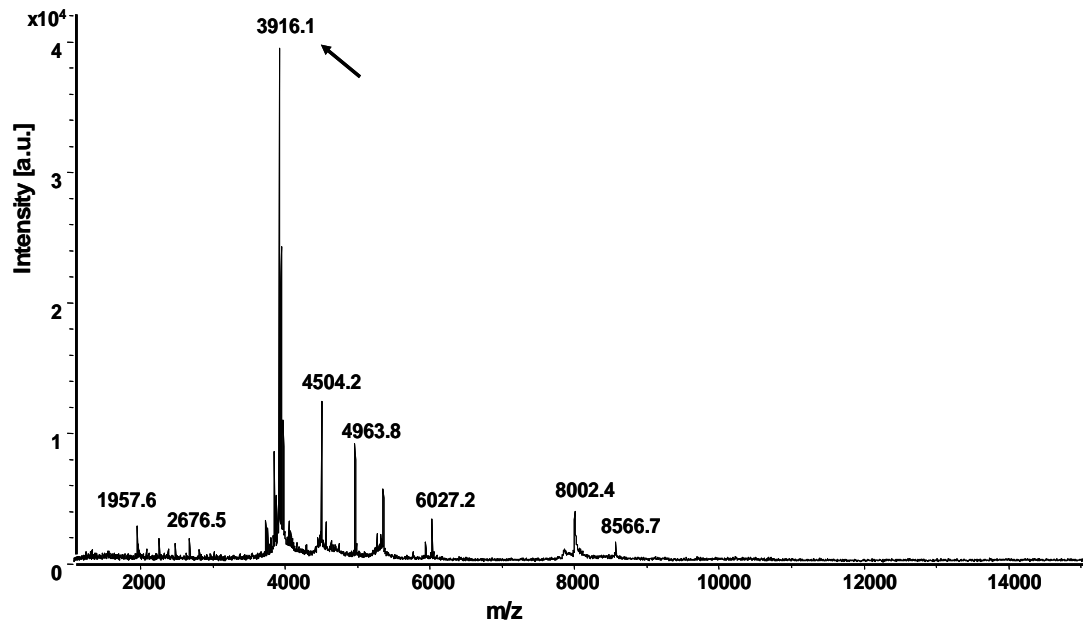


Figure 3.

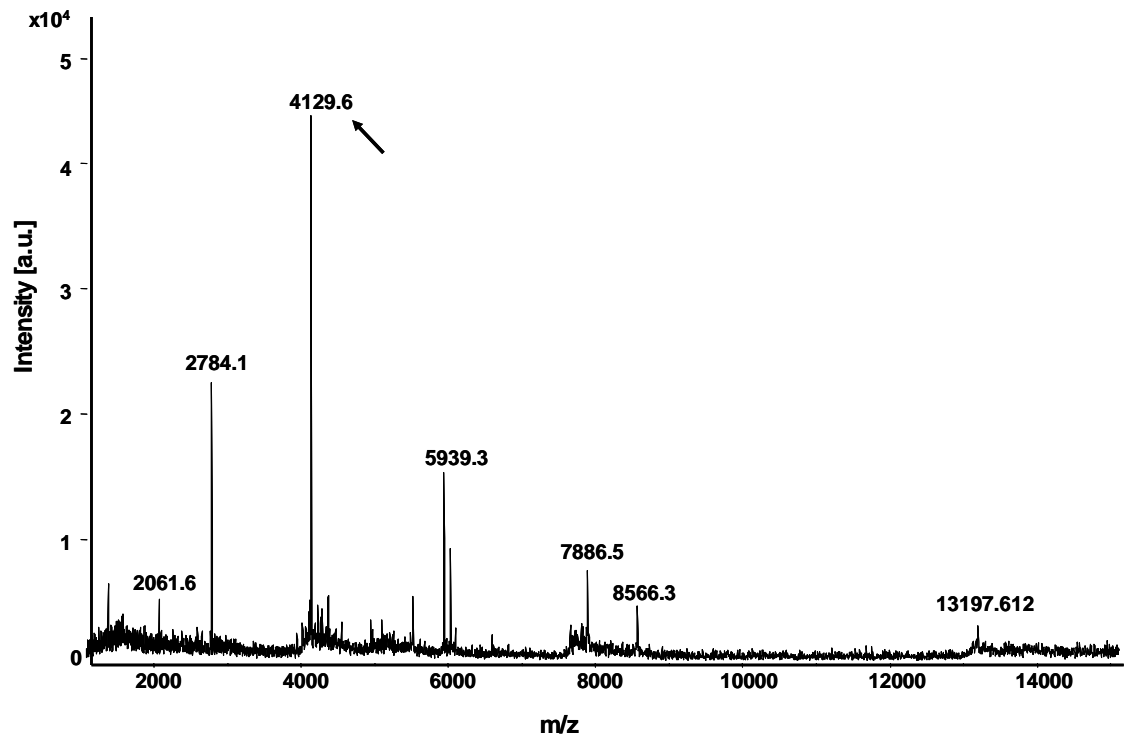


Figure 4.

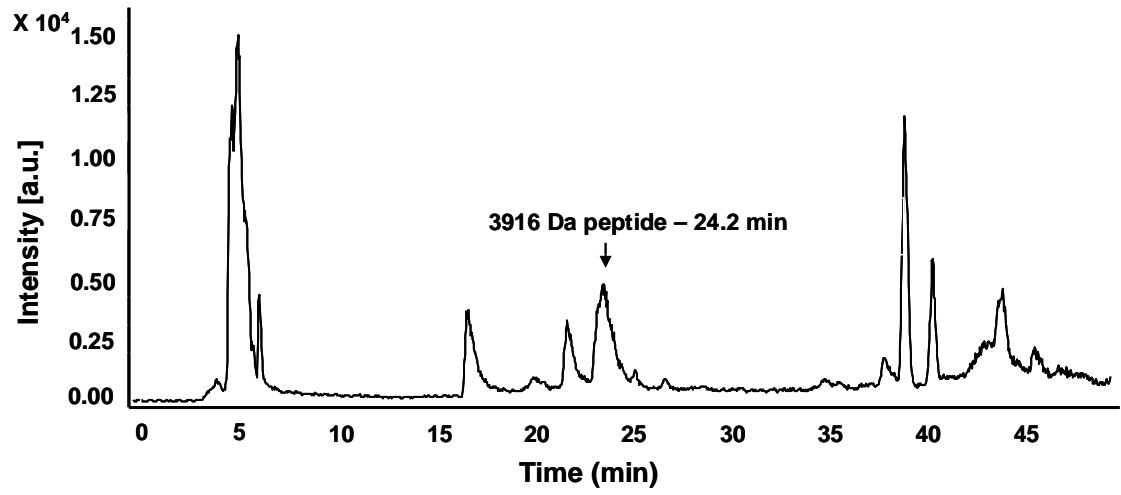


Figure 5.

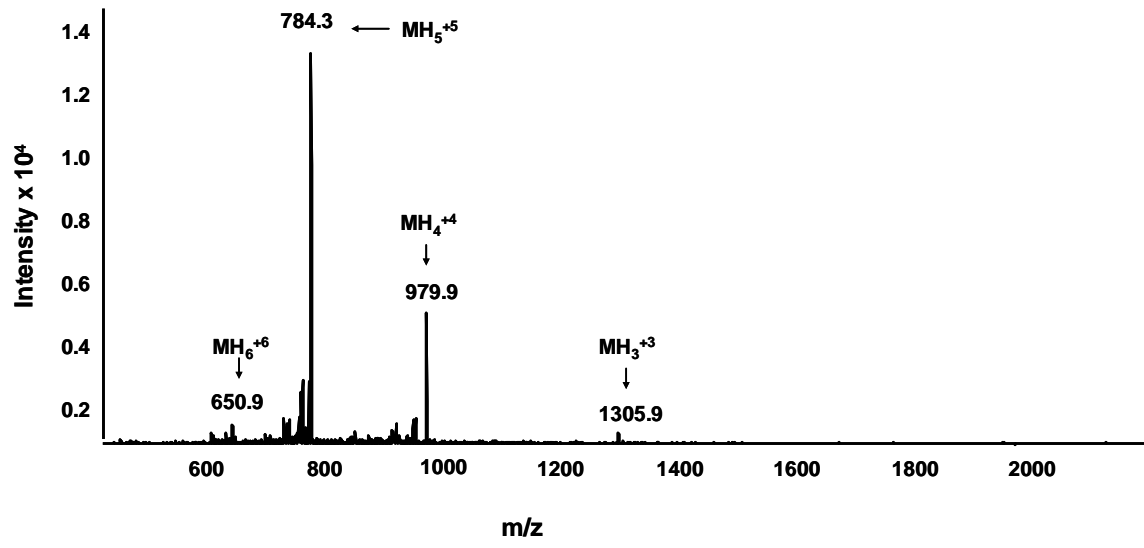
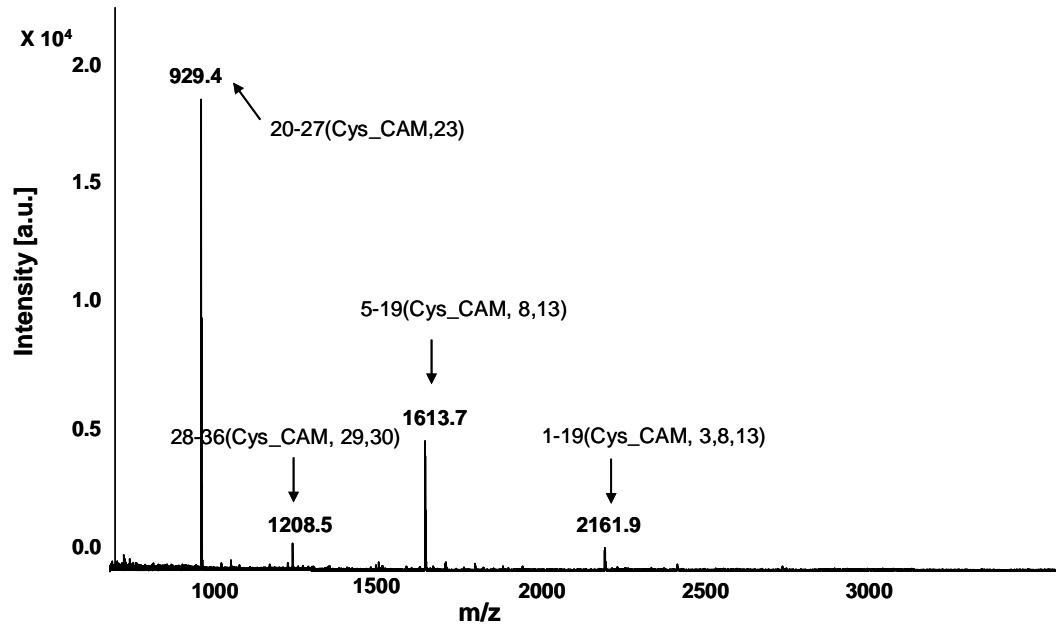


Figure 6.



CHAPTER 4

A DIRECT MALDI MS STUDY TO IDENTIFY AVIAN BETA DEFENSIN 2 (AVBD2) FROM PHEASANT AND QUAIL HETEROPHILS

A DIRECT MALDI MS STUDY TO IDENTIFY AVIAN BETA DEFENSIN 2 (AVBD2) FROM PHEASANT AND QUAIL HETEROPHILS

L. Kannan^{1,2,3}, R. Liyanage⁴, J. O. Lay, Jr⁴, N. B. Anthony³, N. C. Rath¹

¹PPPSRU/Agricultural Research Service/USDA, ²Cell and Molecular Biology Program, ³Department of Poultry Science, ⁴Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701

Address of correspondence:

Narayan C. Rath, Ph.D.
Agricultural Research Service, USDA,
Poultry Science Center, University of Arkansas,
Fayetteville, AR 72701
Email: narayan.rath@ars.usda.gov
Tel: 479-575-6189
FAX: 479-575-4202

Submitted: Journal of Proteome Research

Manuscript style: Full-length Research Article

Abbreviations: AU= arbitrary units; ACN= acetonitrile, AvBD= avian beta defensin; CAM= carbamidomethylation; 1,5 DAN= 1,5 diamionaphthalene; DHB=2,5 dihydroxy benzoic acid; DTT=dithiothreitol; ESI-MS = electrospray ionization mass spectrometry; FA= formic acid; FITC= Fluorescein isothiocyanate; IAA= Iodoacetamide; ISD= in source decay; MALDI-TOF-MS= Matrix assisted laser desorption ionization time-of-flight mass spectrometry; m/z = mass/charge; PI= propidium iodide; RP-HPLC= reverse phase-high pressure liquid chromatography; TOF = time of flight

ABSTRACT

Defensins are antimicrobial peptides containing six cysteine residues bridged internally by 3 characteristic disulfide bonds. Only beta defensin isoforms occur in birds. Avian beta defensin 2 (AvBD2) is the major isoform present in heterophils. In this study, we are reporting a direct MALDI approach to screen and identify avian beta defensins from crude heterophil and bone marrow extracts leading to the deduction of amino acid sequences for pheasant and quail AvBD2. Taking advantage of the characteristic three-disulfide bonds in defensins by reduction and alkylation, using dithiothreitol and iodoacetamide, this approach was able to differentiate these peptides from others with different numbers of disulfide bonds. Before reduction/alkylation, MALDI-TOF mass spectra of heterophil extracts of chicken, turkey, pheasant, and quail showed high intensity peaks corresponding to m/z 3916, 4129, 4114, and 4163 respectively, but after reduction/alkylation, these peaks were shifted by 348 Da consistent with the presence of six cysteine residues. Metastable ion decay studies of corresponding reduced/alkylated ions by MALDI LIFT-TOF/TOF showed the presence of a conserved stretch of 4 amino acids, 'LFCK' at the N-terminal, indicative of AvBD2. Comparing with known sequences of chicken and turkey AvBD2, the pheasant and quail peptide sequences were deduced assuming 90% homology. MALDI in source decay (ISD) fragmentation was performed for the respective peptides, purified by reverse phase HPLC from their bone marrow extracts. This was a parallel approach to verify the sequence obtained from direct MALDI by MS/MS (MALDI LIFT-TOF/TOF) which showed the sequences to be congruent.

Key words: *avian beta defensin, mass spectrometry, tandem mass spectrometry, MALDI*

Introduction

Defensins are cationic, cysteine-rich antimicrobial peptides that are one of the most evolutionarily conserved components of innate immunity occurring in many vertebrate and invertebrate species.¹⁻⁵ The interaction between positive charges on defensins and negatively charged sites on microbial membranes forms the basis of their antimicrobial effects against a broad range of bacteria, fungi, protozoa and enveloped viruses.^{2,6,7} Vertebrate defensins are classified into three main categories, namely α , β and θ defensins, based on disulfide bond pairing of 6 conserved cysteine residues as Cys1-Cys6, Cys2-Cys4, Cys3-Cys5 for α defensins, Cys1-Cys5, Cys2-Cys4, Cys3-Cys6 for β defensins and Cys1-Cys4, Cys2-Cys5, Cys3-Cys6 for θ defensins.^{4,8-10} The absence of α and θ defensins in sub mammalian vertebrates, such as birds and fish, suggests that defensin subfamilies possibly evolved from an ancestral β defensin gene by duplication, polymorphism, and diversification.¹¹ The major sources of these defensins are myeloid cells such as neutrophils (heterophils in birds) and epithelial cells.^{8,12-15}

While over 14 isoforms of beta defensins have been identified in various chicken tissues only a few have been reported from other species of birds.¹⁶⁻¹⁹ The functional and mature forms of these peptides are the result of signal and other tissue specific peptidases. Although genomics and bioinformatics have facilitated prediction of defensin structures, they cannot unambiguously predict the functional and mature forms of the peptide therefore, requiring experimental verifications. In recent years, mass spectrometry has been successfully applied to identify and characterize many cell and tissue associated peptides in several invertebrate and vertebrate species.^{20,21,22} This strategy can reveal tissue associated mature and functional peptides, sometimes even with

minimal pre-analysis sample preparation. In previous studies, using direct screening of chicken and turkey heterophil extracts, by MALDI-TOF-MS followed by HPLC purification, we identified β defensins respectively in their mature forms, which on reduction and alkylation showed a 348 Da mass shift.^{23, 24} Hence, the current study was undertaken to extend our prior work to identify AvBD2 from two other avian species, pheasant and quail, using a rapid and a simple approach to increase the specificity with minimal impact on the method's sensitivity. Reduction and alkylation was incorporated to our general MALDI approach to selectively screen peptides having 6 cysteine residues followed by mass spectrometric fragmentation to sequence these peptides. This 'top down' fragmentation was introduced in place of a relatively time consuming 'bottom up' enzymatic digestion method which involves HPLC isolation and MALDI peptide mass fingerprinting (PMF). Herein, we report for the first time the mature peptide sequence of pheasant and quail AvBD2.

Experimental Procedures

Chemicals and Reagents. One Step PolymorphTM gradient (Accurate Chemicals Co, Westbury, NY), K-EDTA Vacutainer tubes (BD Bioscience, Franklin, NJ), C18 ZipTip micropipette tips (Millipore, Billerica, MA), Bio-Rad dye binding reagent (Bio-Rad, Hercules, CA), peptide calibration standards (m/z 500-5000 Da, Bruker Daltonics, Bremen, Germany), Bio wide Pore C₈ reverse phase HPLC column (15 cm x 4.6 mm, 5 μ m) (Supelco, St. Louis, MO) were purchased from respective vendors. All other reagents including fluorescein isothiocyanate (FITC), propidium iodide (PI), 1,4-

dithiothreitol (DTT), 2-iodoacetamide (IAA), 2, 5-dihydroxybenzoic acid (DHB), 1,5-diaminonaphthalene (DAN) were purchased from Sigma Aldrich (St. Louis, MO).

Isolation of Heterophils. Heterophil granulocytes were isolated from K-EDTA anti-coagulated peripheral blood using One Step Polymorph™ gradient centrifugation.²³ The cells were washed with RPMI media successively and the granulocyte enrichment was determined for purity by staining the cells with FITC and PI.²⁵ Following bleeding, birds were euthanized and bone marrow collected from tibia as described previously.²³ Bone marrow samples were frozen at -20°C until extraction.

Sample Preparation and Direct MALDI Screening. Approximately, 1×10^6 heterophils or ~100 mg of bone marrow from chicken, turkey, pheasant and quail were homogenized in 1 mL of 70% methanol containing 0.2% acetic acid, allowed to precipitate overnight at 4°C, and centrifuged at 21,000 X g for 10 min to obtain the supernatant. For preliminary screening, the crude supernatants were mixed in equal volumes with 1M DHB prepared in 90% methanol, 0.1% formic acid. Spotting was done using the standard dried droplet method by allowing 1-2 microlitres of a 1:1 matrix/analyte mixture to air dry on a Bruker MTP 384 ground stainless steel MALDI target. Mass spectra were acquired on Reflex III MALDI-TOF (Bruker Daltonics GMBH, Bremen, Germany) operated in the positive-ion reflectron mode. Carbamidomethylation was performed by reducing the extracts for 1 hr with 10 mM 1,4-dithiothreitol (DTT) followed by alkylation with 20 mM 2-iodoacetamide in the dark for 25 min at 37°C.²⁴ High intensity mass spectral peaks showing the expected carbamidomethylation of six

cysteines, as indicated by a 348 Da mass shift (after alkylation), were tentatively identified as beta defensins and subjected to subsequent MS based sequencing.

MALDI-MS Sequencing. Presumed beta defensins were sequenced using two different MS approaches. Main approach proposed here for quick and direct analysis involved metastable parent ion selection followed by LIFT-TOF/TOF to study fragment ions produced by its gas phase unimolecular dissociation²⁶. The other method used for verification utilizes an indirect approach involving HPLC purification followed by matrix enhanced MALDI-ISD fragmentation. These experiments were performed on an Ultraflex II MALDI TOF/TOF (Bruker Daltonics GMBH, Bremen, Germany). The amino acid sequence was derived from differences between the selected parent ion's mass and the masses of the metastable ion fragments in the MS/MS spectrum. For ISD studies, peptides were first purified by reverse phase HPLC chromatography (Hewlett 1100; Hewlett Packard, Palo Alto, CA) from their respective bone marrow extracts using a method described previously.²³ The HPLC fractions corresponding to these peptides were collected and tested for purity by MALDI-TOF-MS before and after subjecting to reduction and alkylation. The experiments relying on enhanced ISD employed a saturated solution of DAN prepared in 50% ACN/ 0.1% FA as the matrix.^{27, 28} The purified and reduced/alkylated peptides were each mixed with DAN in equal volumes and spotted on the MALDI target plate. Then the MALDI-ISD mass spectra were used to verify the sequence derived from the metastable ion decay studies.

