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Initial Mapping of the Chicken Blood Plasma Proteome[1, 2]

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Abstract: Proteomics is the study of the entire protein compliment of an organism. The blood plasma is the only tissue in which an organism's entire proteome may be potentially represented. First results toward mapping the broiler plasma proteome are presented here. Blood was taken from eight 18 day-old representative commercial broiler chickens. Plasma was isolated from each sample and pooled. For initial sample fractioning a 0.4 µl aliquot of the pooled plasma was run on one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Based on relative amounts of protein, the gel was divided into three fractions. The proteins were in-gel digested with trypsin. Two-dimensional liquid chromatography in-line with electrospray ionization tandem mass spectrometry was then used for "shot-gun" qualitative plasma proteomics. The resulting tandem mass spectra were then searched against the non-redundant chicken protein database. Generally accepted high stringency statistical criteria for protein identification were used. Eighty-four chicken proteins were identified. Our work demonstrates the future potential for plasma proteomics for identifying biomarkers of disease and production in chickens.

Key words: Blood plasma, broiler, electrospray ionization, mass spectrometry, proteomics

Introduction

The complete annotated chicken genome sequence will soon be available. This genome sequence provides the foundation for understanding the molecular basis of chicken-normo- and patho-physiology (Burgess, 2003). However, it is the protein products of the genome that execute the majority of metabolic functions and constitute the largest proportion of cell structures. Proteomics, which is the study of the entire protein compliment of the genome (i.e. the proteome), promises to have considerable application to all aspects of poultry production in the future. Having an annotated genome sequence makes the chicken the first livestock species for which proteomics is a viable proposition.

Plasma is the liquid part of the blood. Because plasma is the only tissue in which every single protein in the body (postulated to be at least 500 thousand proteins, Anderson and Anderson, 2002) could potentially be present, it holds immense potential for diagnosing, understanding and treating human and animal disease (Yudell *et al*., 2002; Tirumalai *et al*., 2003). Plasma is currently the most common tissue used for diagnosis of disease and nutritional status. Yet despite this, only a tiny fraction of the information present in plasma is currently available. Nucleic acid techniques such as cDNA microarray analysis, PCR and serial analysis of gene expression are inappropriate for plasma. Plasma is a collection of proteins; the only nucleic acids present will be the leakage products from cells or from cell free pathogens. Functional genomic analysis of plasma can only be done by proteomics.

For any species that has an annotated sequenced genome, high through-put identification of plasma biomarkers by proteomic techniques is desirable and is becoming possible (Anderson and Anderson, 2002). Defining the human plasma proteome is the priority for the Human proteome organization (Bunk, 2003). Because the proteome is context dependent, it is critical to define the exact state of the animal from which the plasma was taken. Furthermore, to increase the utility of plasma proteomics for identifying biomarkers, it is desirable to include micro-organism genomes in the analysis. In our study, two-dimensional liquid chromatography electrospray ionization tandem mass spectrometry (2-D LC ESI MS/MS) is described for the proteomic analysis of 0.4 µl of pooled plasma from eight representative 18-day-old commercial broiler chickens raised under environmental and nutritional conditions typical of those in a commercial production setting.

Materials and Methods

Chickens and plasma sampling: Fifteen commercial Ross x Ross 508 broiler day-old male chicks were obtained from a commercial hatchery after being given an antibiotic (Naxcel)[5] and vaccinated against Marek's Disease (HVT/SB1)[6] by in ovo injection at Day 18, and Newcastle disease (B1 type, B1 strain)[6] and infectious bronchitis (Massachusetts and Connecticut types, live)[6] by aerosol at hatch. The chicks were placed in floor pens, on fresh pine shavings, at the Poultry Science Research Farm, Mississippi State University. The pen was equipped with one tube feeder, and a nipple line (3

nipples/pen). Pen density was 0.07m^2/bird (0.9 x 1.2 m/pen), comparable with industry guidelines (Aviagen, 2003). A gas brooder kept the pen at thermoneutral conditions, and lighting was continuous. The diet was formulated to meet nutritional recommendations (NRC, 1994) for optimal growth (Table 1) and was fed in mash form. At 18 d of age, eight chicks closest to the pen mean body weight and in good health were removed and a blood sample (1 ml) was immediately collected by cardiac puncture and centrifuged (4000 rpm, 10 min). Plasma was removed from each sample, pooled, mixed, divided into 500 µl aliquots and immediately frozen at -80 °C until use.