Data Analysis. Chicken and turkey AvBD2 peptide sequences^{13,29} were used as references to determine the sequences of the pheasant and quail peptides. Bruker Flex Analysis 2.4, 3.0 softwares were used to generate the peak list from the MS/MS and MS spectra. For MS/MS the average isotopic mass values were used whereas for MS with enhanced ISD the monoisotopic mass values were used for sequence determining calculations. Typically *a*, *b* and *y* ions were observed in metastable ion decay studies by MALDI LIFT-TOF/TOF (MS/MS) whereas *c* and *z*+2 ions dominated the matrix-enhanced ISD experiments. Spectra generated from reduced/alkylated forms of chicken and turkey AvBD2 were compared with their corresponding theoretical fragment ions using Bruker's Biotools 3.1 software. Ions generated from the reduced/alkylated pheasant and quail peptides were likewise compared with those of chicken and turkey AvBD2 to identify differences indicative of possible mutations in their amino acid sequences.

Results and Discussion

Screening Beta Defensin like Peptides by Direct MALDI. Direct screening of tissue extracts by MALDI-TOF-MS has been shown to be a promising strategy not only to identify and characterize novel peptides but also to distinguish cell specific proteins and peptides.³⁰⁻³⁴ Direct MALDI-TOF-MS of crude heterophil extracts from chicken, turkey, pheasant and quail, showed distinct spectral profiles attributable to their individual peptide constituents (Figure 1). Not surprisingly, bone marrow extracts from all four species also contained peaks similar to those present in the corresponding heterophil MALDI-TOF-MS profiles (data not shown). The most prominent and distinct

peaks were m/z 3916 in chicken, m/z 4129 in turkey, m/z 4114 in pheasant and m/z 4163 in quail. The chicken m/z 3916 and turkey m/z 4129 corresponded to their mature AvBD2 peptides having four amino acid differences but otherwise more than 90% sequence homology.^{13,23,29} By analogy, high intensity peaks from pheasant and quail heterophil extracts obtained under similar conditions, could likely represent respective beta defensins. A simple way to provide additional evidence regarding the identity of the peptides in these samples was selective alkylation of cysteine residues. Direct MALDI-TOF mass spectral profiles obtained for reduced/alkylated heterophil extracts of each species showed that the specific peptide peaks were shifted by 348 Da upon carbamidomethylation indicative of six cysteines, characteristic of beta defensins. Figures 2a and b compare direct MALDI-TOF-MS profiles of chicken before and after reduction/alkylation. The most intense peak at m/z 3916 (AvBD2) and the peak at m/z 4503, which corresponds to chicken AvBD1, were shifted by 348 Da. In turkey, the intense peak at m/z 4129 identified as AvBD2²³ also shifted by 348 Da (Figure 2c,d) whereas peak at m/z 2784 did not show a mass shift suggesting that it has no cysteine residues. The Figures 2e,f for pheasant and 2g,h for quail, illustrate shifts of two respective high intensity peaks by 348 Da. By analogy with chicken and turkey AvBD2, it was likely that the reduced/alkylated peaks at m/z 4463 and 4512 correspond to pheasant and quail AvBD2 respectively.

Identification of Avian Beta Defensin 2. The ions at m/z 4264, 4478, 4463 and 4512 from chicken, turkey, pheasant and quail respectively, were examined by MS/MS (MALDI LIFT-TOF/TOF) to obtain sequence information. Fragment masses from

chicken and turkey were compared with pheasant and quail to identify similarities and differences for the conserved or mutated regions in the amino acid sequences. The observed fragment ions in Table 1, for chicken and turkey AvBD2 were consistent with the known sequences of these species.

A systematic approach was used to compare certain common ions of pheasant and quail with those of chicken and turkey starting with N- and/or C- termini fragments (a_n , b_n and y_n series). For instance, fragment ions at m/z 550 and 522 were found common in all four species (Table 1). Therefore, based on the chicken and turkey sequences and conservancy with other avian species^{12, 13, 18, 19, 35, 36} these b_4 and a_4 ions correspond to the N-terminal amino acid stretch of LFCK (position 1-4), a characteristic of AvBD2 (Figure 3). Table 1 shows a series of ions in pheasant identical to y_n in chicken up to $n=24$ which indicates that the amino acid stretch from position 13 to 36 in pheasant are same as in chicken corresponding to sequence 'CPSHLIVGSCFGFRSCCKWPWNA'. The observed accurate intact monoisotopic mass difference between chicken and pheasant peptide showed 198 Da (chicken, m/z 3913.7 and pheasant, m/z 4111.8). Since the amino acid sequences in pheasant peptide of 1-4 and 13-36 of pheasant are the same as chicken AvBD2, the 198 Da mass differences could be nested in the N-terminal stretch of amino acids between 5 and 12 due to specific positional mutations. Because, the amino acids at position 6 and 8-11 are reported to be highly conserved in AvBD2, across species such as chicken, turkey, duck, and ostrich, (Figure 3), the most likely mutations in pheasant would be at positions 5, 7 or 12. Replacing glycine (G) at position 5 and 12 in chicken AvBD2 with arginine (R) matched the mass difference of 198 Da between chicken and pheasant peptides leading to a possible sequence of

'LFCKRGSCHEFGRCPSHLIKVGSCFGFRSCCKWPWNA' which was in agreement with other observed a_n and b_n ions (Table 1).

Likewise, a series of fragment ions similar to y_n in chicken were found in quail up to y_{24} , all of which were shifted by a constant mass of 49 Da (Table 1). For instance, peak at m/z 253 in quail was 49 Da higher than the y_2 ion of chicken. The peak at m/z 3118 of quail was 49 Da more than the y_{24} of chicken. This is coherent with possible mutation of asparagine (N) to tyrosine (Y) at position 35 in quail. The fact that the same series of ions, mentioned above, showed 48 Da differences compared with y_n series up to y_{24} in turkey, further supports the possible mutation of aspartic acid (D) to tyrosine (Y) transition at position 35 in quail (Table 1). As noted above, the amino acid sequence of 1-4, 6 and 8-11 are reported to be highly conserved in AvBD2 across the species. Therefore, mutations are most likely present at positions 5, 7 or 12. Replacing serine (S) at position 7 and glycine (G) at position 12 of chicken AvBD2 with glutamic acid (E) and asparagine (N) respectively, matched the exact mass of the quail peptide (m/z 4160.7) leading to a possible sequence of 'LFCKRGECHFGNCPSHLIKVGSCFGFRSCCKWPWYA'. This was further in agreement with other observed fragment ions a_n and b_n (Table 1).

The most probable mutations consistent with the data and the corresponding sequences for pheasant and quail peptides are given in Figure 4. The annotations in Figure 5 and masses listed in table 1 represent some of the observed and calculated fragment ions of chicken, turkey, and the proposed pheasant, and quail AvBD2 sequences.

Sequences predicted by MALDI LIFT-TOF/TOF were evaluated by MALDI-MS/MS fragmentation enhanced by DAN as a matrix. While matrix selection and other

approaches can be used to enhance the low level of ISD inherent in MALDI mass spectra, associating any fragment with a specific parent ion remains difficult. Therefore, the peptides were purified by HPLC and their purity was verified using MALDI-TOF-MS with DHB prior to enhancement of ISD using the DAN matrix. The use of ISD fragments in MALDI mass spectra as a means of sequencing is a ‘top down’ approach similar to metastable ion decay analysis by MALDI LIFT-TOF/TOF. The MALDI-ISD and MALDI LIFT-TOF/TOF data are complementary to one another and both provide better sequence information than a typical ‘bottom up’ enzymatic digestion method as evident from Figures 5, 6 and Table 1, 2. Although reduced/alkylated ISD fragments are used for sequencing, this approach can also be used without reduction and alkylation (Figure 7). It is customary to do reduction/alkylation to yield better fragmentation and sequence specific information for peptides containing disulfide bonds.^{27,28} As seen in Figure 6 and Table 2 (reduced/alkylated pheasant peptide) and Figure 7 and Table 2 (non reduced pheasant peptide), the ISD fragmentation appears to be more extensive after alkylation than before. However, in both these cases, most of the observed c_n and $z+2_n$ ISD fragment ions covered a wider range of the overall sequence. While ISD data presented herein, are based on the proposed sequence (Figure 4), the observations made with c_n and $z+2_n$ ions also suggests the possibility to derive the sequences directly and independently (Table 2, Figure 6 and 7). For example, c_n series ions from $n=8-12$ and $14-28$, and $z + 2_n$ series ions from $n=10-22$ were observed for reduced/alkylated form of pheasant peptide based on the sequence obtained from MALDI LIFT-TOF/TOF, (Figure 6, Table 2). This observation not only supports the proposed N-terminus sequence, 1-28, and the C-terminus sequence, 14-36 (Table 2), but also confirms the presence of amino

acid sequence tags that correspond to ‘HFGR’, ‘SHLIKVGSCFGF’ and ‘FGFCSGVKILHS’ (Figure 6). Similar observations were made for MALDI-MS/MS spectra obtained from non reduced pheasant peptide which showed c_n series ions $n=8-25$, 27, 28 and $z+2_n$ series ions $n=10-21$ and 25-28. This supports the N-terminus sequence 1-28 and C-terminus sequence 8-36 (Table 2), and proves the existence of stretches of amino acids corresponding to ‘HFGRCPSHLIKVGSCFGF’, ‘FGFCSGVKILH’, and ‘GFH’ (Figure 7). Quail MS/MS data showed c_n series ions from $n=8-17$, $z+2_n$ series ion from $n=19-27$ for reduced/alkylated form supporting N-terminus sequence 1-17 and C-terminus sequence 9-36, obtained by the MALDI LIFT-TOF/TOF. Similarly, the MS/MS data obtained for non-reduced quail peptide showed c_n series ions $n= 8-16$ and 22-26, and $z+2_n$ series ion $n=10-14$, 21, 22, and 25-28 further supporting N-terminus sequence 1-26 and C-terminus sequence 8-36 (Table 2). The combined information from MS/MS data obtained from non reduced and reduced/alkylated quail peptide confirms ‘HFGNCPSSL’ and ‘CFGF’ sequence tags. It should be noted that complementary information was obtained from both the fragmentation methods. Typical b_n and y_n ions observed in metastable ion decay studies by MALDI LIFT-TOF/TOF were derived from CO-NH bond cleavage whereas c_n and $z+2_n$ ions observed in MS/MS fragmentation were produced by NH-CH bond cleavage. Besides, MALDI LIFT-TOF/TOF method depends primarily on the mass selection of the parent ion, whereas the MS/MS requires pre MS purification that gives better fragmentation.

Conclusion

In conclusion we report the identity of two new avian AvBD2 orthologs in pheasant and quail for the first time. The AvBD2 sequences determined are: 'LFCKRGSCHEFGRCPSHLIKVGSCFGFRSCCKWPWNA' and 'LFCKRGECHFGNCPSHLIKVGSCFGFRSCCKWPWYA', for pheasant and quail respectively. This study demonstrates a direct MALDI approach to identify beta defensin like peptides in complex mixtures without any elaborate separation processes such as the 1D/2D gel electrophoresis, multidimensional HPLC chromatography, or enzymatic/ chemical digestion processes. Identification is based on the finding of 6 cysteine residues by cysteine-specific alkylation followed by the determination of the amino acid sequences by MALDI LIFT-TOF/TOF. ISD fragmentation using DAN as the MALDI matrix verified the sequences obtained from MALDI LIFT-TOF/TOF data.

Acknowledgements. Thanks are due to Sonia Tsai and Scott Zornes for technical assistance.

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Table 1. MS/MS fragmentation ions obtained by MALDI LIFT-TOF/TOF showing theoretical and observed masses of selective a_n , b_n and y_n ions of the reduced/alkylated chicken, turkey, pheasant and quail peptides.

| Ion types | Chicken | | Turkey | | Pheasant | | Quail | |
|------------|---------|--------|--------|--------|----------|--------|--------|--------|
| | Theo | Obsv | Theo | Obsv | Theo | Obsv | Theo | Obsv |
| a4 | 521.7 | 520.5 | 521.7 | 520.8 | 521.7 | 520.2 | 521.7 | 522.7 |
| a6 | 635.8 | N/D | 734.9 | 735.1 | 734.9 | 735.1 | 734.9 | 734.7 |
| a8 | 883.1 | N/D | 996.2 | 998.6 | 982.2 | 982.5 | 1024.2 | 1025.6 |
| a10 | 1167.4 | 1168.4 | 1280.5 | 1281.5 | 1266.5 | 1267.2 | 1308.6 | 1308.9 |
| a15 | 1625.9 | 1626.3 | 1838.2 | 1837.8 | 1824.1 | 1823.1 | 1824.1 | 1825.3 |
| a16 | 1763.0 | 1763.5 | 1975.3 | 1975.7 | 1961.3 | 1962.7 | 1961.2 | 1960.1 |
| a18 | 1989.3 | 1989.9 | 2201.6 | 2202.3 | 2187.6 | 2188.4 | 2187.6 | 2186.3 |
| a26 | 2872.4 | 2873.6 | 3084.7 | 3084.6 | 3070.6 | 3071.4 | 3070.6 | 3070.2 |
| a35 | 4147.8 | N/D | 4361.1 | N/D | 4346.1 | 4344.1 | 4395.1 | 4394.1 |
| b1 | 114.2 | 113.6 | 114.2 | 113.27 | 114.2 | 113.3 | 114.2 | 114.1 |
| b2 | 261.3 | 260.9 | 261.3 | 260.99 | 261.3 | 261.2 | 261.3 | 260.6 |
| b3 | 421.5 | 422.4 | 421.5 | N/D | 421.5 | 421.6 | 421.5 | 421.0 |
| b4 | 549.7 | 549.0 | 549.7 | 549.0 | 549.7 | 549.3 | 549.7 | 550.9 |
| b7 | 750.9 | N/D | 864.0 | N/D | 850.0 | 850.2 | 892.1 | 892.6 |
| b8 | 911.1 | 911.8 | 1024.2 | N/D | 1010.2 | 1010.9 | 1052.3 | 1053.6 |
| b9 | 1048.2 | 1048.9 | 1161.4 | 1162.0 | 1147.4 | 1147.5 | 1189.4 | 1188.7 |
| b10 | 1195.4 | 1196.2 | 1308.6 | N/D | 1294.5 | 1294.5 | 1336.6 | 1337.0 |
| b11 | 1252.4 | 1253.5 | 1365.6 | 1366.5 | 1351.6 | 1352.5 | 1393.6 | 1394.4 |
| b12 | 1309.5 | 1309.7 | 1521.8 | 1522.9 | 1507.8 | 1508.5 | 1507.7 | 1508.1 |
| b14 | 1566.8 | N/D | 1779.1 | 1781.0 | 1765.1 | 1767 | 1765.0 | 1765.0 |
| b15 | 1653.9 | N/D | 1866.2 | 1864.3 | 1852.2 | 1853 | 1852.1 | 1853.2 |
| b18 | 2017.3 | N/D | 2229.6 | 2230.6 | 2215.6 | 2217 | 2215.6 | 2215.4 |
| b20 | 2244.6 | N/D | 2456.9 | 2456.9 | 2442.9 | 2443 | 2442.9 | 2242.2 |
| b36 | 4246.9 | 4247.9 | 4460.2 | 4460.6 | 4445.2 | 4446.5 | 4494.2 | 4493.6 |
| y1 | 90.1 | N/D | 90.1 | N/D | 90.1 | 90.5 | 90.1 | 89.0 |

| | | | | | | | | |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|
| y2 | 204.2 | 204.4 | 205.2 | 204.3 | 204.2 | 204.3 | 253.3 | 253.3 |
| y4 | 487.5 | 487.9 | 488.5 | 488.9 | 487.5 | 487.9 | 536.6 | 538.3 |
| y7 | 962.1 | N/D | 963.1 | 963.1 | 962.1 | 964.3 | 1011.2 | 1009.5 |
| y13 | 1717.0 | 1717.4 | 1718.0 | 1718.6 | 1717.0 | 1717.9 | 1766.0 | 1765.2 |
| y14 | 1877.2 | 1877.6 | 1878.1 | 1878.2 | 1877.2 | 1877.3 | 1926.2 | 1925.1 |
| y15 | 1964.2 | 1964.6 | 1965.2 | N/D | 1964.2 | 1962.7 | 2013.3 | 2012.0 |
| y16 | 2021.3 | 2021.6 | 2022.3 | 2022.0 | 2021.3 | 2021.7 | 2070.4 | 2071.2 |
| y18 | 2248.6 | 2248.7 | 2249.6 | 2247.5 | 2248.6 | 2249.1 | 2297.7 | 2299.4 |
| y21 | 2612.1 | 2612.5 | 2613.0 | N/D | 2612.1 | 2612.9 | 2661.1 | 2660.8 |
| y23 | 2796.2 | 2796.8 | 2797.2 | 2797.9 | 2796.2 | 2796.8 | 2845.3 | 2845.3 |
| y24 | 2956.4 | 2957.0 | 2957.4 | 2958.1 | 2956.4 | 2956.9 | 3005.5 | 3005.1 |
| y25 | 3013.5 | 3014.5 | 3113.6 | 3114.1 | 3112.6 | 3113.7 | 3119.6 | 3118.9 |
| y26 | 3070.5 | 3071.8 | 3170.7 | 3171.5 | 3169.7 | 3170.6 | 3176.7 | 3176.0 |
| y27 | 3217.7 | 3218.9 | 3317.8 | 3318.6 | 3316.9 | 3318.4 | 3323.8 | 3324.7 |
| y28 | 3354.9 | 3355.8 | 3455.0 | 3456.1 | 3454.0 | 3455.3 | 3461.0 | 3461.6 |
| y29 | 3515.1 | 3516.2 | 3615.2 | 3616.3 | 3614.2 | 3614.8 | 3621.2 | 3621.4 |
| y33 | 3844.4 | 3845.0 | 4057.7 | N/D | 4042.7 | 4042.3 | 4091.7 | 4091 |
| y36 | 4264.9 | 4264.9 | 4478.2 | 4477.8 | 4463.2 | 4463.4 | 4512.2 | 4511.2 |

Theo= theoretical; Obsv= observed; N/D= not detected

Table 2. Fragmentation ions obtained by MALDI ISD showing theoretical and observed masses of selective c_n and $z+2_n$ ions of pheasant and quail AvBD2 before and after reduction/alkylation.

| Ion types | Reduced/alkylated | | | | Non reduced | | | |
|----------------|-------------------|--------|--------|--------|-------------|--------|--------|--------|
| | Pheasant | | Quail | | Pheasant | | Quail | |
| | Theo | Obsv | Theo | Obsv | Theo | Obsv | Theo | Obsv |
| c8 | 1026.5 | 1026.4 | 1068.5 | 1068.5 | 912.5 | 912.6 | 954.5 | 954.7 |
| c9 | 1163.6 | 1163.5 | 1205.6 | 1205.6 | 1049.5 | 1049.7 | 1091.5 | 1091.8 |
| c10 | 1310.6 | 1310.6 | 1352.6 | 1352.7 | 1196.6 | 1196.8 | 1238.6 | 1239.0 |
| c11 | 1367.6 | 1367.7 | 1409.7 | 1409.7 | 1253.6 | 1253.8 | 1295.6 | 1296.0 |
| c12 | 1523.7 | 1523.8 | 1523.7 | 1523.7 | 1409.7 | 1409.9 | 1409.7 | 1410.2 |
| c14 | 1780.8 | 1780.8 | 1780.8 | 1780.8 | 1609.8 | 1610.0 | 1609.7 | 1610.2 |
| c15 | 1867.9 | 1867.9 | 1867.8 | 1867.9 | 1696.8 | 1697.0 | 1696.8 | 1697.3 |
| c16 | 2004.9 | 2004.9 | 2004.9 | 2003.8 | 1833.9 | 1834.1 | 1833.8 | 1833.3 |
| c17 | 2118.0 | 2118.0 | 2118.0 | 2118.0 | 1946.9 | 1947.2 | 1946.9 | N/D |
| c22 | 2602.3 | 2602.2 | 2602.3 | N/D | 2431.2 | 2431.6 | 2450.2 | 2450.6 |
| c23 | 2762.3 | 2762.2 | 2762.3 | N/D | 2534.3 | 2534.7 | 2553.2 | 2553.5 |
| c24 | 2909.4 | 2909.3 | 2909.4 | N/D | 2681.3 | 2681.8 | 2700.2 | 2700.5 |
| c25 | 2966.4 | 2966.3 | 2966.4 | N/D | 2738.3 | 2738.8 | 2757.3 | 2757.4 |
| c26 | 3113.5 | 3113.3 | 3113.4 | N/D | 2885.4 | N/D | 2904.3 | 2904.5 |
| z+2(10) | 1349.6 | 1349.6 | 1398.6 | N/D | 1235.5 | 1235.7 | 1265.5 | 1264.9 |
| z+2(11) | 1496.7 | 1496.7 | 1545.7 | N/D | 1382.6 | 1382.8 | 1412.6 | 1412.2 |
| z+2(12) | 1553.7 | 1553.7 | 1602.7 | N/D | 1439.6 | 1439.9 | 1469.6 | 1469.1 |
| z+2(13) | 1700.7 | 1700.8 | 1749.8 | N/D | 1586.7 | 1586.9 | 1616.7 | 1616.2 |
| z+2(21) | 2595.2 | 2595.2 | 2644.2 | 2644.2 | 2424.2 | 2424.5 | 2473.1 | 2473.5 |
| z+2(22) | 2682.3 | 2682.2 | 2731.3 | 2731.2 | 2511.2 | N/D | 2560.1 | 2559.4 |
| z+2(25) | 3095.4 | 3095.3 | 3102.4 | 3102.3 | 2867.4 | 2867.7 | 2874.2 | 2874.4 |
| z+2(26) | 3152.5 | N/D | 3159.4 | 3158.3 | 2924.4 | 2923.9 | 2931.3 | 2931.3 |
| z+2(27) | 3299.5 | 3299.3 | 3306.5 | 3307.3 | 3071.4 | 3071.3 | 3078.3 | 3078.2 |
| z+2(28) | 3436.6 | N/D | 3443.5 | N/D | 3208.5 | 3209.7 | 3215.4 | 3215.1 |

Theo= theoretical; Obsv= observed; N/D= not detected

FIGURE LEGENDS

Figure 1. MALDI-TOF mass spectra of crude heterophil extracts of chicken, turkey, pheasant and quail.

Figure 2. MALDI-TOF mass spectra of non-reduced and reduced/alkylated heterophil extracts of chicken (a and b), turkey (c and d), pheasant (e and f), and quail (g and h). Arrows indicate the intact and modified forms of AvBD.

Figure 3. Mature AvBD2 peptide sequences of chicken, turkey, duck and ostrich aligned with respect to their conserved regions.

Figure 4. Proposed sequences of mature AvBD2 of pheasant and quail aligned with chicken and turkey counterparts. The highlighted amino acids are the proposed mutations.

Figure 5. MALDI LIFT-TOF/TOF MS/MS spectra of (a) chicken, (b) turkey, (c) pheasant and (d) quail showing a_n , b_n and y_n ion fragments.

Figure 6. MALDI MS ISD fragmentation profile of purified and alkylated pheasant AvBD2, showing c_n and $z+2_n$ ions.

Figure 7. MALDI MS ISD fragmentation profile of purified and non-alkylated pheasant AvBD2, showing c_n and $z+2_n$ ions.

Figure 1.

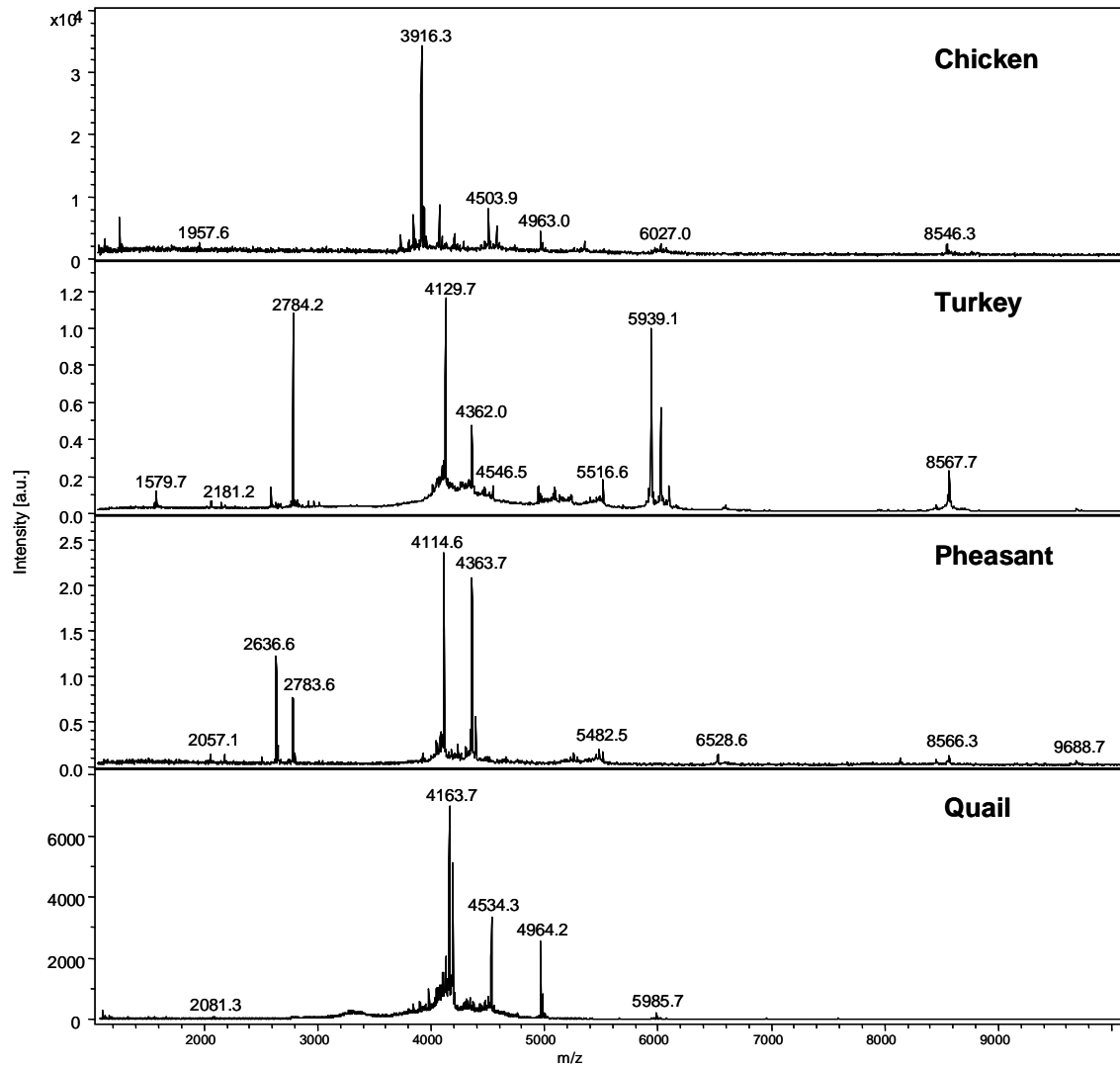
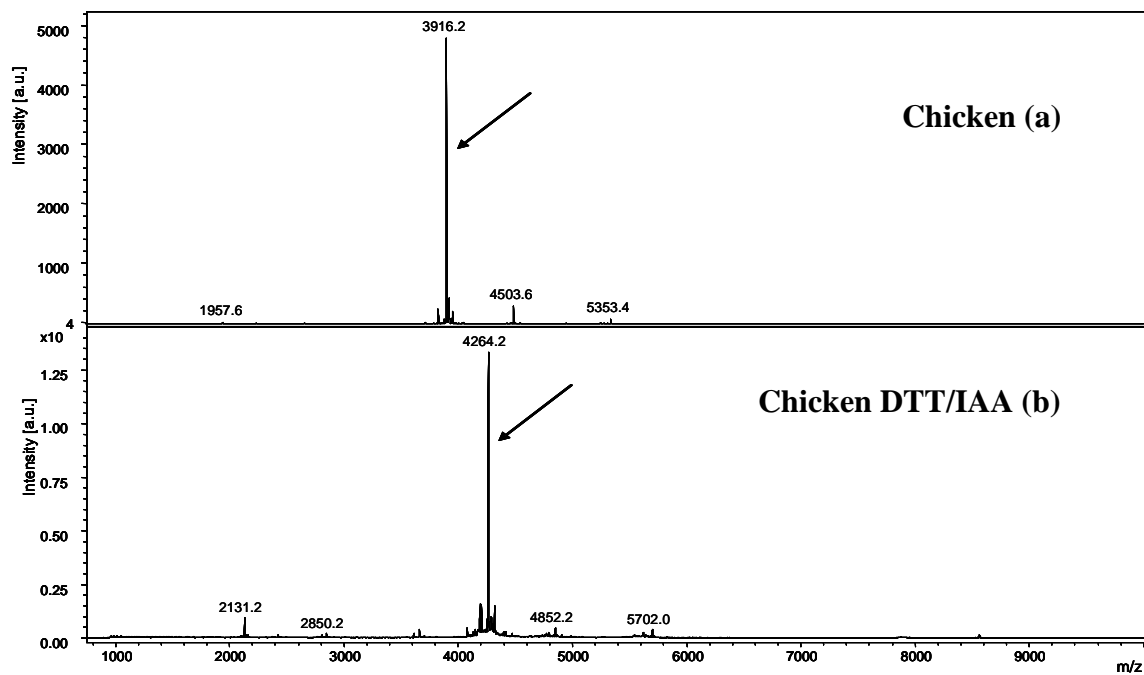
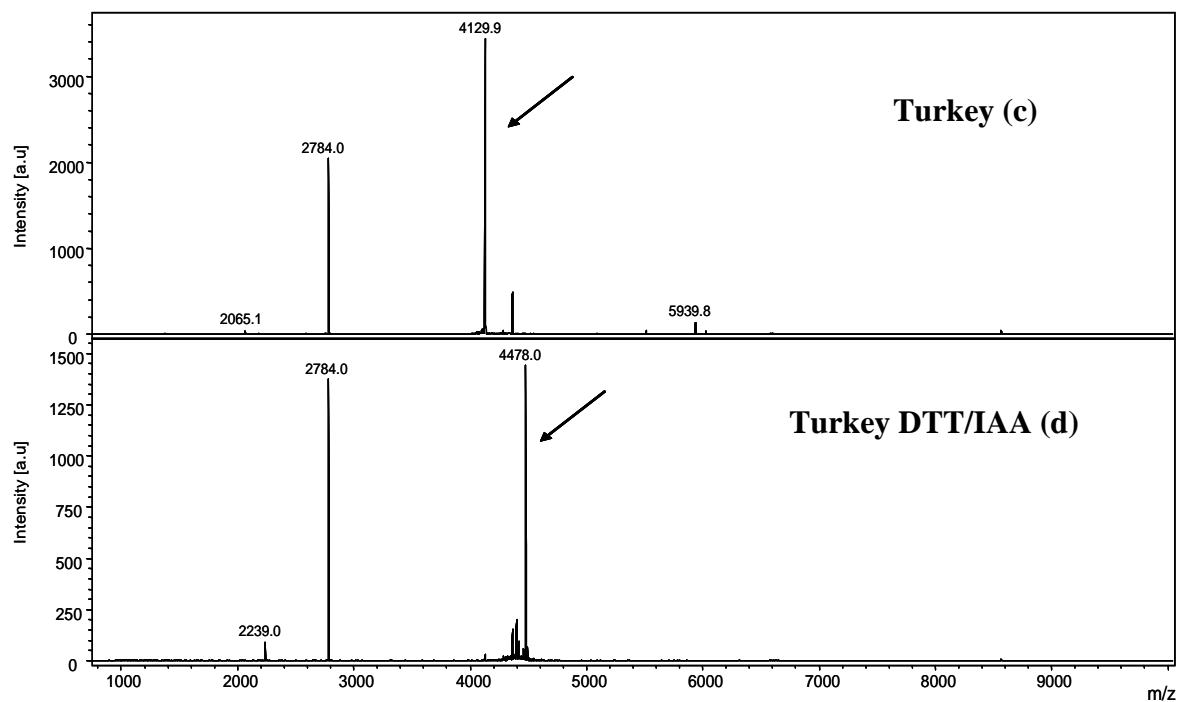
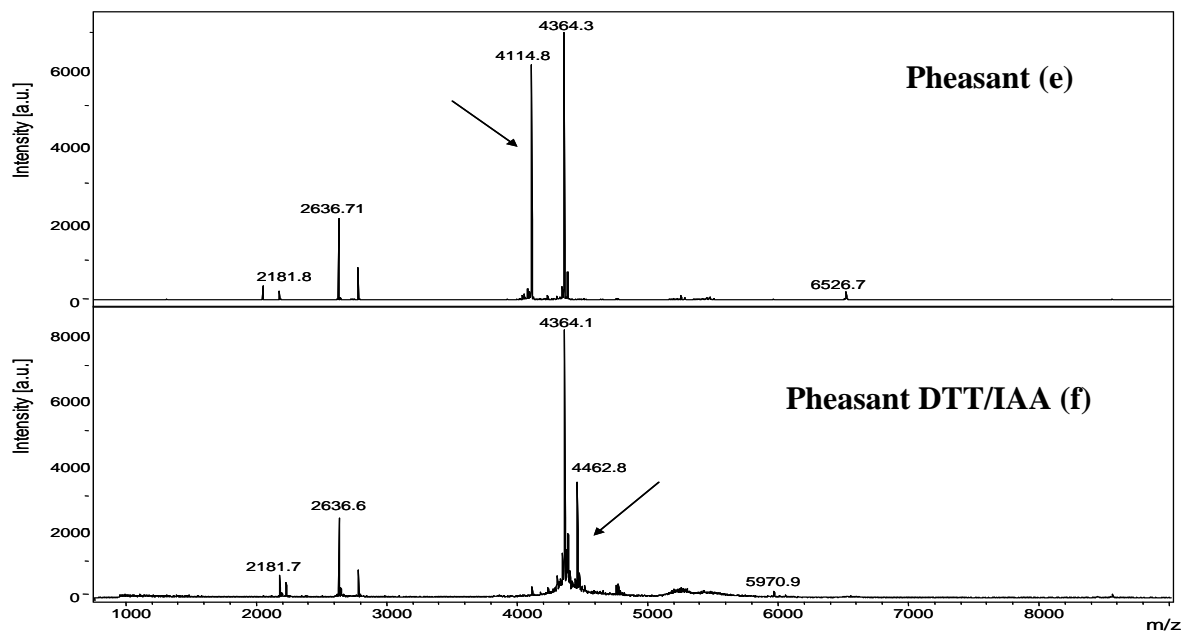


Figure 2.







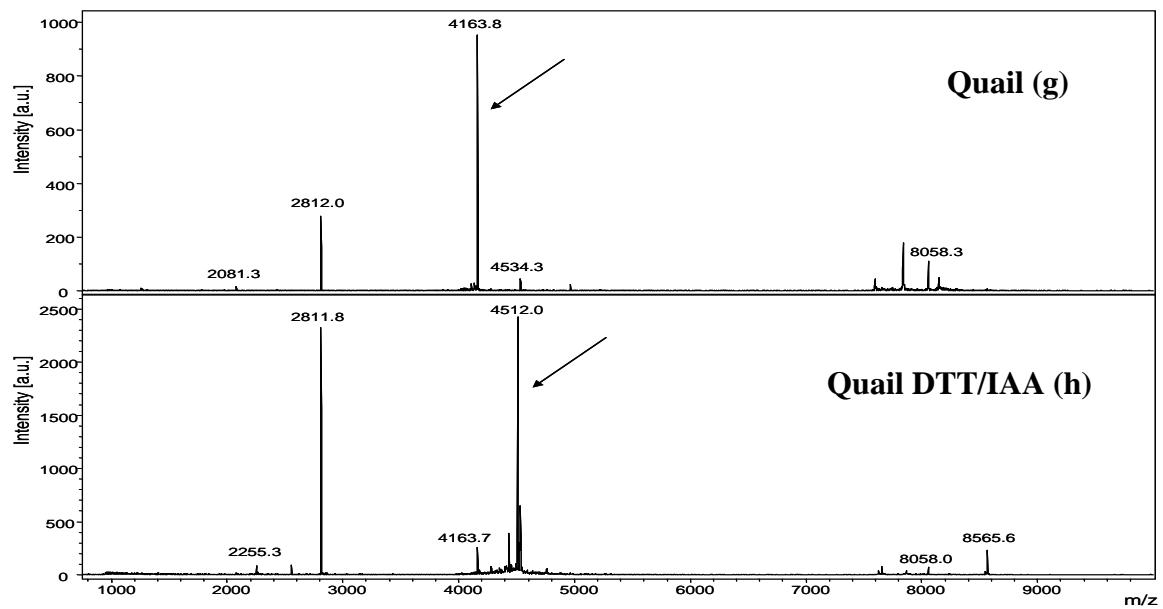


Figure 3.

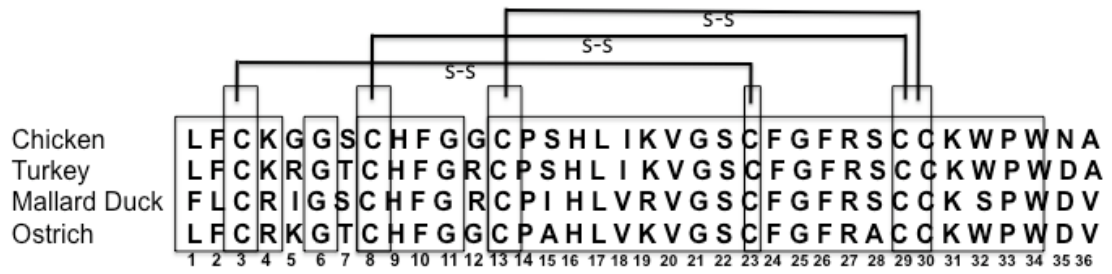


Figure 4.