One-dimensional SDS PAGE : One dimensional SDS-PAGE was used as an initial fractionation step. One plasma sample was thawed on ice and a 5 µl aliquot removed and diluted 1/25 using ddH_2O. Ten µl of the diluted plasma sample (equivalent to 0.4 µl plasma) was run on a non-reducing one-dimension SDS polyacrylamide gel (1.5 mm) by electrophoresis. After electrophoresis the gel was stained (Fig. 1) with Coomassie brilliant blue[7]. Three groups of proteins were identified and cut from the gel using a scalpel. Group 1 contained the most abundant proteins (the two darkest staining discrete bands); group 2, the next most abundant proteins (pale staining discrete bands); and group 3 contained the proteins in the remaining gel in the lane (i.e., the faintest staining portion much of which was invisible to the naked eye). The gel containing the group three proteins was cut into 1mm slices and put into a 15 ml tube (Falcon)[8].

Table 1: Composition of diet

Ingredient	g/kg
Yellow corn	628.47
Soybean meal 48%	248.34
Corn gluten meal	50.00
Poultry oil	26.27
Dicalcium phosphate	18.28
Limestone	10.97
Salt	4.60
DL-Methionine	2.72
Vitamin/mineral premix[1]	2.50
Coban	0.75
Other ingredients	7.00
Calculated composition	
ME (Kcal/kg)	3,100
CP (%)	20.5
Lys (%)	0.95
TSAA (%)	0.97
Ca (%)	0.90
Available P (%)	0.45
DEB (meq/kg)[2]	178.6
Analyzed composition	
CP (%)	21.3

[1]The vitamin and mineral premix contained per kg of diet: retinyl acetate, 2,654 µg; cholecalciferol, 110 µg; dl-α-tocopherol acetate, 9.9 mg; menadione, 0.9 mg; B_{12}, 0.01 mg; folic acid, 0.6 µg; choline, 379 mg; d-pantothenic acid, 8.8 mg; riboflavin, 5.0 mg; niacin , 33 mg; thiamin, 1.0 mg; d-biotin, 0.1 mg; pyridoxine, 0.9 mg; ethoxiquin, 28 mg; manganese, 55 mg; zinc, 50 mg; iron, 28 mg; copper, 4 mg; iodine, 0.5 mg; selenium, 0.1 mg. [2]Dietary electrolyte balance; Na+K-Cl.

In-gel trypsin digestion: All volumes used ensured that the gel pieces were covered (group 1 and 2 = 50 µl; Group 3 = 150 µl). All incubations were at ambient room temperature unless stated. For Coomassie brilliant blue destining, equal volumes of ammonium bicarbonate (100mM)[9] and HPLC grade acetonitrile (ACN)[10] were added to the gel factions and incubated (ambient temperature, 10 min). The ammonium bicarbonate/ACN was aspirated and discarded. Then ACN was added to dehydrate the gels (5 min). The ACN was then aspirated and discarded. To reduce the proteins, dithiothreitol (10mM)[7] was added, incubated (30 min), aspirated and discarded. To alkylate the proteins, iodoacetamide was added (55mM)[9], incubated (20 min), aspirated and discarded. ACN dehydration was then repeated 3 times (5 min incubation each), aspirated and discarded. Residual ACN was removed by drying in a vacuum centrifuge. Sequencing grade modified trypsin10 was then added and incubated (12 h at 37 °C). A peptide extraction solution (1% trifluoroacetic acid9, 2% ACN) was added to the tubes, incubated (30 min), aspirated and transferred to a new tube. Extraction solution (12 µl) combined with dehydration solution (12 µl) were added to the tubes, incubated (30 min), aspirated and transferred to new tubes. The samples were then resuspended in 0.1% formic acid.