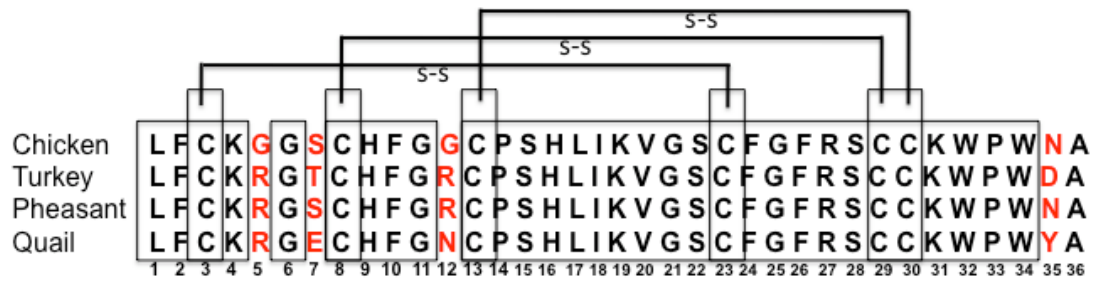
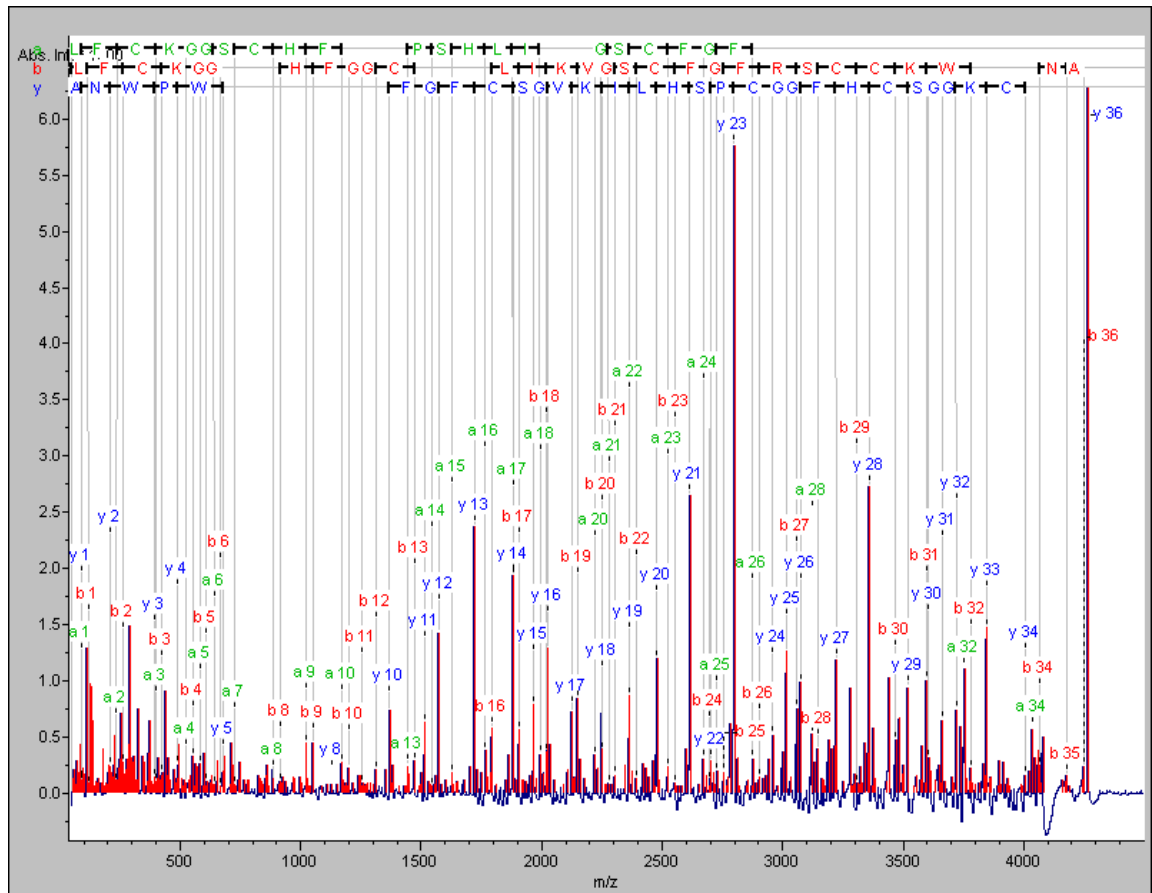
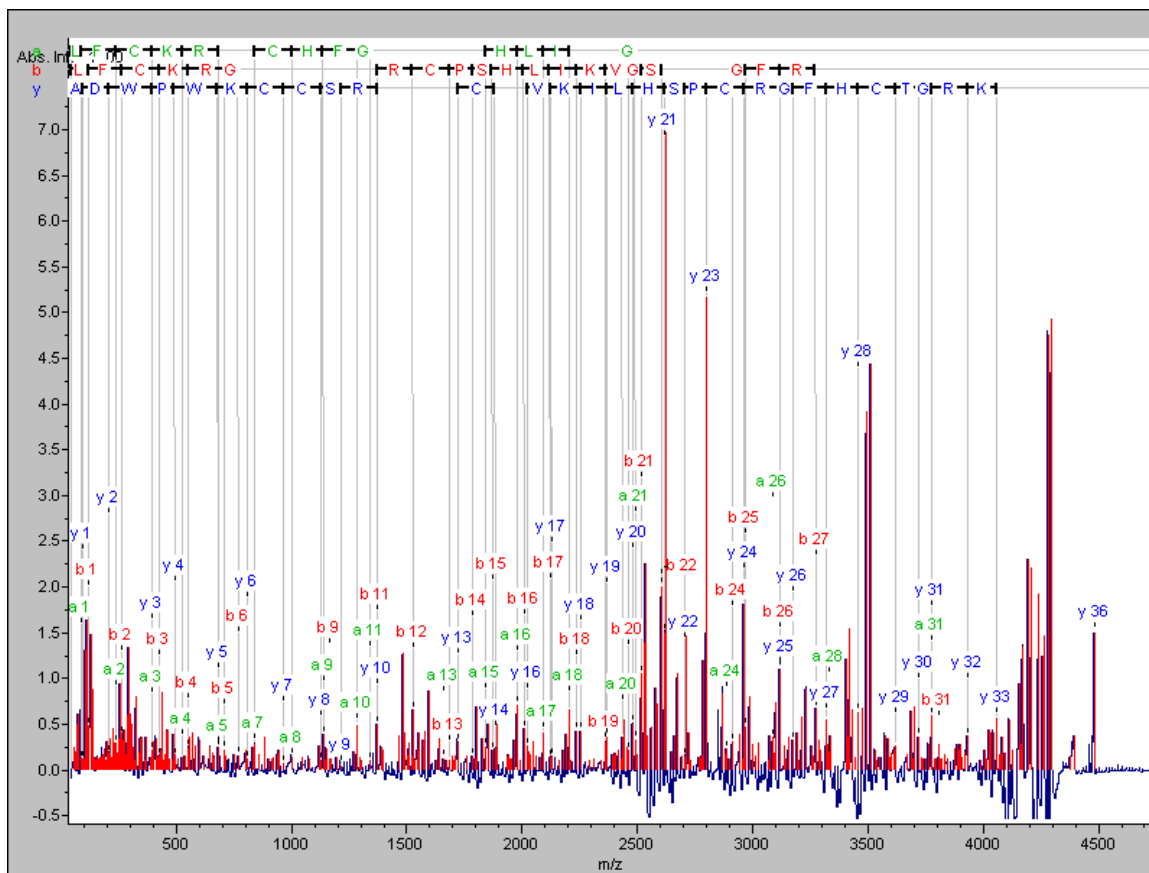


Figure 5.

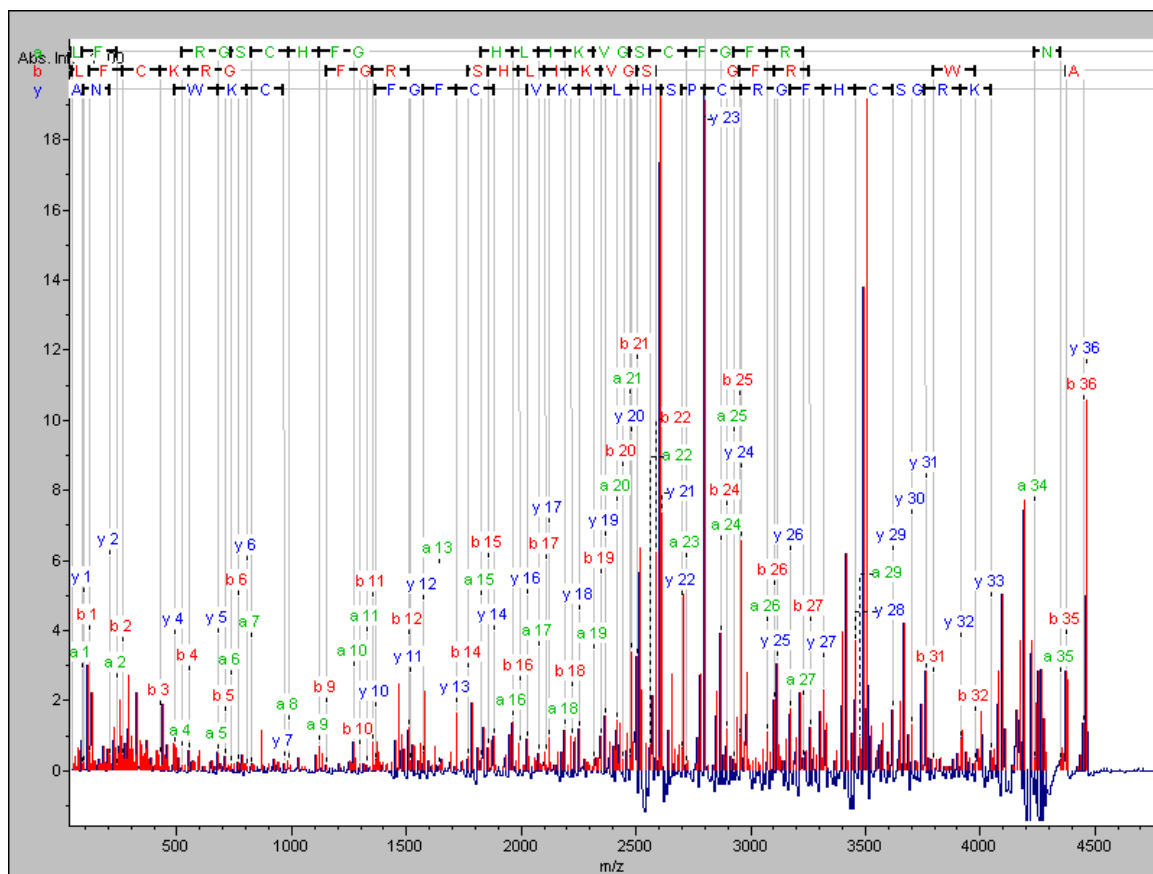
(a) Chicken



(b) Turkey



(c) Pheasant



(d) Quail

Figure 6.

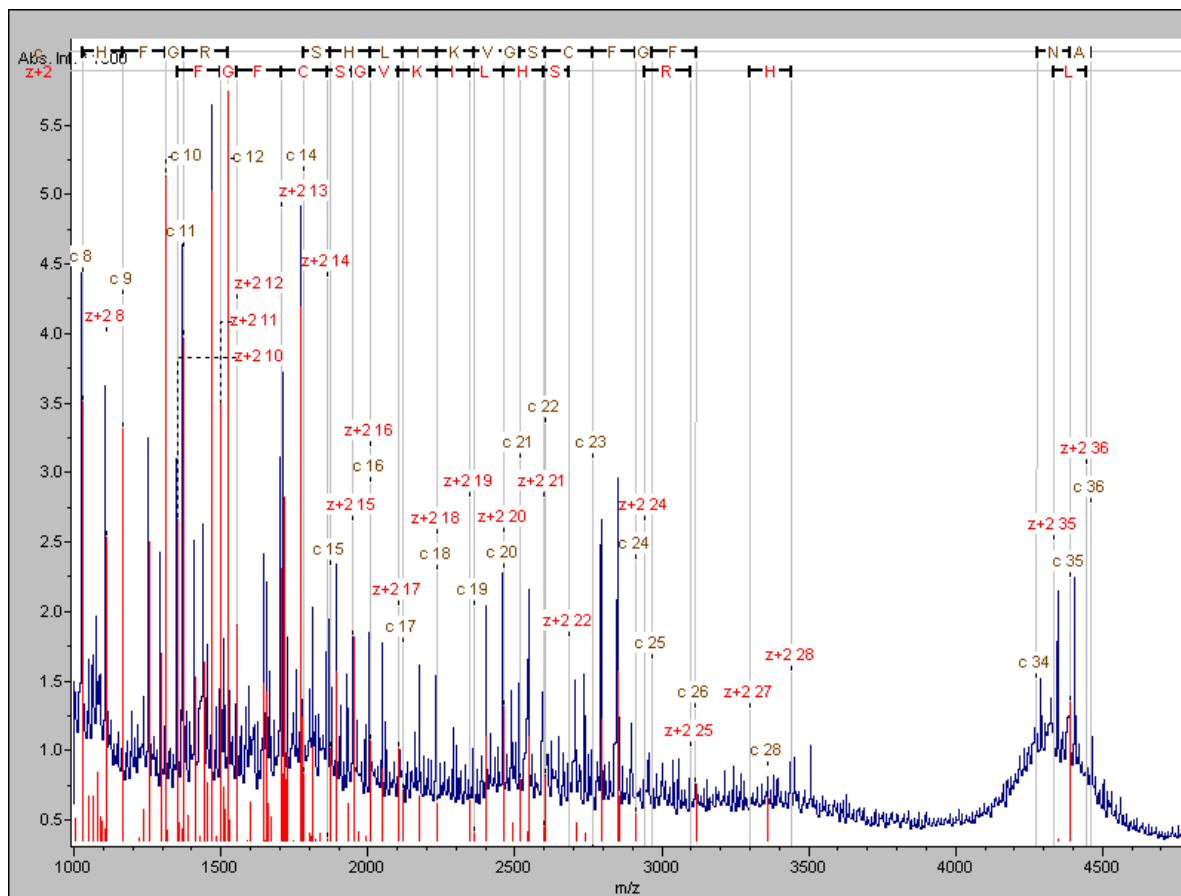
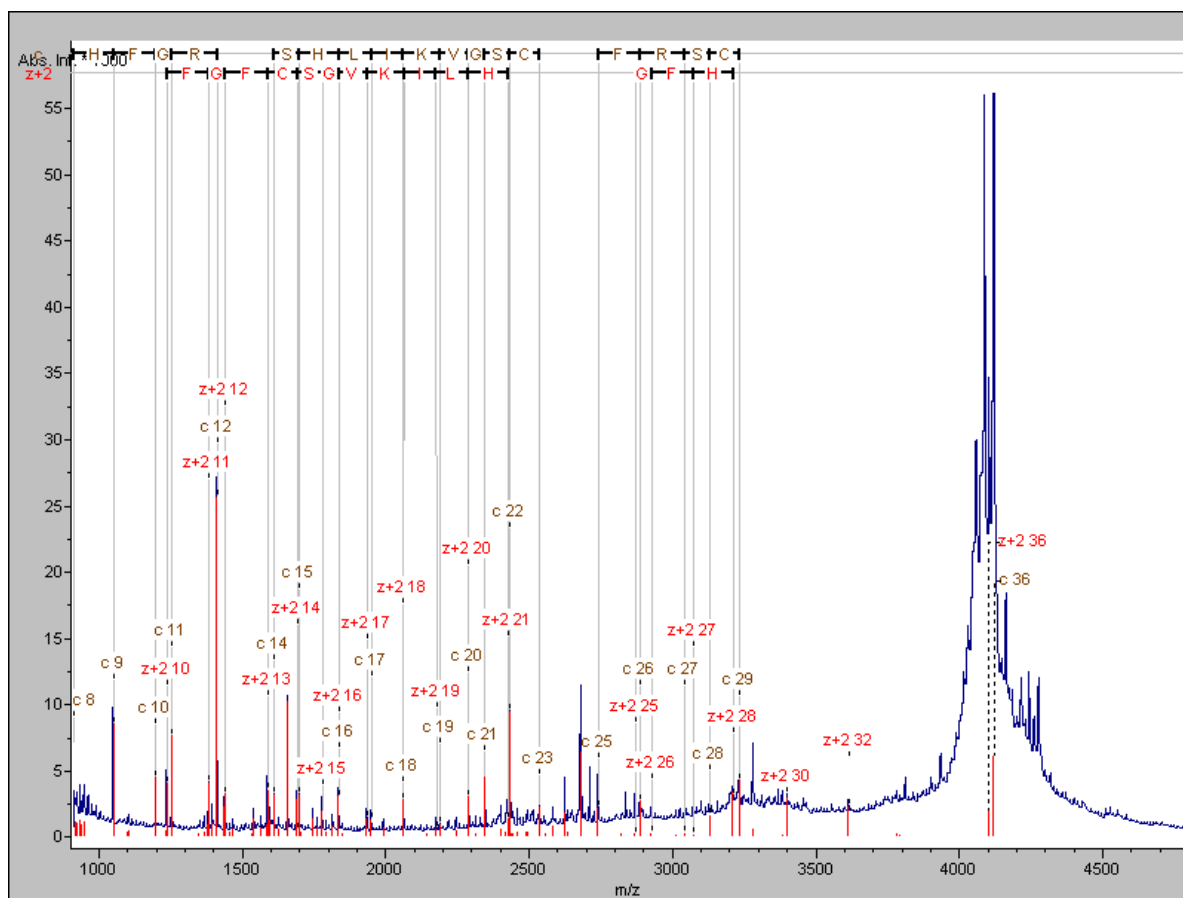


Figure 7.



CHAPTER 5

EFFECT OF TOLL-LIKE RECEPTOR AGONISTS ON THYMOSIN BETA 4 PRODUCTION BY CHICKEN MACROPHAGES

EFFECT OF TOLL-LIKE RECEPTOR AGONISTS ON THYMOSIN BETA 4 PRODUCTION BY CHICKEN MACROPHAGES

Lakshmi Kannan^{1,2,3}, Narayan C. Rath¹, Rohana Liyanage⁴, Jackson O. Lay, Jr⁴

¹PPPSRU/Agricultural Research Service/USDA, Fayetteville, AR 72701,

²Cell and Molecular Biology Program, ³Department of Poultry Science, University of Arkansas, Fayetteville 72701, ⁴Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701.

Address of correspondence:

Narayan C. Rath, Ph.D.
Agricultural Research Service, USDA,
Poultry Science Center, University of Arkansas,
Fayetteville, AR 72701
Email: narayan.rath@ars.usda.gov
Tel: 479-575-6189
FAX: 479-575-4202

To be submitted: Journal of Leukocyte Biology

Manuscript Style: Full-length Research Article

Abbreviations: AU= arbitrary units; CpG-ODN= CpG oligodeoxynucleotide; DHB= 2,5- dihydroxybenzoic acid; FGN= flagellin; H= heavy isotope ¹³C-lysine labeled; L= light lysine labeled; LOX= loxoribine; LPS= lipopolysaccharide; MALDI-TOF= matrix assisted laser desorption ionization-time of flight; MS= mass spectrometry; *m/z*= mass/charge; PGN= peptidoglycan; poly (I:C)= poly (inosinic:cytidilic acid); qPCR= quantitative polymerase chain reaction; RT-PCR= reverse transcription-polymerase chain reaction; SILAC=stable isotope labeling of amino acids in cell culture; TLR = toll-like receptor

ABSTRACT

Thymosin beta 4 (T β 4) is a 5 kDa actin sequestering intracellular peptide which has been shown to possess a variety of extracellular functions including chemotaxis, wound healing, tissue remodeling, and angiogenesis. However, the regulation of T β 4 production and the mechanism of its secretion to extracellular environment are not understood. Previously, we found that the chicken macrophage is a rich source of T β 4. Since macrophages play a central role in innate immunity including their participation in wound healing and angiogenesis, the objective of this study was to find whether activation of macrophages through appropriate toll-like receptors (TLR) would promote T β 4 synthesis and secretion. We treated chicken macrophages with different TLR agonists and studied their effects on cellular and extracellular T β 4 levels at 6 and 24 h time points. Real time PCR was used to determine changes in gene expression while SILAC followed by mass spectrometry was used to monitor T β 4 content in cells and conditioned media. The results show that while certain TLR activators, induced the expression of reference genes (interleukins-1 β , -6, and nitric oxide synthase) indicative of macrophage activation, none caused any significant change on T β 4 gene activation. The SILAC data however, showed a decrease in the cellular T β 4 content along with its corresponding detection in cell culture supernatants at 24 h by PGN, PAM and LPS. No changes in T β 4 level were detectable at 6 h time point. In the absence of any change in T β 4 gene expression that would indicate its intracellular replenishment, the decrease in cellular T β 4 levels at 24 h could likely relate to macrophage death induced by those agonists. Using lactate dehydrogenase (LDH) activity of the conditioned media, as an indicator of cell death, our results show that those agonists which showed depletion of cellular T β 4 also caused

higher LDH levels in the conditioned media. These results imply that T β 4 in the extracellular fluid likely originate from dying cells.

Keywords: *macrophage, SILAC, thymosin beta 4, toll-like receptor*

INTRODUCTION

Thymosin beta 4 (T β 4) is a highly conserved polypeptide, originally identified as T cell maturation factor in the thymus gland and later found to occur ubiquitously in many cells and tissues [1, 2]. T β 4 binds to the cytoplasmic G-actin preventing its polymerization to F-actin thus, regulating cytoskeletal organization, and cell motility [3, 4]. Extracellularly, T β 4 promotes a variety of functions including chemotaxis, angiogenesis, wound healing, and down regulate inflammation [5-8]. Additionally, T β 4 also possesses antimicrobial activity, facilitates antigen presentation, and is one of the major genes up regulated following immune activation [9, 10]. Although many of its diverse effects on different cells and tissues have been understood using exogenous T β 4, the mechanism of its synthesis and secretion to extracellular environment is not well understood [7, 11].

In the course of screening for bioactive peptides in phagocytic cells, we identified T β 4 as an abundantly occurring peptide in chicken macrophages [12]. The macrophages are a major component of innate immunity, which participate in many functions attributed to T β 4 such as, wound healing, angiogenesis, and tissue remodeling [13-15]. The macrophages recognize various microbial pathogens and their products through a series of 'pattern recognition receptors (PRR)' called toll-like receptors (TLR) [16] that cause their activation leading to the synthesis and secretion of various cytokines, chemokines, metabolites, and enzymes which in turn mediate different biological effects

[13, 17]. Therefore, the objective of this study was to find whether TLR activation would lead to the production of T β 4 by the macrophages, which could then participate in post-inflammatory healing processes.

MATERIALS & METHODS

Reagents and chemicals

Dialyzed fetal bovine serum (FBS) and SILAC RPMI-1640 cell culture media (L-arginine and L-lysine depleted) were purchased from Pierce (Rockford, IL). $^{13}\text{C}_1$ labeled lysine (**H**) was obtained from Cambridge Isotope Laboratories (Andover, MA). BCA protein assay kit (Pierce, Rockford, IL), RNAeasy mini and on-column DNA digestion kit (Qiagen Corp, Chatsworth, CA), Retroscript reverse transcriptase kit (Ambion, Austin, TX), Multiplex PCR kit (Qiagen, Valencia, CA), SYBR green PCR master mix (Applied Biosystems, Austin, TX), and lactate dehydrogenase (LDH) cytotoxicity kit (Promega, Madison, WI) were all purchased from the respective vendors. All other chemicals including L-lysine (**L**), L-arginine, non-enzymatic cell dissociation medium, antibiotic antimycotic solution, were obtained from Sigma-Aldrich (St. Louis, MO).

Toll-like Receptor ligands

The peptidoglycan-polysaccharide polymers PG-PS 10S (PGN), a sonicated cell wall preparation of *Streptococcus pyrogenes*, was a gift from BD Bioscience (San Jose, CA). The concentration of the product, 5mg equivalent rhamnose/ml 0.85% saline, was used to dilute to required concentration. A synthetic lipoprotein Pam3CSK4 (palmitoyl- 3-cysteine-serine-lysine-4; PAM), *Salmonella typhimurium* flagellin (FGN) and guanine

analog loxoribine (LOX) were purchased from Invivogen (San Diego, CA). The CpG-oligodeoxynucleotide (CpG-ODN 2006), corresponding to the sequence TCGTCGTTTTGTCGTTTTGTCGTT [18] was synthesized by Invitrogen (Carlsbad, CA). *Salmonella typhimurium* lipopolysaccharide (LPS) and dsRNA analog, polyI:C were purchased from Sigma-Aldrich (St. Louis, MO). All the above ligands were prepared as stock solutions in sterile physiological water.

Preparation of SILAC media

L-Lysine (**L**) and L-arginine were dissolved in sterile PBS at a stock concentration of 10g/L and 50g/L, respectively and filtered using a 0.2 μ m filter. A stock concentration (10g/L) of $^{13}\text{C}_1$ -lysine (**H**) was similarly prepared and filtered. The “Light (**L**)” SILAC medium for the control cells was prepared adding 2 mL of stock solution of L-arginine and 4 mL of lysine (**L**) to 500 ml of depleted RPMI 1640 medium. The “Heavy (**H**)” SILAC medium was prepared in a similar manner by adding 2mL of arginine and 4 mL of $^{13}\text{C}_1$ -lysine (**H**) stock solutions to 500 ml of depleted RPMI 1640 medium. Both ‘**L**’ and ‘**H**’ SILAC media were filtered and supplemented with 10% dialyzed FBS, 20 mM glutamine, and 1X concentration of antibiotic antimycotic solution.

Macrophage culture and activation by TLR agonists for SILAC studies

Transformed chicken macrophage cell line HTC [19] cultured in RPMI-1640 media containing 10% FBS and 1X concentration of antibiotic antimycotic solution were initially grown in normal RPMI 1640 medium. At 80% confluence, the media was aspirated and the cells were washed with PBS and detached using a non-enzymatic

dissociation medium for 5 min, and washed with PBS three times by successive centrifugation at 380 X g for 8 min each [12]. One half of the detached cells were seeded into the 'L' SILAC RPMI medium and the other half into the 'H' SILAC RPMI medium, prepared as described above. Metabolic labeling of cells were done according to Ong and Mann (2006) with 6 successive passages of cells in respective media that theoretically labels the entire proteome of the cells with 90-100 % efficiency [20]. Both light and the heavy isotope labeled HTC cells were detached and plated in triplicates at a concentration of 1×10^6 cells / ml in 12 well culture plates in their respective media and grown overnight incubated at 37°C under 5% CO₂. The H labeled cultures was then stimulated with different TLR agonists for 6 and 24 h. Each TLR agonist treatment consisted of a triplicate culture consisting of: TLR2, gram positive bacterial peptidoglycan (PGN), TLR3, poly inosinic, cytidylic (poly I:C), TLR4, gram negative LPS, TLR5, flagellin, TLR2/1, PAM3CSK4, TLR7, Loxoribine; and TLR9, CpG-ODN [16]. The final concentrations of the agonists were PAM (1 µg/ml), PGN (1 µg rhamnose equivalent /ml), FGN (100 µg/ml), poly I:C (1 µg/ml), LOX (100 µM), CpG-ODN (5 µM) and LPS (1 µg/ml) respectively. The concentrations of the TLR agonists were based on values recommended by the suppliers and used in earlier literatures [21, 22].

Sample preparation for MALDI-MS and SILAC studies

After specified periods, 6 and 24 h of stimulation with TLR agonists, the plates were centrifuged at 300 X g for 10 min and the conditioned media from both control and treatment group were removed. Aliquots of the conditioned media free of cells were mixed with equal volumes of 100% methanol containing 2% acetic acid to denature and

precipitate proteins over a 16 h period at 4°C, and centrifuged at 21,000 X g for 10 min to obtain the supernatant extract containing Tβ4. The adhered cells were washed with Dulbecco's PBS twice, lysed with 200 μl of 0.1% n-octyl β-glucopyranoside (OβG by repeated pipetting, and centrifuged at 21,000Xg for 10 min to obtain the cell lysate. The cell lysate and conditioned media extract from each control (C) consisted of **L** and **H** lysine labeled samples (C_L and C_H) whereas the TLR agonist treated groups (T) consisted of only **H** lysine labeled samples (T_H) based on the *in vivo* incorporation of stable isotopes (Fig.1). Protein concentrations of both supernatant extract and cell lysates were determined using micro BCA protein assay to equalize the protein concentrations in case of variations between different samples. All the treatment samples (cell lysate and supernatant extract) were used individually but the **L** labeled control samples (cell lysate and supernatant extract) were pooled and used for all mixing experiments.

Cell lysate and supernatant extracts were mixed individually with an equal volume of one molar 2, 5-dihydroxybenzoic acid (DHB) in 90% methanol containing 0.1 % formic acid and spotted onto a Bruker MTP 384 stainless steel MALDI target. MALDI-TOF spectra were acquired over the *m/z* range 1-10 kDa in the positive ion reflector mode using Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonik GMBH, Bremen, Germany) [12, 23]. The data were processed using Bruker Flex Analysis 2.4/3.0 software. Initial screening of the supernatant extract and cell lysate samples of C_L, C_H and T_H by MALDI-TOF-MS was done to detect the presence of Tβ4 (*m/z* 4963, protonated molecule in C_L and *m/z* 4972, protonated molecule in C_H and T_H). Following preliminary screening, pooled control C_L and individual C_H were mixed 1:1 to determine the intensity ratios of **L** and **H** Tβ4 in the cell lysate and conditioned media by

MALDI-TOF-MS as above. Based on the peak intensities observed in the mass spectrum for **L** and **H** T β 4, the intensity ratios (C_H/C_L) for controls and (T_H/C_L) were calculated for all treatment groups. The final fold changes or the relative changes in the levels of intracellular C and released T β 4 were calculated by dividing the intensity ratio T_H/C_L over C_H/C_L . The mean and standard error of the mean (SEM) was calculated for control and each treatment group using measurements obtained from individual set of experiments repeated three times. The results were evaluated using Students *t*-test and a *p* value ≤ 0.05 was considered significant.

Activation of macrophages by TLR agonists for other analysis

HTC cells of 1×10^6 / mL were seeded and grown separately in triplicates in normal RPMI 1640 medium and treated with different TLR agonists as above for 6 and 24 h for gene expression and other analyses. For nitrite and LDH determination, the conditioned media was collected from all samples at both time periods, centrifuged, and saved at -20°C until assay.

Gene expression studies

The macrophage activation induced changes in the expression of T β 4 and other reference genes were assessed at 6 h stimulation as follows: Total RNA was extracted from control and TLR agonist treated macrophage cultures using RNeasy Mini Kit. Any contaminating DNA was subjected to on column digestion with RNase-free DNase 1 according to the manufacturer's instructions. The cDNA was synthesized using Retroscript reverse Transcriptase kit. The gene-specific primers for T β 4, IL-6, IL-1 β ,

iNOS and β -actin were designed with the Primer 3 software program (<http://frodo.wi.mit.edu/>) (Table 1). The expression of genes for IL-6, IL-1 β and iNOS were used as positive controls indicative of macrophage activation, and β -actin as the housekeeping gene. Q-PCR was used to determine the expression of target genes relative to the levels of the housekeeping gene β -actin. Following optimization of primer concentrations and volumes (what does it mean?), real-time PCR was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Austin, TX). Each mRNA sequence was amplified in duplicate in 25 μ L reactions containing SYBR green PCR master mix; cDNA corresponding to 1 μ g of reverse transcribed RNA and 200 nM (optimized concentration) of forward and reverse primers. Both a no template and no reverse transcriptase control were included for each amplification reaction, and the homogeneity of the amplified products were confirmed routinely by melting curve analysis. The results were analyzed by the standard curve method and normalized to β -actin as an endogenous control. Then the relative fold change in the expression of target genes (T β 4, IL-6, IL-1 β and iNOS) were assessed between the non stimulated control or TLR agonist stimulated macrophages.

Nitrite and LDH measurement

The nitrite content of all conditioned media was measured as described earlier using Griess reagent [19]. The LDH activity of the conditioned media was used as an indicator of cell damage or death [24]. The assays were done using control and treatment culture supernatants at both 6 and 24 h time points. The LDH activity was measured using a cytotoxicity assay kit according to the manufacturer's protocol.

Statistics

The quantitative results were evaluated using students t test. A p value ≤ 0.05 was considered significant.

RESULTS

TLR activation induced T β 4 release

MALDI-MS profiles of 1:1 mixtures of '**L**' control cell lysate and '**H**' cell lysates and similarly treated conditioned media extracts are shown in Figs 2-4. Protonated ion, m/z 4963 corresponds to T β 4 with '**L**' lysine (^{12}C) label, while m/z 4972 corresponds to T β 4 with '**H**' lysine (^{13}C). Signal intensities of L and H T β 4 were monitored at 6 h and 24 h time period. MALDI spectrum in Fig 2a shows **L** and **H** labeled T β 4 in the 1:1 mixture of control cell lysates whereas Figs. 2b-h represent spectra from 1:1 mixture of control (**L**) and respective TLR agonist stimulated **H** cell lysates at 6 h. The signal intensity of **L** and **H** labeled T β 4 remained approximately same in all groups in cell lysates at 6 h and in the corresponding conditioned media extract it was below the detection limit. At 24 h time point, the cell-associated T β 4 decreased significantly in PGN, PAM, and LPS treated cells (Fig 3). Both CpG and FGN treatments showed a moderate decrease but neither poly I:C nor LOX showed any change in cellular T β 4 (Fig 3). Maximal decrease in the cellular T β 4 was detected in PGN, PAM and LPS treated groups which was consistent with its presence in their respective conditioned media along with their oxidized forms (**L**: T β 4 sulfoxide m/z 4979, **H**: m/z 4988; Fig 4). It is note worthy that the oxidized form appeared to be the predominant form of the observed T β 4 in the conditioned media (Fig

4). The relative changes in cellular T β 4 in macrophages treated with different TLR agonists at 6 and 24 h are shown in Fig 5. Whereas at 6 h no change in cellular T β 4 was observed, at 24 h PGN, PAM and LPS showed significant decrease in cellular T β 4 ($p \leq 0.001$) along with CpG and FGN treated groups that also showed a lower albeit statistically significant decrease in cellular T β 4 ($p \leq 0.05$).

Gene expression

qPCR results for the mRNA expression in macrophage T β 4, IL-6, IL-1 β , iNOS relative to β -actin reference is shown in Fig 6. T β 4 mRNA expression in both control and in all TLR agonist treatment groups remained unchanged. By contrast, PAM, PGN, LPS, CpG, and FGN induced a significant up-regulation of IL-1 β , IL-6, and iNOS (Fig. 3) compared with the non-stimulated controls. However, FGN produced lesser effect on comparison with other agonists mentioned above. Both poly I:C and LOX agonists showed very minimal effect that was not statistically different from control.

Functional activation of macrophages by TLR agonists

Groups treated with TLR agonists PGN, LPS, PAM, FGN and CpG showed a moderate nitrite release at 6 h time point that was significantly enhanced at 24 h. Except for poly I:C, and LOX all treatment groups showed a significant increase in nitrite levels at 24h time points albeit some which showed very high levels of nitrite (Fig. 7).

LDH changes

There were no differences in LDH activity between control and the different TLR agonist treated culture media at 6 h time point but at 24 h, the LDH activity was significantly higher in all treatment groups except for poly I:C and LOX. The 24 h conditioned media including that of control groups had higher LDH activities at 24 h point compared with 6 h (Fig 8).

DISCUSSION

Thymosin β 4 is a ubiquitously occurring peptide in eukaryotic cells which has been shown to exert a variety of extracellular effects including wound healing, angiogenesis, and tissue remodeling [6, 7, 25, 26]. Despite its diverse extracellular actions, the mechanism of its release into the extracellular fluid remains intriguing because the peptide lacks a signal sequence, which is essential of most secretory proteins and peptides [11]. Nonetheless, there are a handful of reports suggestive of its secretion to extracellular environment using bone marrow endothelial cells and myocardial cells in culture and T β 4 is present in significant quantities in human wound and blister fluids [27-29]. Our rationale to study T β 4 release from macrophages was its abundance in these cells [12, 30, 31] and these cells when subjected to immunomodulation by TLR-agonists increase their secretory activities [32]. SILAC has been a very sensitive and quantitative mass spectrometric method to measure the changes in peptide levels using corresponding MALDI MS signal ratio of light (**L**) and heavy (**H**) labeled peptide ions [33, 34]. Labeling with **H** (^{13}C) and **L** (^{12}C) lysine showed 9 Da difference consistent with the 9 lysine residues present in mature chicken T β 4 as expected and the ratio of the MALDI

signal of these ions were monitored to determine the expression differences as shown in Fig 4 [12]. Our results however, showed any discernible change in neither cellular nor conditioned media associated T β 4 at 6h time point in any treatment group. By contrast, a significant decrease in cellular T β 4 was evident at 24 h time point in cultures treated with PGN, LPS, PAM, and CpG. Both **L** and **H** T β 4 as well as their corresponding oxidized forms (T β 4 sulfoxide) with additional mass difference of 16 Da due to oxidation of methionine [35] were detected in the conditioned media after 24 h treatment. Interestingly the T β 4 sulfoxide appeared to be the dominant form in the conditioned media. The T β 4 sulfoxide formation may be related to macrophage respiratory burst resulting from their activation (Rath et al., 2003). T β 4 sulfoxide ostensibly acts as an antioxidant that appeases inflammation [35-37].

Based on 24 h result, it was clear that at least some TLR agonists are able to induce release of T β 4 into the culture media implying that similar mechanisms may be operative *in vivo* leading to its accumulation in extracellular fluids under inflammatory conditions. Hence, we asked if T β 4 was released by secretion induced by the above agonists, then it should be replenished in the cells that could be evident by assessing the changes in T β 4 gene expression. Along with T β 4, we also monitored the expression of Il-1 β , Il-6, and iNOS genes at 6 h time point as the measures of macrophage activation, and the changes in nitrite levels of cell culture supernatant at 24 h. The results showed that none of the agonists were able to modulate the expression of T β 4 gene whereas PGN, LPS, PAM and CpG caused significant stimulation in the expression of Il-1 β , Il-6, and iNOS genes and induced nitrite release that was consistent with the macrophage activation [19]. The changes in mRNA expression are generally considered to reflect the

changes in their respective proteins or peptides. In the absence of any detectable changes in T β 4 gene expression, at the same time the observed intracellular and extracellular changes in the levels of this peptide suggested that cell damage may be a factor leading to its release into the cell conditioned media. LDH leakage into the extracellular medium is used as an indicator for the loss of cell viability which is caused by cytoplasmic membrane damage leading to cell death [24]. Therefore, we measured the changes in LDH levels in cell culture media, which showed a significant increase in culture media in those groups which showed the loss of cellular T β 4 at 24 h. It is likely that macrophage apoptosis is induced at 24 h by the action of those TLR agonists causing the leakage of T β 4 into the conditioned medium. In fact, there are several reports, which show that both gram-positive and gram-negative microbial products such as PGN and LPS induce macrophage apoptosis [38-40].

In conclusion our studies demonstrate that immunomodulatory and inflammatory agents affect the release of T β 4 into extracellular environment that is induced by cellular damage. We believe this finding provides a mechanism for the release of T β 4 into wound environment facilitating healing of injured or damaged tissues.

ACKNOWLEDGEMENTS

Thanks are due to Sonia Tsai, Scott Zornes, and Dana Bassi for technical assistance.

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Table 1: Primer sequences used in Q-PCR [12, 19].

| RNA target | Forward primer (5' to 3') | Reverse primer (5' to 3') | Accession number |
|-------------------|----------------------------------|----------------------------------|-------------------------|
| β - actin | CCATGAAACTACCTTCAACTCCA | GATTCATCGTACTCCTGCTTGCT | L08165 |
| IL-6 | CTCCTCGCCAATCTGAAGTC | TCATAGAGACGCTGCTGCCAG | AJ309540 |
| iNOS | AGGCCAAACATCCTGGAGGTC | TCATAGAGACGCTGCTGCCAG | U46504 |
| IL-1 β | GGCTCAACATTGCGCTGTAC | CCCACTTAGCTTGTAGGTGGC | Y15006 |
| T β 4 | GCCGAGATCGAGAAATTTGA | GAAGGCAATGCTTGTGGAAT | NM_001001315 |

FIGURE LEGENDS

Figure 1. A flow chart illustrating SILAC method to study the effects of different TLR ligands on T β 4 production by HTC cells.

Figure 2. MALDI-MS profiles of 1:1 cell lysate mixtures containing light (**L**) and heavy (**H**) lysine labeled T β 4 after 6 h stimulation with TLR agonists.

Figure 3. MALDI-MS profiles of 1:1 cell lysate mixtures containing light (**L**) and heavy (**H**) lysine labeled T β 4 after 24 h stimulation with TLR agonists.

Figure 4. MALDI-MS profiles of 1:1 supernatant extract mixtures containing light (**L**) and heavy (**H**) lysine labeled T β 4 after 24 h stimulation with TLR agonists.

Figure 5. Relative changes in the cellular T β 4 induced by TLR agonists at 6 and 24 h. Data represent the mean value \pm SEM for triplicates samples in each groups from three independent experiments (n=9). ** Indicates $p \leq 0.0001$ and * is $p \leq 0.005$.

Figure 6. Q-PCR gene expression results after 6 h stimulation of HTC cells by different TLR agonists. The results represent an average of duplicate experiments.

Figure 7. Changes in nitrite production at 6 and 24 h time point induced by TLR agonists in HTC cells. * Indicates $p \leq 0.0001$.

Figure 8. Cell viability changes measured by LDH activity at 6 and 24 h. ** Indicates $p \leq 0.0001$ and * is $p \leq 0.005$.

Figure 1.