Plasma protein identification: The peptides obtained from the in-gel digestion were desalted using a peptide microtrap[12], eluted in 95% ACN, 0.1% trifluoroacetic acid, dried using a vacuum centrifuge and then resuspended in 0.1% formic acid (20 µl). The samples were analyzed by strong cation exchange (SCX), followed by reversed-phase liquid chromatography coupled directly in-line with electrospray ionization ion trap tandem mass spectrometry. The peptide sample was loaded directly into the sample loop of the HPLC (Proteome X workstation)[13] using an LC gradient ion exchange system consisting of a quaternary gradient pump (Thermo Separations P4000)[13] equipped with BioBasic SCX, 0.32 x 100 mm and BioBasic C18, 0.18 x 100 mm columns[14]. The salt gradient was in steps of 10, 23, 37, 51, 70, 99, 300 and 700 mM ammonium acetate in 5% ACN, 0.1% formic acid. The reversed phase gradient used ACN in 0.1% formic acid starting at 5% and using a linear gradient going to 30% in 30 min, then from 30%

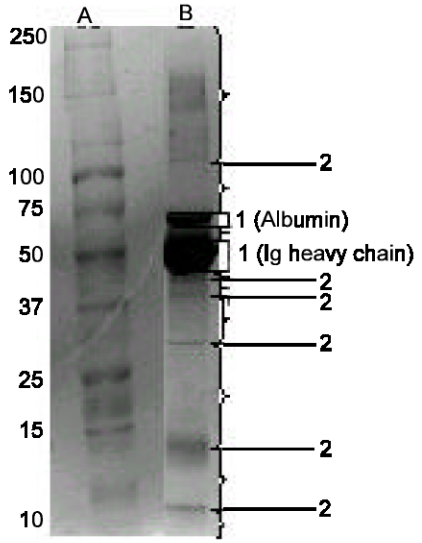

Fig. 1: One-dimension Coomassie brilliant blue-stained sodium dodecyl sulphate polyacrylamide gel electrophoretogram of pooled chicken plasma. Lane A: molecular weight standards (Mw in kDa). Lane B: 0.7 µl of 18 day old broiler blood plasma pooled from 8 chickens. Three groups were of bands cut from the gel: groups one and two are labeled, group three contained all proteins in the remaining parts of the gel after groups one and two were removed (marked as).

and using a linear gradient going to 65% in 9 min, then 95% ACN for 5 min followed by 5% ACN in 15 min. The spectrum collection time was 59 min for every SCX step. The LCQ Deca XP ion trap mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by alternating between a single full MS scan followed by three MS/MS scans on the three most intense precursor masses (as determined by Caliber mass spectrometer software in real time) from the full scan. Dynamic mass exclusion windows were 2 min. In addition, MS spectra for all samples were measured with an overall mass/charge (m/z) range of 200-2000.

Resultant mass spectra and tandem mass spectra were used to search the non-redundant protein database downloaded from the National Center for Biotechnology Information (NCBI; September 21, 2003) using TurboSEQUESTTM Bioworks Browser 3.1[13]. TurboSEQUESTTM cross-correlates experimentally acquired mass spectra with theoretical mass spectra generated in-silico. The idealized spectra were weighted with b and y fragment ions. For example, fragments resulting from the amide linkage bonded from the N and C termini. Trypsin digestion was applied to generate the "parent ions", and the database searched against had plant protein sequences that were removed, but included mass changes due to cystine carbamidomethylation and methionine oxidation. The peptide (MS "parent") ion mass tolerance was set to 2.5, and the fragment ion (MS^2) tolerance was set to 0.0. For a protein to be considered to be identified in the sample, it had to meet one of the following criteria:

A. More than two peptides with X-correlation >1.5 (+1 charge), >2.5 (+2 charge), >3.5 (+3 charge); delta correlation value >0.1; preliminary score >200; Rank/ preliminary score <11.
B. One or more peptides with X-correlation >3.5 (+1 charge), >4.0 (+2 charge), >4.5 (+3 charge).
C. One peptide that was uniquely present in only one protein with X-correlation >1.5 (+1 charge), >2.5 (+2 charge), >3.5 (+3 charge).