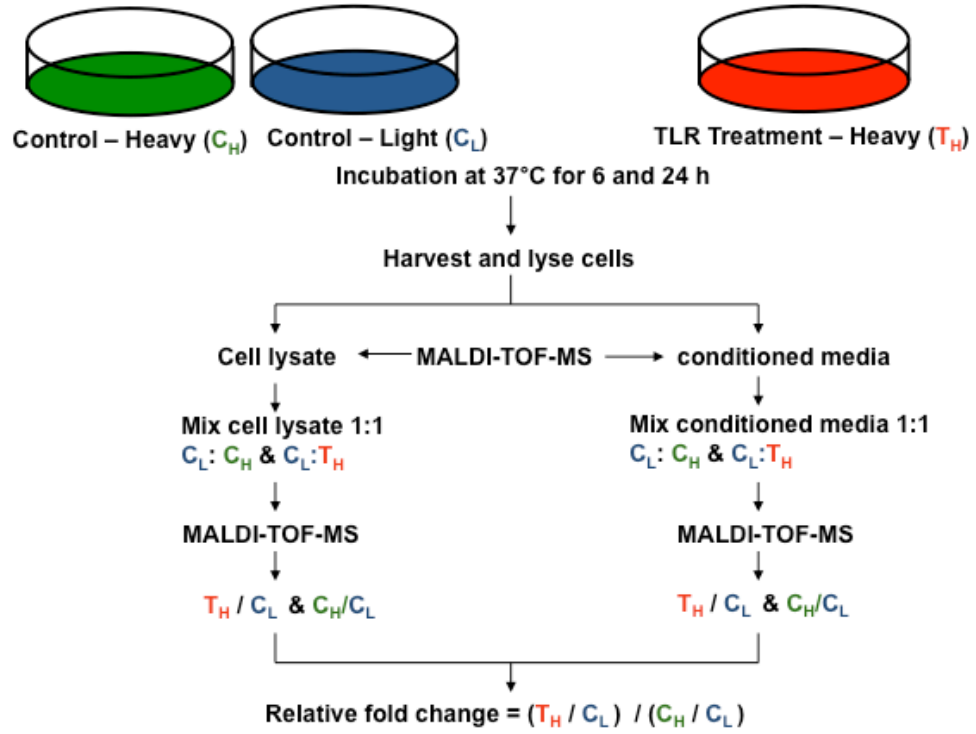


Figure 2.

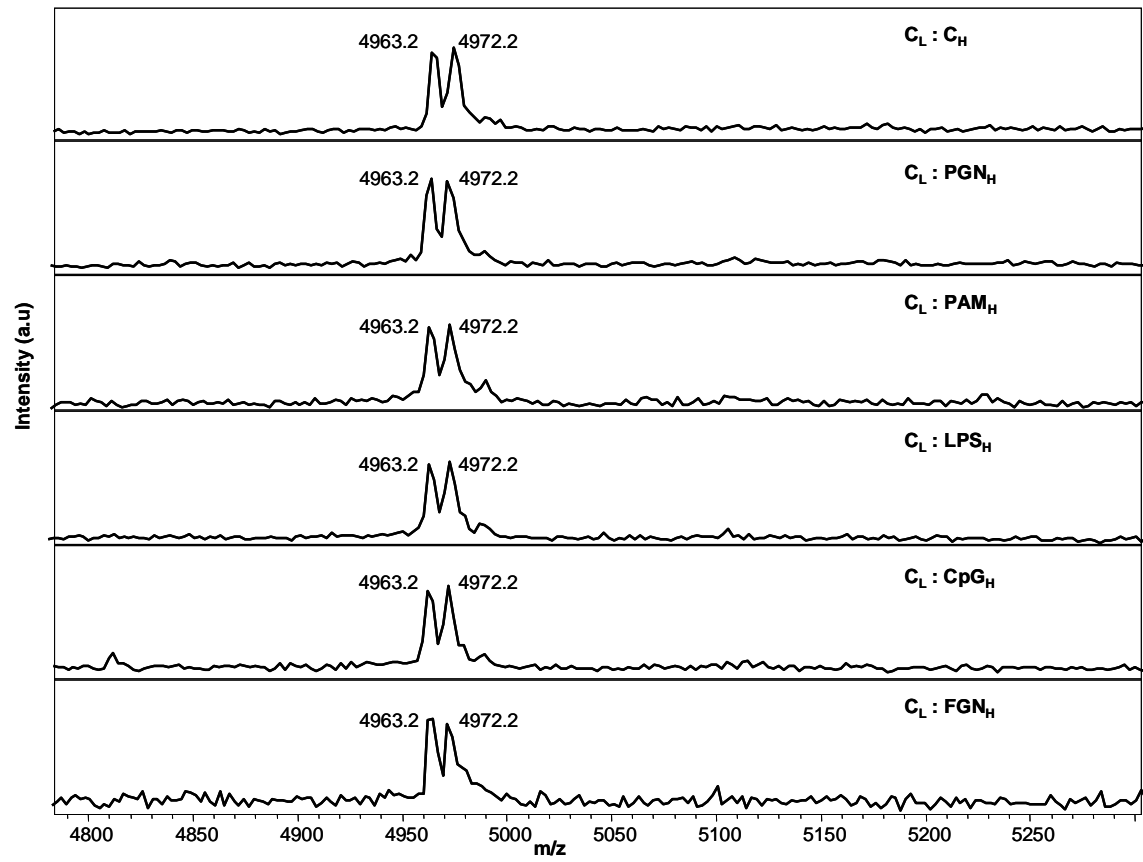


Figure 3.

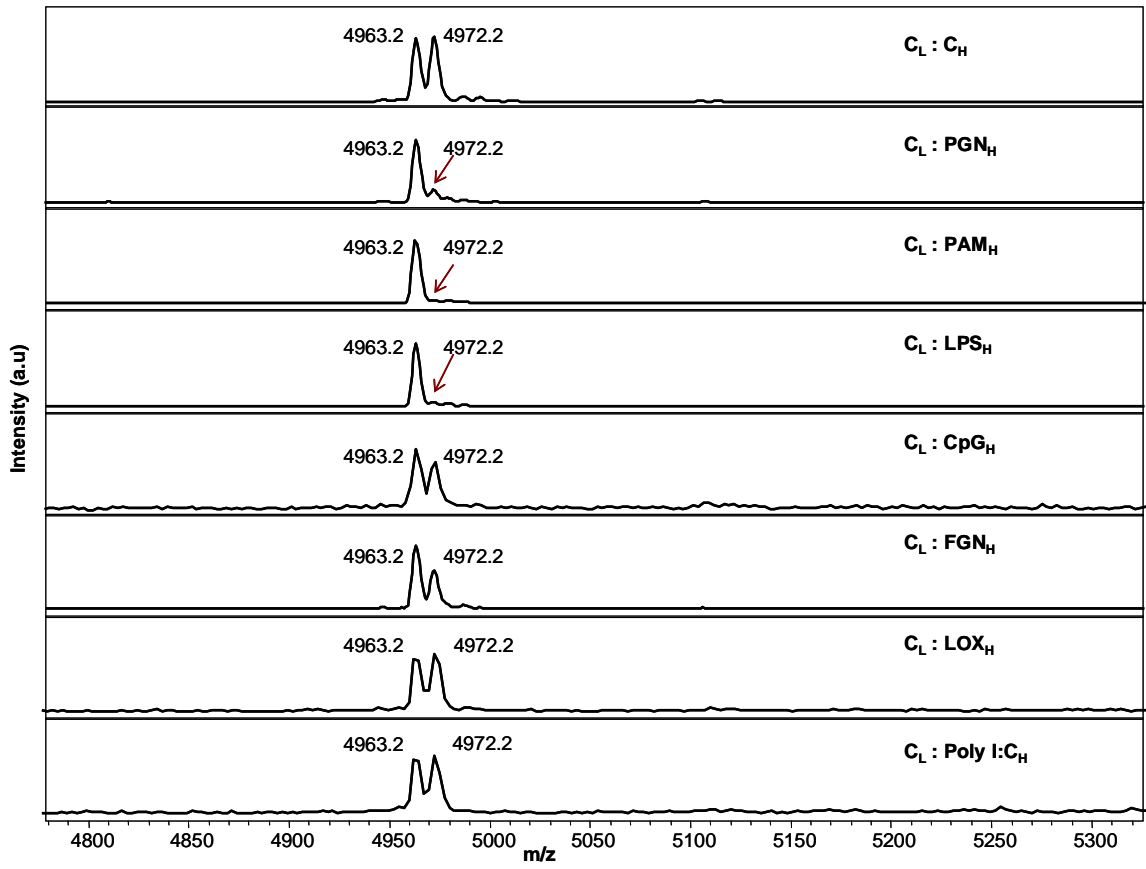


Figure 4.

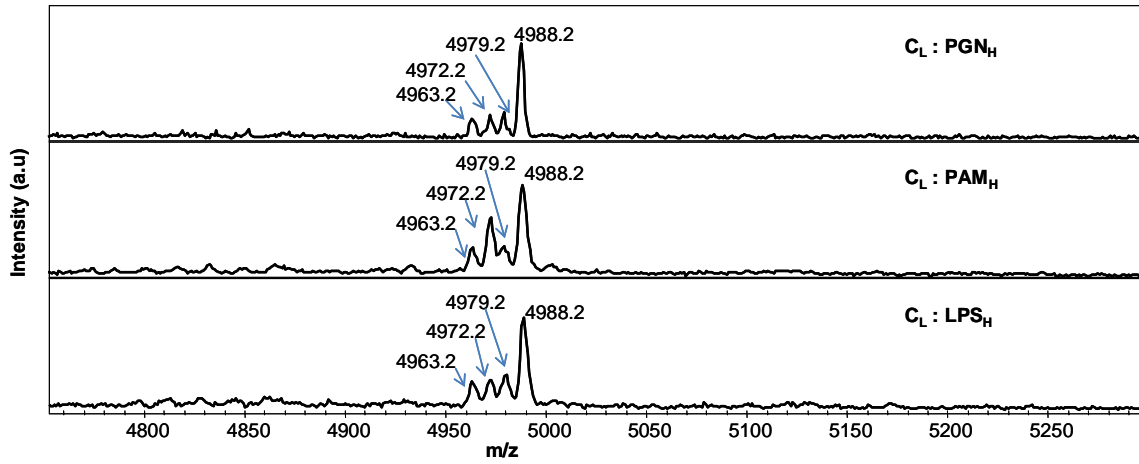


Figure 5.

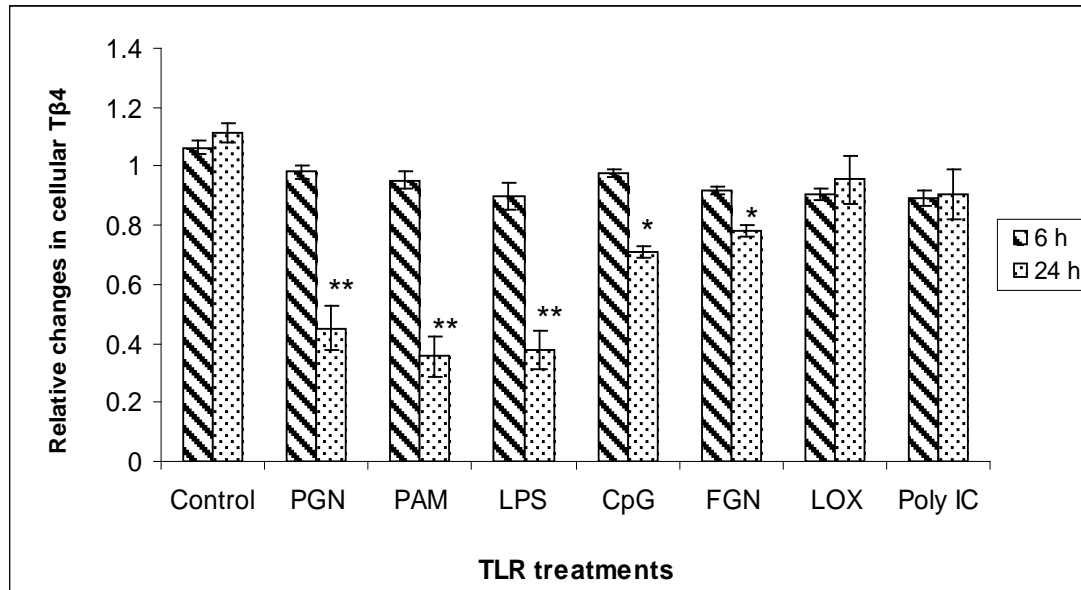


Figure 6.

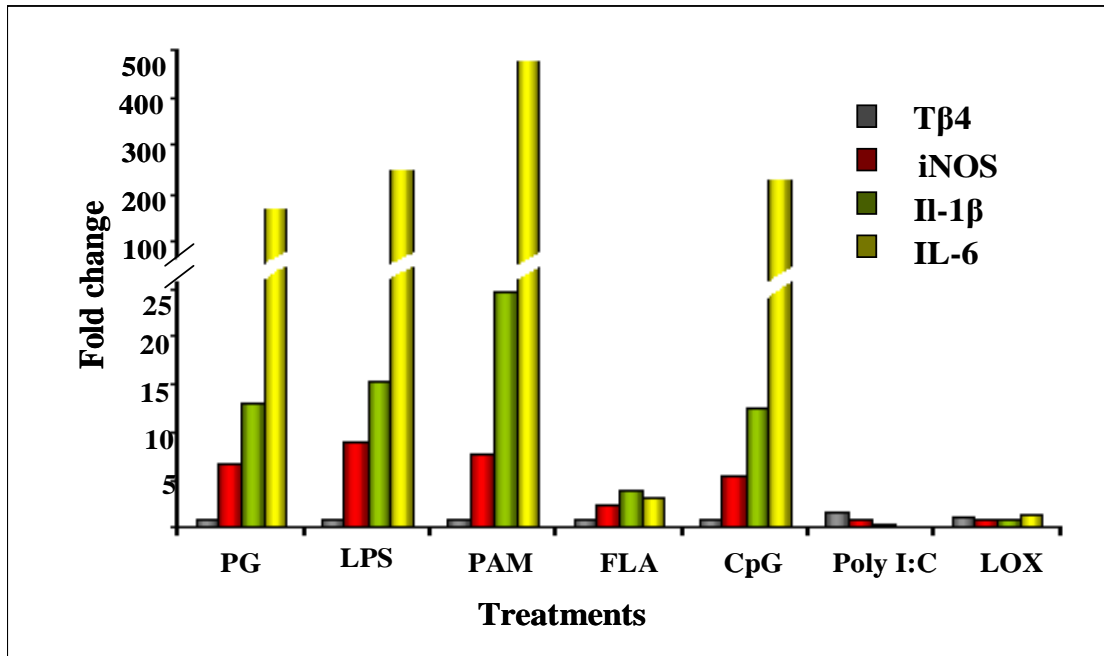


Figure 7.

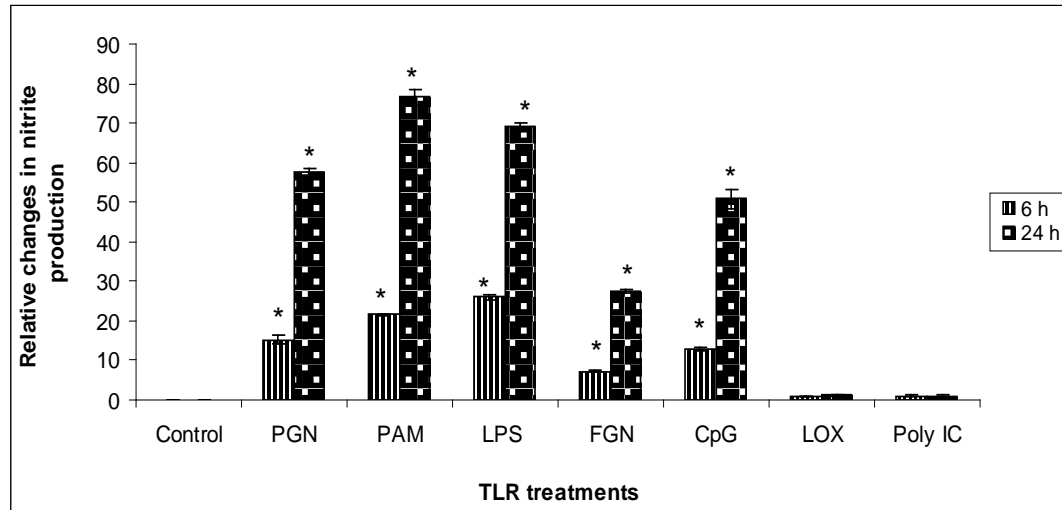
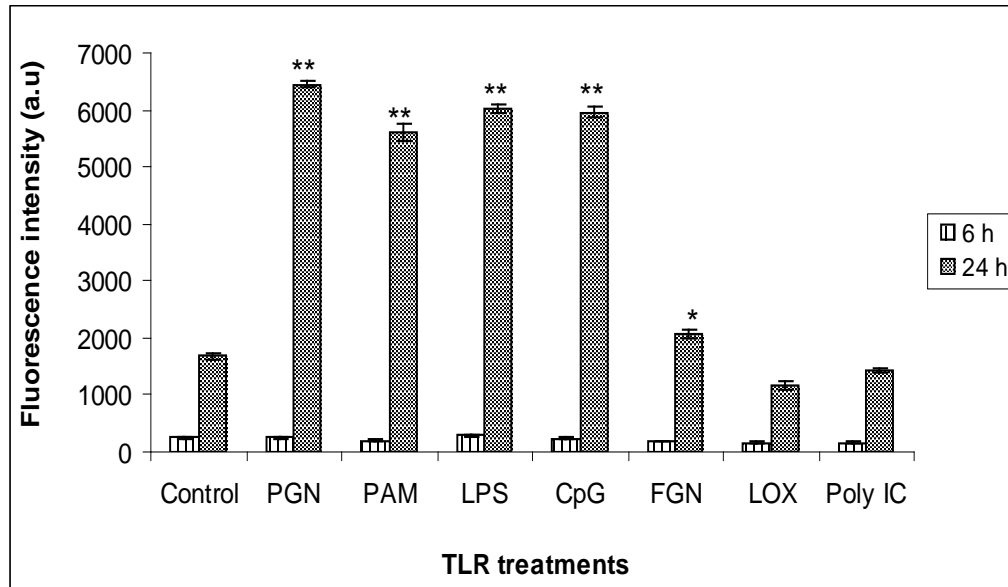


Figure 8.



CHAPTER 6

EVALUATION OF BETA DEFENSIN 2 PRODUCTION BY CHICKEN HETEROPHILS USING DIRECT MALDI MASS SPECTROMETRY

**EVALUATION OF BETA DEFENSIN 2 PRODUCTION BY CHICKEN
HETEROPHILS USING DIRECT MALDI MASS SPECTROMETRY**

Lakshmi Kannan^{1,2,3}, Rohana Liyanage⁴, Jackson O. Lay, Jr⁴, Narayan C. Rath¹

¹PPPSRU/Agricultural Research Service/USDA, ²Cell and Molecular Biology Program,
³Department of Poultry Science, ⁴Department of Chemistry and Biochemistry,
University of Arkansas, Fayetteville, AR 72701

Address of correspondence:

Narayan C. Rath, Ph.D.
Agricultural Research Service, USDA,
Poultry Science Center, University of Arkansas,
Fayetteville, AR 72701
Email: narayan.rath@ars.usda.gov
Tel: 479-575-6189
FAX: 479-575-4202

Published: Molecular Immunology, 46(15):3151-56, 2009

Manuscript Style: Short Communication Research Article

Section: Defensins

Abbreviations: AU= arbitrary units; AvBD= avian beta defensin; BD= beta defensin;
CAM= carbamidomethylation; CpG-ODN= CpG oligodeoxynucleotide; DHB= 2,5-
dihydroxybenzoic acid; DTT= 1,4- dithiothreitol; FA= formic acid; FGN= flagellin;
FITC= Fluorescein isothiocyanate; IAA= 2- iodoacetamide; LOX= loxoribine; LPS=
lipopolysaccharide; MALDI-TOF= matrix assisted laser desorption ionization time-of-
flight; MS= mass spectrometry; m/z = mass/charge; PGN= peptidoglycan; poly (I:C)=
poly (inosinic:cytidilic acid); RT-PCR= reverse transcription-polymerase chain
reaction ;TLR = toll-like receptor

ABSTRACT

Beta defensins (BD) are cysteine rich, cationic antimicrobial peptides (AMP) produced mainly by epithelial and myeloid cells such as neutrophils. In birds, the neutrophil equivalent heterophils produce avian beta defensins (AvBD) of which AvBD2 is the major isoform. Heterophils recognize pathogens or their derived products through a series of pattern recognition receptors called toll-like receptors (TLR) leading to their antimicrobial activities. This work is the first report of TLR modulation of AvBD2 expression in chickens. To measure the effect of TLR activation on AvBD2 production, the heterophils were cultured with different TLR agonists for 6 h. Modulation of AvBD2 levels by TLR activation was measured using direct MALDI mass spectrometry without stable isotopic labeling or chromatographic separation. Chemical modification of the conditioned media was performed using reduction/alkylation with dithiothreitol and iodoacetamide to distinguish TLR treated AvBD2 (reduced/alkylated) from controls (non-reduced). Changes in corrected ion intensity ratios were assumed to reflect AvBD2 modulation in heterophils upon activation with different TLR agonists. In general, TLR agonists increased AvBD2 production with LPS showing the greatest induction and CpG-ODN showing little or no effect. These data show that the direct MALDI-MS coupled with reduction/alkylation may provide a rapid relative quantitative approach to the measurement of agonist-induced differential expression of AvBD2.

Key words: *avian beta defensin 2, heterophil, MALDI-TOF-MS, toll-like receptors*

1. INTRODUCTION

Antimicrobial peptides are the efferent arms of innate immunity that play a vital role in host defense mechanisms by neutralizing microbial pathogens (Boman, 1995; Hancock, 2001). The defensins are an important class of these peptides that have been identified and characterized in many species including plants, invertebrates, and vertebrates (Brogden et al., 2003; Ganz, 2005; Zasloff, 2002). The vertebrate defensins contain three pairs of intramolecular disulfide bonds and are classified into subfamilies of α , β , and θ depending on the positions of their cysteine residues and the disulfide bond connectivity (Ganz, 2005; Klotman and Chang, 2006; Selsted and Ouellette, 2005). However, the avian species only express beta defensins and to date, there are 14 known isoforms of avian beta defensins in chicken and few orthologs in other species of birds (Lynn et al., 2004; Lynn et al., 2007; Sugiarto and Yu, 2006; van Dijk et al., 2008; Xiao et al., 2004). Whereas these AvBDs have been predicted using genomic and bioinformatics approaches, only a few corresponding peptides have been isolated (Evans et al., 1994; Harwig et al., 1994; Kannan et al., 2009; Sugiarto and Yu, 2006; Thouzeau et al., 2003). The avian leukocyte, heterophils, play a central role in innate immunity during acute phase response and microbial infection (Harmon, 1998; Kaiser, 2007). These cells use their surface receptors, called 'Toll-like receptors' (TLR) to discriminate microbial motifs called 'pathogen-associated molecular patterns' (Janeway and Medzhitov, 2002; Sabroe et al., 2003). TLR activation triggers immune responses resulting in the expression of effectors such as cytokines, chemokines, and antimicrobial factors including beta defensins (Boyd et al., 2007; Kogut et al., 2005; Kogut et al., 2006). Although there are several studies on the expression profiles and antimicrobial activities

of AvBDs, there is a limited knowledge of TLR activation and resultant beta defensin expression. Most studies on the regulation of AvBD have employed RT-PCR which can detect gene expression, but not subsequent peptide production (Akbari et al., 2008; Milona et al., 2007; Subedi et al., 2007; van Dijk et al., 2008). To date, we know of no study on the TLR activation induced expression of BD at the peptide level in avian species. Our earlier studies using direct screening of phagocytes and bone marrow of chickens and turkeys, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), showed avian beta defensin 2 (AvBD2) as an abundant peptide present in heterophils (Kannan et al., 2009). Thus, the objective of the current study was to find whether TLR activation would induce changes in the production of AvBD2 by heterophils. In recent years, MS has been used to measure differential expression of proteins and peptides. MS approaches include direct intensity based measurements and methods based on differential labeling of proteins using stable isotopes such as SILAC (stable isotope labeling of amino acids in cell culture), isotope-coded affinity tag (ICAT), and isobaric tag for relative and absolute quantitation (iTRAQ) reagents (Gygi et al., 1999; Ong and Mann, 2007; Wiese et al., 2007; Yan and Chen, 2005). Herein, we report a simple and an inexpensive method to determine the changes in AvBD2 production by heterophils induced by TLR agonists, using differential chemical labeling (reduction/alkylation) followed by MALDI-TOF-MS.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

One Step PolymorphTM medium (Accurate Chemicals Co, Westbury, NY), K-EDTA Vacutainer tubes (BD Bioscience, Franklin, NJ), RPMI-1640 medium (Mediatech Inc., Herndon, VA), C18 ZipTip micropipette tips (Millipore, Billerica, MA), peptide calibration standards of 500-5000 Da (Bruker Daltonics, Bremen, Germany), BioRad dye binding reagent (Bio-Rad, Hercules, CA), TLR agonists such as synthetic lipoprotein Pam3CSK4 (palmitoyl-3-cysteine-serine-lysine-4; PAM), *Salmonella typhimurium* flagellin (FGN), and guanine analog loxoribine (LOX) (Invivogen, San Diego, CA) were purchased. The Streptococcal *pyrogenes* peptidoglycan (PGN) was a gift from BD Bioscience (San Jose, CA). The CpG-oligodeoxynucleotide (CpG-ODN 2006), corresponding to the sequence TCGTCGTTTTGTCGTTTTGTCGTT (Xie et al., 2003), was synthesized by Invitrogen (Carlsbad, CA). All other reagents including *Salmonella typhimurium* lipopolysaccharide (LPS), synthetic dsRNA analog poly I:C, bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), propidium iodide (PI), 1,4-dithiothreitol (DTT), 2-iodoacetamide (IAA), and 2,5- dihydroxybenzoic acid (DHB) were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Isolation of chicken blood heterophils

All animal procedures were approved by the University of Arkansas animal care and use committee. Blood was obtained from ten three week-old broiler chickens by cardiac puncture using K-EDTA Vacutainer tubes prior to killing. The K-EDTA anti coagulated

blood was subjected to centrifugation over One Step Polymorph™ gradient at 500 X g for 20 min. After removing the mononuclear cell layer at the inter phase of serum and the density gradient medium, the cells were further centrifuged for another 30 min to band the heterophils in the gradient and pellet red blood cells to the bottom. The bands rich in heterophil granulocytes, were washed following manufacturer's suggested protocol, pooled, and finally suspended in RPMI-1640 media containing 0.2% BSA. The viability and the purity of heterophils were determined by trypan blue exclusion and staining of cytocentrifuged cells with fluorescein isothiocyanate and propidium iodide respectively (Rath et al., 1998). The viability of the cells was determined to be more than 98%.

2.3. Activation of heterophils by TLR agonists

The heterophils were distributed at a concentration of 1×10^5 cells/ ml/ well in 12 well plates and cultured in triplicate with different TLR agonists for 6 h at 37°C. Different ligands, TLR1/2: peptidoglycan (PGN), TLR3: poly inosinic cytidylic acid (poly I:C), TLR4: gram negative bacterial lipopolysaccharide (LPS), TLR5: bacterial flagellin (FGN), TLR1/6: PAM3CSK4, TLR7: loxoribine; and TLR9: CpG-ODN, were prepared at 100X concentrations in RPMI-1640 medium and added to the cultures. TLR agonists may elicit a response by different mechanisms and they have different active concentrations. In order to be reasonably certain that these agonists would elicit heterophil activation and also the differential expression of AvBD2, concentrations of the TLR agonists were selected based on values recommended by the suppliers and in the literature (Kogut et al., 2005; Xie et al., 2003). The final concentrations of the TLR agonists were PAM (1µg/ml), PGN (1 µg/ml), FGN (1 µg/ml), poly I:C (1 µg/ml), LOX

(100 μ M), CpG-ODN (1 μ M) and LPS (1 μ g/ml). The control cultures received the diluent media only.

2.4. Sample preparation

After 6 h of stimulation, the conditioned media were aspirated and spun at 1,000 X g for 5 min to obtain supernatants free of cells and frozen at -20⁰C for subsequent use. Aliquots of the conditioned media were mixed with equal volumes of 100% methanol containing 2% acetic acid to denature and precipitate proteins over a 16 h period at 4⁰C, and centrifuged at 21,000 X g for 10 min to obtain the supernatant extract containing AvBD (Kannan et al., 2009). The residual protein concentrations of the media were measured using a BioRad dye binding assay in order to equalize the protein content of different samples if necessary.

2.5. Chemical modification

Aliquots of supernatant extract from different TLR treatment (T) groups were subjected to reduction for 1 h with 10 mM 1,4-dithiothreitol (DTT) followed by alkylation with 20 mM 2-iodoacetamide (IAA) in dark for 25 min at 37⁰C. Equivalent aliquots of both control (C) and treated samples were also subjected to similar conditions but without DTT/IAA additions. Thus, each group consisted of non-reduced (nr) and reduced/alkylated (r) samples based on their chemical treatments, C_{nr}, C_r, T_{nr}, and T_r respectively, (Fig. 1). They were used not only to calculate correction factors (CF) for individual groups but also to determine the relative changes in AvBD2 production due to treatments as described below.

2.6. Direct MALDI-MS analysis

For MALDI measurements, microliter volumes of sample were mixed 1:1 with 1M DHB, prepared in 90% methanol containing 0.1 % formic acid. Two μl of this mixture was spotted onto a Bruker MTP 384 MALDI target. Calibrated MALDI-TOF spectra were acquired over the m/z range from about 1-10 kDa in the positive ion reflector mode using either a Bruker Reflex III MALDI-TOF or a Bruker Ultraflex II MALDI-TOF-TOF MS (Bruker Daltonik GMBH, Bremen, Germany). Samples were analyzed in both manual and automation modes. The data were processed using Bruker Flex Analysis 2.4/3.0 and ClinProTools 2.2 softwares.

The samples were initially screened for the presence of AvBD2 by searching for ion signal at m/z 3916 indicated by its protonated intact ion. After confirmation of the presence of AvBD2, aliquots of the TLR agonist treated samples were reduced/alkylated to produce a characteristic mass shift of 348 Da to produce m/z 4264 that would differentiate them from the controls. The efficacy of this chemical modification was also confirmed by MALDI-MS. The control and treated samples were then mixed 1:1 for the determination of differential expression of AvBD2.

2.7. Determination of correction factor (CF)

Our procedure compares the signal intensities of AvBD2 peptides that are reduced/alkylated or are not. This approach assumes that the peak heights for the protonated molecules reflect the quantity of these AvBD2 species in modified and non-modified samples. However, the ionization efficiency of the two peaks can be affected by the complex environment, necessitating the use of correction factors (CF) estimated for

each group, CF_C for control and CF_T for treatments. Therefore, each sample split consisting of equal amounts of reduced/alkylated and non-reduced AvBD2 were mixed so that the theoretical intensity ratios would be 1. This 1:1 mixture was then subjected to direct MALDI-MS analysis and the actual intensity ratios of reduced/alkylated over non-reduced AvBD2 peaks (C_r/C_{nr} & T_r/T_{nr}) were measured for controls and each of the individual treatments as described in Fig. 1. Accordingly, the correction factors were calculated based on six mass measurements obtained from triplicate samples, $CF_C = C_{nr}/C_r$ and $CF_T = T_{nr}/T_r$. CF_C corrects for the differences in ionization efficiency due to reduction/alkylation, whereas CF_T corrects for the differences due to reduction/alkylation and the presence of TLR agonists. Since CF_T takes care of both the factors that may influence the ionization efficiency of AvBD2, we consider the use of only CF_T in the formula for calculating the relative changes in AvBD2 production.

2.8. Relative changes in AvBD2

Based on the peak intensities observed in the mass spectrum for reduced/alkylated and non-reduced AvBD2, the intensity ratios (T_r/C_{nr}) were calculated for all treatment groups. Relative changes in AvBD2 production were determined by multiplying the intensity ratio (T_r/C_{nr}) with appropriate correction factors CF_T for each treatment group (Fig. 1). The mean and standard error of the mean (SEM) was calculated from six mass measurements obtained using triplicate samples. The differences were determined using students *t*-test and a *P*-value ≤ 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

Defensins are important mediators of innate immunity; hence, there is a considerable interest in measuring their changes under a variety of conditions which challenge immunity. However, the occurrence of different isoforms of beta defensins which in chicken alone numbers upto 14 variants (Lynn et al., 2007; van Djik et al., 2008), has precluded the development of some common immunoassays that may be broadly applicable to all of them. In fact, commercially available immunoassays of defensins are largely limited to mammalian species (Gardner et al., 2009; Ghosh et al., 2007).

Consequently, RT-PCR has been the method of choice for most AvBD related studies, which measure the changes in mRNA populations. However, incongruity in the expression levels of mRNA and proteins that may relate to alternative splicing, post transcriptional gene silencing and protein modifications can preclude accurate estimation of changes at the peptide level. Therefore, the current method may be suitable to measure the changes in BD levels using simple chemical modification of their highly conserved cysteine residues rather than more expensive stable isotope methods.

Our results show that the heterophils constitutively produce AvBD2 as evident from the protonated ion observed in direct MALDI-MS spectra (Fig. 2) (Kannan et al., 2009). Generally, in mass spectrum the signal intensity of the peaks reflect the quantity of materials present; however, the experimental conditions and chemical modifications can alter the ionization efficiency of the peaks (Guerrera and Kleiner, 2005; Yan and Chen, 2005). Therefore, we checked to determine whether the ionization efficiency of the reduced/alkylated and non-reduced AvBD2 were identical. AvBD2 containing samples (treated and controls) were split and half of the sample was reduced/alkylated, before

remixing to measure the different signal levels for nominally identical quantities of both forms of AvBD2 in the same sample. The prominent ion corresponding to AvBD2 (m/z 3916) shifted in reduced/alkylated samples by 348 Da, showing complete carbamidomethylation of 6 cysteine residues resulting in the mass of 4264 (Fig. 3a, b). In general, carbamidomethylation is an efficient and reliable chemical modification method widely used in many proteomic studies (Simpson, 2003). In this experiment, we confirmed the labeling to be 100%, with no trace of non-reduced AvBD2 in the TLR agonist treated samples. Fig. 4a shows a MALDI spectrum for a 1:1 mixture of non-reduced and reduced/alkylated forms of AvBD2 in the control sample whereas Fig. 4b to 4h show spectra for a 1:1 mixture of the control (non-reduced) and TLR agonist treated (reduced/alkylated) samples. The peak at m/z 3916 represents AvBD2 in untreated control group whereas the peak at m/z 4264 corresponds to the reduced/alkylated AvBD2 from the treated sample. Changes in relative intensity ratios of m/z 4264 and m/z 3916 are taken to reflect the changes in AvBD2 production induced by different TLR agonists. However, it is evident that the intensity ratio obtained directly from the MALDI spectra does not truly represent AvBD2 populations. Therefore, correction factors were estimated to calculate the changes in the ionization efficiency of AvBD2 due to both reduction/alkylation and presence of TLR agonists. The average CF and the SEM for different groups were as follows; controls, 1.5 ± 0.1 ; PGN, 2.0 ± 0.1 ; Poly I:C, 2.2 ± 0.2 ; LPS, 2.2 ± 0.2 ; FGN, 1.8 ± 0.3 ; PAM, 1.5 ± 0.1 ; LOX, 2.2 ± 0.3 ; and CpG, 2.0 ± 0.1 .

The overall results show that most TLR agonists increased the secretion of AvBD2 by heterophils albeit to different extents except for CpG-ODN which showed no change (Fig. 5). The inability of CpG-ODN to incite production of AvBD2 may be that, it

is not a potent inflammatory agent in the concentration used in this study or due to the absence of TLR 9 receptor in chickens for which it acts as a ligand (Boyd et al., 2007; Dar et al., 2009; Kogut et al., 2006; Temperley et al., 2008). However, many studies using CpG-ODN in poultry models have shown it to activate of both macrophages and heterophils (Xie et al., 2003; Kogut et al., 2006). Therefore, the inability of CpG-ODN to induce AvBD2 remains unexplained. LPS is a potent inflammatory agent, which induced maximal changes in AvBD2 production ($P \leq 0.001$); others such as PGN, PAM, FGN, LOX and poly I:C also showed significant increase in its production ($P \leq 0.05$) consistent with their efficacies to activate heterophils (Kogut et al., 2005).

Quantitative analysis of human defensins by mass spectrometry has been reported using polyacrylamide gel electrophoresis, heavy stable isotope labeling, liquid chromatography, and electro spray ionization (ESI) mass spectrometry (Jorgensen et al., 2006; Thompson et al., 2006; Zhou et al., 2002). The advantage of the current procedure is that the measurement of the relative changes in BD can be achieved without stable isotope labeling or liquid chromatography and the assay can be performed directly and quickly in complex mixtures.

In conclusion, these results demonstrate that the method of chemically modifying AvBD without stable isotope tagging followed by direct MALDI-TOF-MS analysis may be a suitable and inexpensive method to study their differential expression. This general method of relative quantification can likely be adopted for any system where peptide of interest contains at least one cysteine residue.

ACKNOWLEDGEMENTS

We thank Sonia Tsai and Scott Zornes for technical assistance and Drs. Yuchun Du and Mohan Achary for their helpful reviews. This work was supported in part by a grant from Arkansas Biosciences Institute.

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FIGURE LEGENDS

Figure 1: A flowchart showing the experimental protocol to determine the relative changes in AvBD2 production by chicken heterophils upon TLR activation.

Figure 2: MALDI-TOF spectral profiles of heterophil conditioned media extract prepared from control and TLR agonist treated cultures showing AvBD2 peak (m/z 3916).

Figure 3: MALDI-TOF mass spectra of heterophil conditioned media extracts showing AvBD2 peaks (a) without reduction and alkylation and (b) after reduction and alkylation.

Figure 4: MALDI-TOF-MS profiles of 1:1 mixture of non-reduced and reduced AvBD2 forms. (a) control sample showing both non-reduced (m/z 3916) and reduced (m/z 4264) and (b) to (h) represent equivalent mixtures of control non-reduced (m/z 3916) and TLR agonist treated reduced (m/z 4264) AvBD2.

Figure 5: Relative changes in the production of AvBD2 by heterophils induced by different TLR agonists compared with control. Data represents the mean value \pm SEM for six mass measurements from triplicates samples in each groups. * indicates significant statistical differences with control $p \leq 0.05$ and ** represents $p \leq 0.001$.

Figure 1.

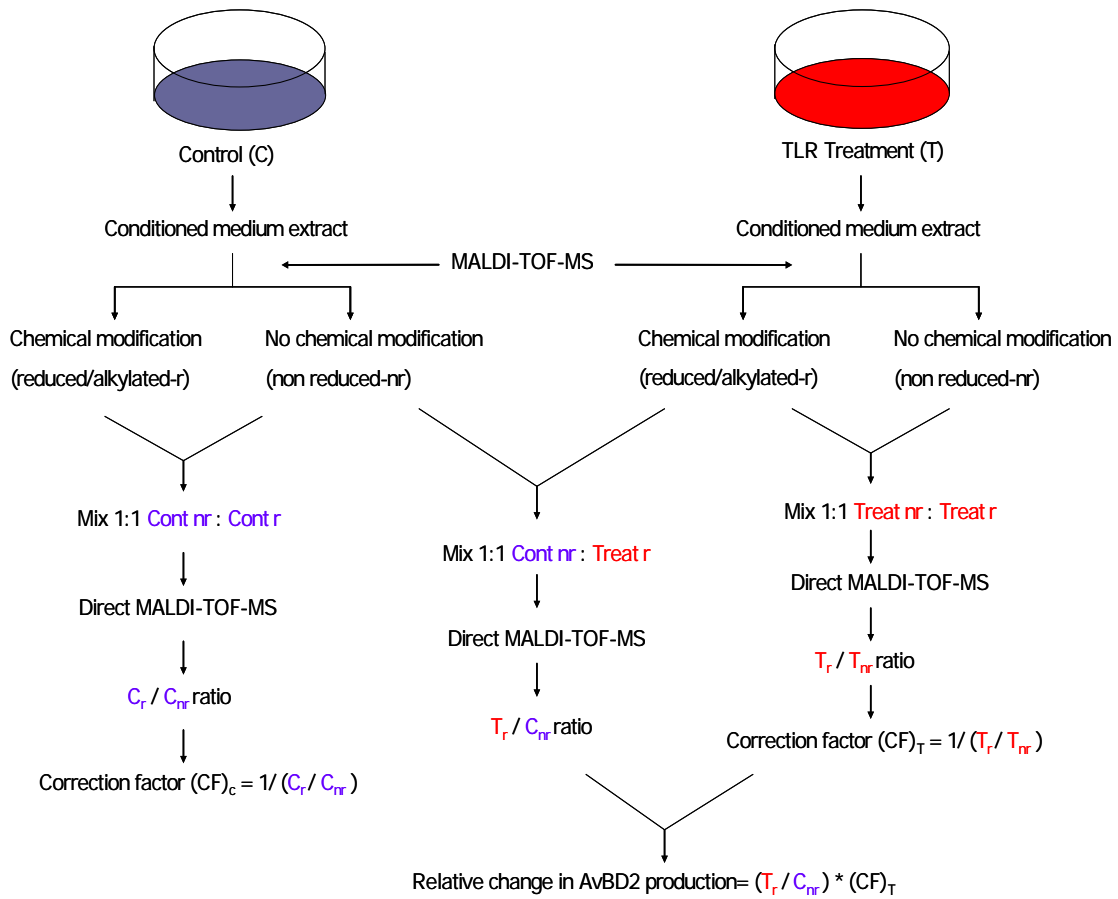


Figure 2.