Results and Discussion

Primary objective of this study was to begin to catalog chicken plasma proteins after optimizing a protocol for identifying plasma proteins by 2-D LC ESI MS/MS. Although the point of 2-D LC ESI MS/MS is to avoid gel-based electrophoretic steps (Burgess, 2003), higher abundance proteins interfere with the identification of lower abundance proteins (Adkins *et al*., 2002). We were seeking a simple method to initially fractionate the plasma so as to increase the dynamic range of the mass spectrometry analysis. The high abundance proteins in plasma include albumin and immunoglobulins (Turner and Hulme, 1971; Burtis *et al*., 2001). Plasma may also have proteins that are present at relatively lower concentrations. These may occur as a result of apoptotic activities, synthesis and/or secretion, among others, from tissues throughout the body (Kennedy, 2001; Schrader and Schulz-Knappe, 2001). Especially during necrosis, apoptosis, and hemolysis, cell contents containing a plethora of proteins are released and enter the blood plasma. Being able to identify these proteins from blood samples is the benefit to using proteomics for identifying biomarkers of disease. Such proteins are already targets for classical clinical pathology testing.

Like clinical pathology, one of the aims of proteomics is to identify relative or absolute changes in protein concentrations, or states, in association with biological processes (such as diseases) to be used as biomarkers. Being able to identify many proteins quickly is fundamental to rapidly screening many plasma/serum samples from many disease processes/physiological states for efficient biomarker discovery and validation.

We fractionated our plasma sample using 1-D electrophoresis. The total plasma proteins were separated into three fractions and 84 separate proteins were identified from the 0.4 µl of plasma loaded onto the gel. Despite the very small volume, to our knowledge

Table 2: Proteins identified from blood plasma, present in the dense fraction of a sodium dodecyl sulphate polyacrylamide gel electrophoretogram

Protein	Accession number	Example peptide sequence
Albumin	113575	LVKDVVDLAQK
Attachment region binding protein	2388805	YDVYLINPQGKAFRSK
Axonin-1	114735	ASVKEAVGDLAIVNAQLK
Bloom's syndrome protein homolog	17366086	IFDVHYFNIFSTSTLK
Brain-derived neurotrophic factor	2118555	RGELSVCDSTSEWVTAAEK
Breast basic conserved protein 1	730527	RRNKSTESLQANVQR
Cation-independent mannose 6 phosphate receptor	15022505	TLLVFNTTSK
Collagen type XX alpha 1 precursor	14280020	GTQPKGSGETM#R
Connectin/titin	11359853	QEAHFTLTVQR
Connexin 31	3746662	KGDQKCEYK
Cyclic nucleotide gated channel	2493752	DDLMEALTEYPDAKAM#LEEK
Cysteine-rich protein 2	1705932	MPNWGGGNKCGACGR
DAN	15823273	MC*PPSPGC*C*R
Delta-crystallin enhancer binding factor	544148	AFKYKHHLK
Dorsal neural-tube nuclear protein	18623510	ALNGGCPIQTDAR
EURL protein	18203020	NC*SKLPC*LQM#GRAGMK
Ferritin repressor protein	2492644	YARLPFSIR
Fibrinogen alpha/alpha-E chain	1706798	ESPSLVDKTSSASSVHR
Fibrinogen beta chain	399491	IQKLENAIATQTDYC*R
Fibrinogen gamma chain	3641556	LSIDGQQHSGGLK
Fibrochimerin	2506307	GEKGDRGDIASQNM#M#R
Fibulin-1	2947316	CLSLEC*PENYRK
Glutathione-requiring prostaglandin D synthase	6225842	NQDVKEKAFNDILTNK
GTPase activating protein	5650776	LINAEYSC*YR
Hemoglobin beta chain	122587	NTFSQLSELHC*DK
Hemoglobin beta-H chain	2118956	LLIVYPWTQR
Hemoglobin epsilon chain	122723	NTYAKLSELHC*DK
Heparin cofactor II	3132623	LNILNANFGFNLYR
Hexokinase 1	23821300	FKASGVEGADVVK
Homeobox protein Hox-B1	399997	VKRNPPKTAK
Homogenin	3688784	EPDNFWVALGGK
Ig lambda chain	125945	VAPTITLFPPSKEELNEATK
Immunoglobulin lambda chain	13990832	APGSAPVTVIYSNNQRPSDIPSR
Interferon gamma	1708496	NEKRIILSQIVSM#YLEMLENTDK
Inversin	18448956	TSFM#WAAGKGSDNVIR
Ip3ka	4584405	LMKDPLRSCVPCFHGVVER
LIM domain interacting RING finger protein	4959046	TC*SVC*ITEYTEGNKLR
LIM domain-containing homeodomain LH-2A	7441992	RFSVQRCARCHLGISASEMVMR
Makorin-2	17017225	AFAFQASQDKVC*SICMEVVYEK
Matrilin-1	115555	AAVKKM#AYMEK
Myosin heavy chain	86369	SRAIRQAKDER
Myosin light chain kinase	2851396	GQTSTSWLLTVKRPK
Neurogenic differentiation factor	6685684	ERTRM#HGLNDALDNLR
ORF1	391638	SSAKTM#SESFKSK
Ovotransferrin chain	1127086	ATYLDC*IK
Ovotransferrin precursor	1351295	KDSNVNWNNLK
Pericentriolar material-1	23320951	VFSKKNREQLEK
PIT 54	13434994	QLGC*GTALSSPKK
Proto-oncogene tyrosine-protein kinase	462471	PGTM#SPEAFLEEAQIMKR
Pyruvate carboxylase	22128505	ALLGAWRLPLLR
Regulator of G protein signaling 3 isoform	32263639	KFCILYLAEK
Restricted expression proliferation associated protein-100	28071277	NKLTVPSTPTM#LKR
Retinoic acid receptor rxr-gamma	133700	VLTELVSKMKDMQMDK