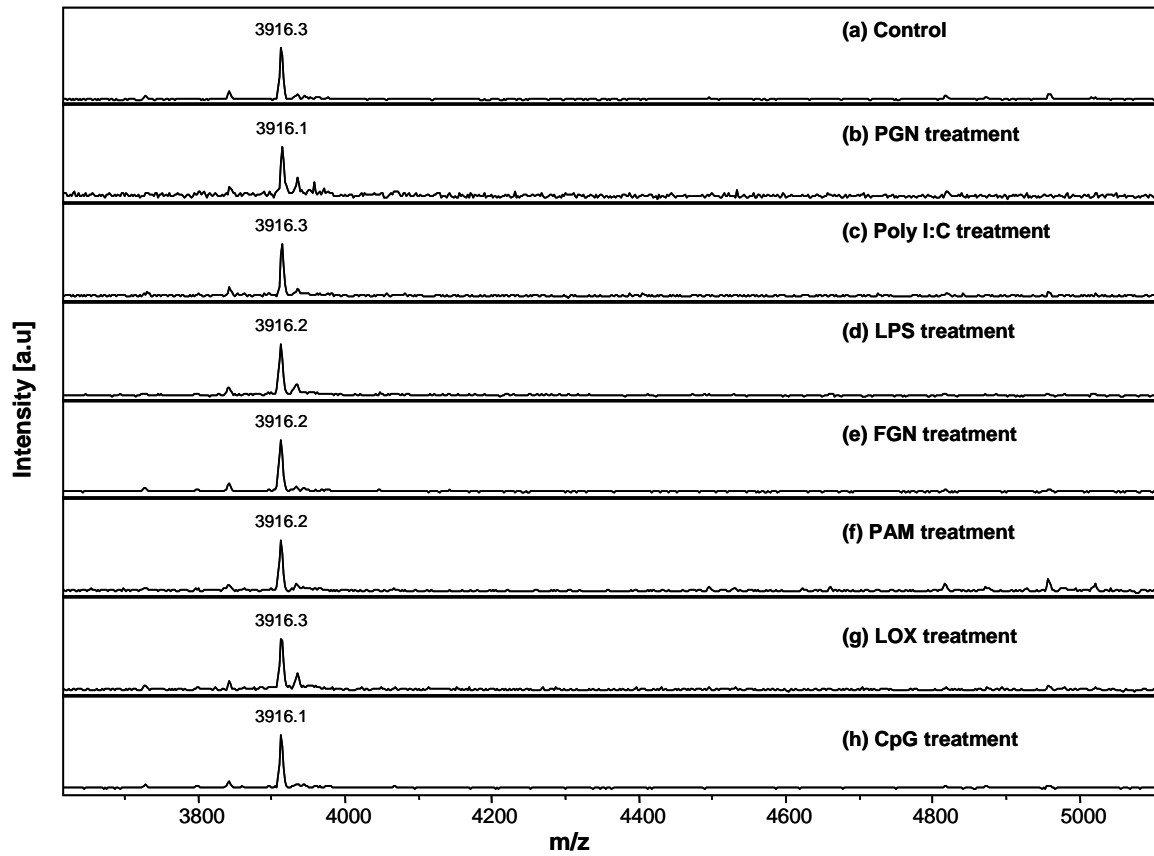


Figure 3.

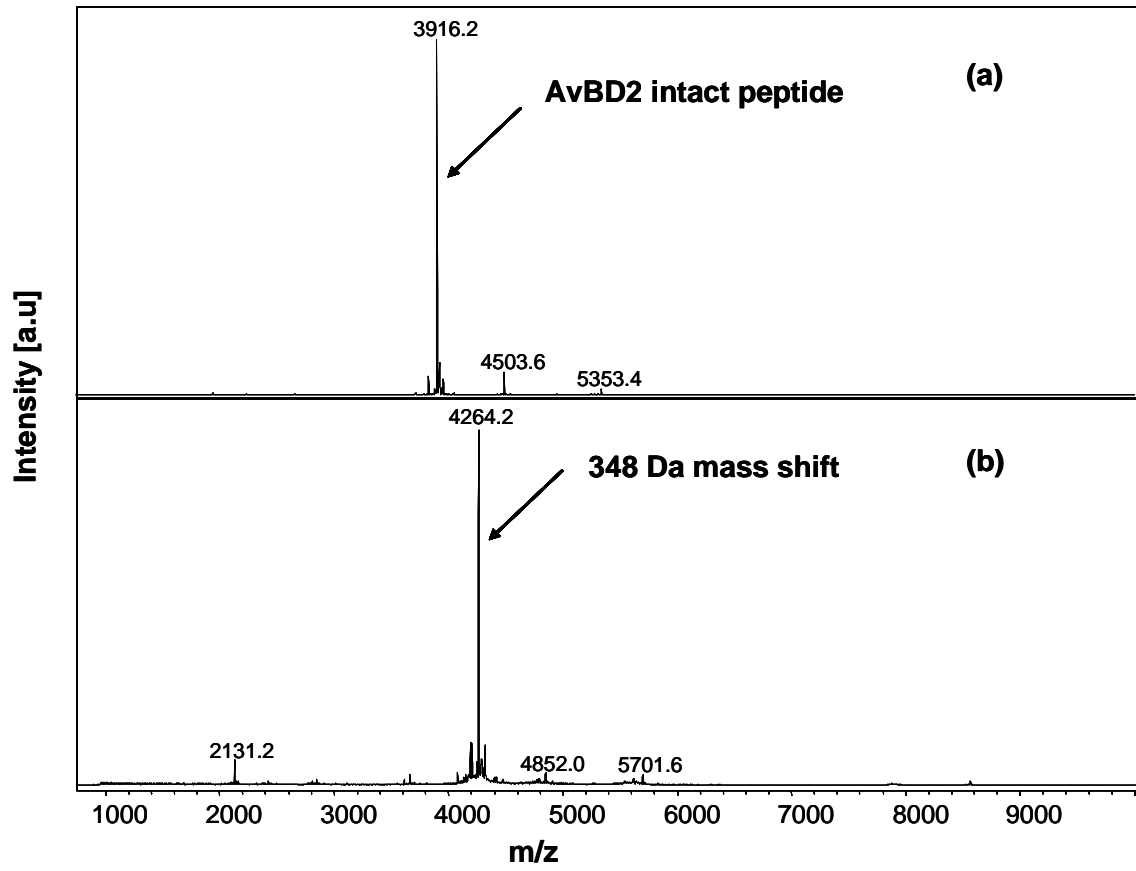


Figure 4.

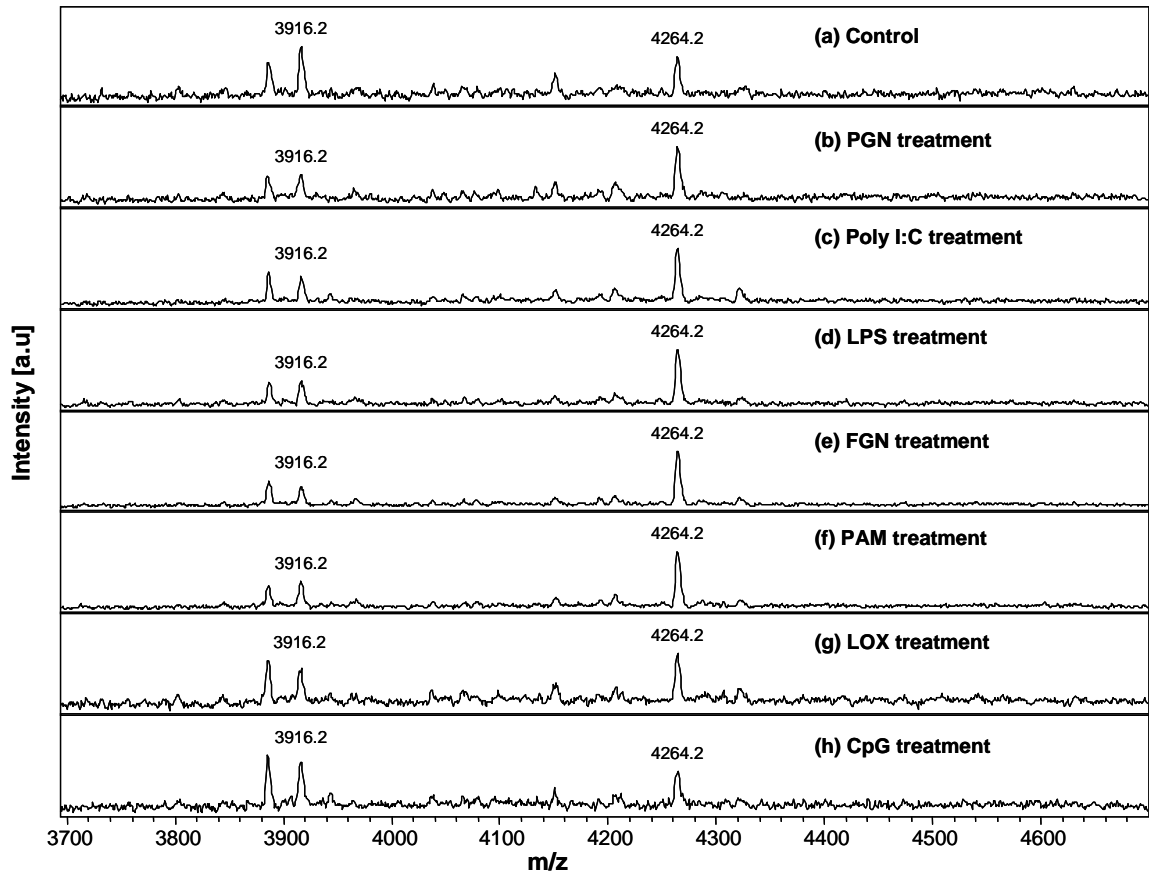
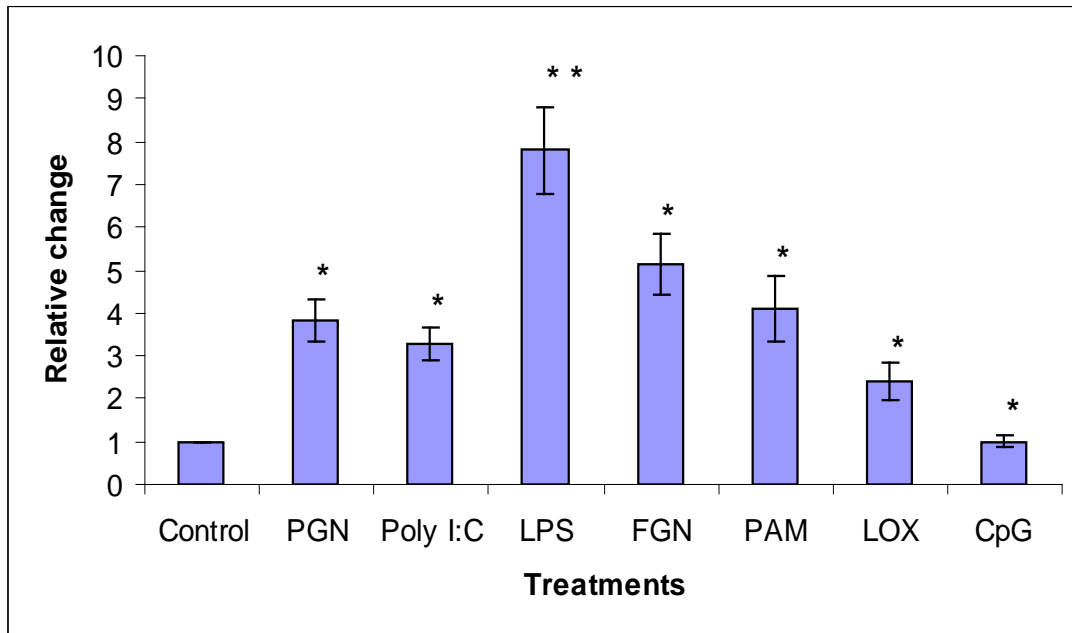


Figure 5.



CONCLUSION

Owing to the ubiquitous role of peptides in many physiological processes as hormones, neurotransmitters, growth and antimicrobial factors, we hypothesized that many such peptides occur as native components of cells as part of its specialized functions and peptide levels are likely regulated under physiological demand.

We used avian mononuclear cells/macrophages and heterophils as models of specialized cells that are central components of innate immunity. Using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry we screened whole cells and their extracts to determine similarities and differences in their spectral profiles. The high intensity mass spectral peaks were used as the criteria of selection to identify and characterize the corresponding peptides from each of the above cell populations. Then the quantitative changes in peptide levels were studied under TLR activation of these cells mimicking immunomodulation.

Screening mononuclear cells, transformed avian macrophages, and heterophils identified a high intensity spectrum corresponding to m/z 4963 expressed differentially in mononuclear cell but not in heterophil populations which exhibited its specific profiles. Using proteomic approaches such as reverse phase high performance liquid chromatography-electrospray ionization, N-terminal sequencing, and peptide mass fingerprint, m/z 4963 was identified as thymosin beta 4 ($T\beta 4$), an actin binding peptide. The mature form of the peptide identified was found to be modified and had lower mass than that predicted from its corresponding genomic sequence (Chapter 2). Similarly, we identified and characterized avian beta defensin 2 (AvBD2), as an abundantly occurring antimicrobial peptide in heterophils of both chickens and turkeys with 90% sequence

homology (Chapter 3). The study on beta defensins was extended to two other related avian species, pheasant and quail. Chapter 4 deals with reduction and alkylation approach, incorporated to general MALDI screening strategy, to selectively screen peptides having 6 cysteine residues followed by mass spectrometric fragmentation method to sequence the peptide. The approach proved to be rapid in place of time-consuming HPLC isolation and MALDI peptide mass fingerprinting (PMF) method. The study yielded the mature peptide sequences of pheasant and quail orthologs of AvBD2 (Chapter 4). This is the first report of these peptides in pheasant and quail. These results demonstrate that MALDI-TOF is a potential tool to rapidly screen and detect low molecular weight proteins and peptides associated with cells, tissues, and biological fluids that cannot be resolved by 2-D gel electrophoresis. Also, these studies show that a simple, rapid and direct screening of crude extracts by MALDI-TOF-MS can detect important homologous peptides relevant to disease or other peptidomic endpoints, even across species lines.

Tβ4 is an anti inflammatory peptide involved in tissue remodeling, angiogenesis and wound healing while avian beta defensins are antimicrobial peptides although their other physiological roles are not completely understood. AvBD2 has been suggested to act as an immunomodulator and anti inflammatory factor. Thus it postulates that these peptides need to be regulated under conditions, which evoke an innate immune activity. A reasonable assumption with reference to the regulatory mechanisms of these peptides can be related to Toll like receptors (TLR). The TLRs are the primary sensors of innate immunity acting as the pattern recognition molecules on the surface of both monocytes/macrophages and heterophils. Recognition of different ligands derived from

different pathogen sources such as bacteria, virus, parasites, and fungi can elicit both common and separate immune responses. Thus, the recognition of these pathogens would lead to the activation of intracellular signaling pathways to modulate the production of these peptides along with other pro and/or anti inflammatory molecules. We studied to find the effects of different TLR agonists on the expression and production of T β 4 in a chicken transformed macrophage cell line and AvBD2 in chicken blood heterophils using gene expression and peptide quantification as described in Chapters 5 and 6.

Chapter 5 discusses the activation of different TLRs by corresponding agonists and their effects on T β 4 production by chicken macrophages. In view, of its multiple effects such as wound healing, angiogenesis and tissue remodeling, the study was done to understand the mechanism of its release to the extracellular environment. Gene expression studies by real time PCR showed only few agonists, lipopolysaccharide, peptidoglycan, and synthetic lipoprotein PAM3CSK4, that stimulate activation of TLR 2, 4, and TLR2/1 respectively, up-regulated the expression of Il-6, Il-1 β and iNOS but had no effect on T β 4. Stable isotope labeling with amino acids in the cell culture (SILAC) followed by mass spectrometry, to study T β 4 secretion showed these ligands caused a significant decrease in cellular T β 4 content at 24 h. The decrease was accompanied by a corresponding release of T β 4 into the culture medium. In the absence of its synthesis, the release of T β 4 into the culture medium appears to be associated with cell death as indicated by LDH measurements in the culture medium. These results imply that T β 4 in extracellular fluid originates from dying cells rather than active secretions. This contributes to the understanding of the origin of T β 4 in the wound fluids.

Chapter 6 describes a novel method to quantify AvBD2 production by heterophils under immunomodulating conditions of TLR activation. It uses chemical modification and mass spectrometric approach to measure the changes in AvBD2 levels. The results show significant differences among the different TLR agonist treatment groups in eliciting AvBD2 release into the culture medium with LPS having highest effect and the CpG showing no effect. Also peptidoglycan, PAM3CSK4, flagellin, and Poly I:C showed significant secretion of AVBD2. In the absence of an available immuno assay method for AvBDs, the current method which uses chemical modification of cysteinyl residues in BDs provides an approach to measure the relative levels of any AvBD isoforms in complex biological mixtures. This approach of modifying beta defensins and MALDI-TOF-MS analysis appears to be a suitable tool which is simple, cost and time effective.

These studies provide a paradigm of expression peptidomics of cells which likely are consequential for their physiology. Identifying and characterizing all cell or tissue associated peptides will provide better understanding of their functions and their application potentials.

A BIOGRAPHICAL SKETCH- LAKSHMI KANNAN

Education

- Aug 2009 Ph.D, Cell & Molecular Biology, University of Arkansas, Fayetteville, AR
- May 2004 M.Sc., Biotechnology, University of Madras, Chennai, India
- May 2002 B.Sc., Chemistry, University of Madras, Chennai, India

Professional Experience

- Aug 2005- Aug 2009 **Senior Graduate Research Assistant** in the Cell & Molecular Biology Program, University of Arkansas, Fayetteville, AR
- Apr 2003-Sep 2003 **Research Trainee** in Department of Biochemistry, Indian Institute of Science, Bangalore, India

Honors and Awards

Dale Bumpers Distinguished Doctorate Scholar in the Dale Bumpers College of Agricultural, Food and Life Sciences, University of Arkansas (2009); First Place in oral presentation and second place in poster presentation at student research competition sponsored by Gamma Sigma Delta Honor Society, University of Arkansas (2009); received Student Travel Award from Arkansas Biosciences Institute (ABI) to participate and present in the ABI Fall Research Symposium, Little Rock, AR (2008); awarded a Certificate of Excellence for outstanding presentation in the immunology section at Poultry Science Association, Niagara Falls, Canada (2008); received Tom Sullivan Award for First Place in the scientific presentation at the Poultry Science Graduate Association Summer Symposium at Department of Poultry Science, University of Arkansas (2008); recipient of Student Travel Award from the Aegean International Conference to participate and present in the 5th International Conference on Innate Immunity at Crete, Greece (2008); awarded Certificate of Excellence for outstanding achievement in leadership, service, campus activities, and academics by a national program known as Who's Who among Students in American Universities and Colleges, Center for Leadership and Community Engagement, University of Arkansas (2008); awarded First Place in the poster presentation sponsored by Gamma Sigma Delta Honor Society, University of Arkansas (2008); awarded a Certificate of Excellence for outstanding presentation in the immunology section at Poultry Science Association, San Antonio, TX (2007); awarded with a free Registration Grant to participate and present in the First International Symposium on Thymosin at Washington DC (2007); received Merit Scholarship during M.Sc. Biotechnology for the academic years, University of Madras, Chennai, India (200-2004); awarded the Best Outgoing Student during B.Sc. in Chemistry for the Academic years, University of Madras, Chennai, India (1999-2002); received merit of First Place in Physical Chemistry, University of Madras, Chennai, India (2002).

Publications and Scientific Presentations

Published 7 peer-reviewed papers, 3 as primary and 4 as co-author and 2 more as primary author are in the process of review. Number of oral and poster presentations in international, national and regional meeting include 24.

Academic and Professional Memberships

American Association for the Advancement of Science, American Society of Mass Spectrometry, American Association of Immunology, Poultry Science Association, Associate Member of Sigma Xi International Honor Society of Scientific and Engineering Research, University of Arkansas Chapter, Poultry Science Graduate Student Association.