Protein	Accession number	Example peptide sequence
Semaphorin 3E	8134691	MPC*PMQSNIPQVSK
Slit-3	16118485	CLHGKC*MAANVAYTC*K
Tenascin Y	11359854	YQFLRHLQQNPHPK
Teneurin-1	4877313	FSEEGLVNAR
TGF-beta 4	18266257	CPC*EM#GPGHAEEMR
Transcription factor MafF	2497473	QKM#ELEWEVDKLAR
Translocating chain-associated membrane protein	26449182	WREHSSSQPQSVKK
Unnamed protein product	63524	VRHPATNTVVEDHVK
Unnamed protein product	929597	FTC*TVQHEELPLPLSK
Vasotocin VT1 receptor	6580883	QNEYQVTNQK
Virion-associated nuclear-shuttling protein	11993887	QWDQHFRAMK
Vitamin-D binding protein	4572456	LPELQKPTNK
Werner helicase interacting protein	28804790	MLEGGERSLYVARR

C* = carbamidomethylated cystine; M# = oxidized methionine

Table 3: Proteins identified from blood plasma present in light and faint fractions of a sodium dodecyl sulphate polyacrylamide gel electrophoretogram

Protein	Accession number	Example peptide sequence
Albumin[1,2]	113575	LVKDVVDLAQK
Axl-related receptor tyrosine kinase[1]	1572687	RKETRFGHAFGSVVGR
Coagulation factor X (Stuart)[1]	119760	YC*KINNGDCEQFC*SIKKSVQK
Cytoplasmic aspartate aminotransferase[1]	112971	RGLDLQGLLDDM#EK
DNA glycosylase[1]	6492377	KAKATEPK
Ephrin type-A receptor 7[1]	8134447	EIGPLSKK
Fatty acid-binding protein[2]	462065	NCKSVVTLDGDK
Hemopexin[1]	16805334	PHSWEVLPGYPRDLR
Hepatocyte growth factor-like/macrophage stimulating protein[1]	1130676	IIC*GPSESHLVLLK
Kinase[1]	1370092	QAQLRKAK
Laminin gamma 1[1, 2]	14165136	PC*SC*NPAGSTGECNMETGRC*TCK
Lymphoid transcription factor[1]	15387602	QSYQGNSALNPK
Makorin-2[1]	17017225	AFAFQASQDKVC*SICMEVVYEK
Neurogenic differentiation factor[1]	6685684	ARRVKANAR
OL-protocadherin isoform[1]	14210853	SLHSTLER
Ovotransferrin chain[2]	1127086	ATYLDC*IK
Progesterone receptor[1]	130893	NDC*IVDKIR
Ryanodine receptor type 3[1]	1212912	AFFENAAEDLEK
T-cell receptor Vgamma1.1b[1]	2707453	RQM#PAKAPER
Tissue transglutaminase[1]	2148922	DQNLDC*SRR
Vitronectin[2]	1922282	GVPNQLDAAMAGR

[1,2]Superscripts correspond to the stained area of the gel where the proteins were identified; [1]Light (group 2), [2]Faint (group 3). C* = carbamidomethylated cystine; M# = oxidized methionine.

this is the largest number of plasma proteins ever identified and reported in a single assay from chicken plasma. The first fraction (Fig. 1, group 1) contained the two darkest staining bands that are classically associated with albumin and immunoglobulin heavy chain. Many proteins other than albumin and immunoglobulin (62; 70% of the total number of proteins), were identified from group 1 (Table 2). This is because firstly, albumin is a blood transport protein; it binds many biomolecules and drugs including hormones, lipoproteins and amino acids (Ritchie and Navolotskaia, 1996; Beutler and Williams, 2001; Burtis *et al.*, 2001). Secondly, "single" bands on 1-D gels contain many proteins of similar sizes. In contrast, only 18 proteins (20% of the total) were identified from the second fraction (the six group 2 bands; Table 3). The very pale staining part of the gel (group 3) contributed 5 proteins to the total (5.7% of the total number of proteins identified). The few higher abundance proteins in the second fraction (faint bands) may have masked those proteins that were present at similar (low) concentrations to the group 3 proteins.

The proteins that we identified from chicken have a wide array of different functions. These include enzymes,

receptors, binding proteins, coagulation factors, transport and structural proteins, among others, and derive from many different tissues. The chicken plasma proteome identified in our study falls short of a recently reported human blood serum proteome of 490 proteins (Adkins *et al.*, 2002) for four reasons. Firstly, Adkins *et al.* (2002) used 500 µl of plasma whereas we used only 0.4 µl. Secondly, we are reporting the initial optimization of 2-D LC ESI MS/MS for chicken plasma proteomics and did not run the mass spectrometer continuously for the 6 d (120 fractions at 80 min/fraction) that it would have taken to replicate the Adkins *et al.* (2002) work. Thirdly, our liquid chromatography separation was done completely on-line meaning this separation was less intricate than Adkins *et al.* (2002). Finally, the chicken genome sequence project is currently not completed, thus database resources is considerably less at the present time. An annotated chicken genome will dramatically increase the number of chicken blood plasma proteins that can be identified at high stringency. The database matches that are presented here represent a baseline. Once the chicken genome sequence is released, many putative chicken open reading frames (ORF) will be identified by computational biology. Proteomics will be invaluable to confirm that translated products of these putative ORF exist.

Future work requires both increasing the "depth and scope" of chicken plasma proteomics using more complex pre-analytical deconvolution strategies in the "wet-laboratory" and also more sophisticated computer database searches. Plasma is ideal for complex pre-analytical deconvolution because the proteins are already in solution and large volumes of plasma can be easily obtained. Many possibilities exist for expanding the defined human blood plasma proteome (Anderson and Anderson, 2002), and any could be applied to the chicken. Our identification of 88 proteins from only 0.4 µl of chicken blood plasma is an encouraging foundation from which to build.

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[13]ThermoFinnigan Corporation, San Jose, CA.

[14]Thermo Hypersil-Keystone, Bellefonte, PA.

Abbreviation key: ACN (Acetonitrile), ORF (open reading frames), SCX (Strong cation exchange), Two-dimensional liquid chromatography electrospray ionization tandem mass spectrometry (2-D LC ESI MS/MS).