Application and Diagnosis of Diseases in Veterinary Medicine

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Introduction

The proteome is the full complement of proteins expressed by a cell or tissue at any given time or environmental condition. Proteomics is the study and identification of this set or subset of proteins *(Abersold & Man, 2003; Cristea et al., 2004)*. Whereas the genome is composed of all the potential genes that can be expressed as proteins, the proteome is composed of all proteins actually expressed by the genome *(Wasinger et al., 1995)*. Proteomics is often comparative, where differences in protein expression in cell lysates or tissues in various conditions (diseased versus normal) are measured. Its main purpose is to identify proteins and establish their function. To this end, proteomics also involves the analysis and definition of the many protein-protein interactions responsible for biological processes that are regulated by multiple proteins. A better molecular understanding of the physiological role of these processes will enhance our knowledge of the pathological changes cells and tissues are susceptible to. Ultimately, this knowledge will help in the development of new drugs against human and animal diseases.

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Unlike other methodologies that analyze a few proteins at a time, proteomics can analyze thousands of proteins in a single experiment. This ability to analyze thousands of proteins gives the field of proteomics a unique capability to demonstrate how cells dynamically respond to changes in their environment. Therefore, a goal of proteomics is to identify new and potentially unexpected changes in protein expression, interaction or modification as a result of an experimental treatment (Prenni et al., ۲۰۰۷).

Proteomics has rapidly moved from a relatively new technology to a rapidly maturing essential tool in the omics age. Its existence is largely due to the success of the genome projects as well as rapid advancements in commercial mass spectrometers. The field of proteomics could not exist without the success of the genome projects. The genome projects of the various domestic animals will continue to increase our ability to associate desired traits with the necessary genes/proteins. Genome projects have given us the gene sequence and gene expression information with the use of techniques such as real-time PCR and microarray assays. However, understanding the genes is only the beginning of the biological story. Proteomics is giving us information regarding protein expression and post-translational modifications. Together, these techniques have brought animal research to a molecular level.

Proteomics involves the use of analytical methodologies such as liquid chromatography (LC) to separate proteins or peptides, and mass spectrometry (MS), to isolate, identify, and characterize proteins and their associated PTMs. An advantage of proteomics in biomarker discovery is the ability to detect a theoretically unlimited number of proteins in a given sample without the need for antibodies. Bottom-up proteomics including proteolytic digestion of proteins prior to the use of LC to separate peptides coupled with tandem MS (MS/MS) for peptide sequencing, a process commonly referred to as LC-MS/MS, has become the most widely used proteomic approach for the identification of individual proteins in complex mixtures. Additionally, advances in ion fragmentation strategies, including electron transfer dissociation (ETD), have provided superior tools for the characterization of modified peptides. Detection of disease-specific modifications of APP glycopeptides, however, is still an emerging aspect of veterinary biomarker discovery (Boehmer and Olumee-Shabon, ۲۰۱۲).

In essence, a proteomic approach enables an investigator to step back and without prejudice, view the whole picture of cellular functions instead of one particular action of one protein. This type of research enables the discovery of unexpected connections between cellular processes and can serve as a precursor to new hypotheses (Lippolis, & Reinhardt, ۲۰۱۰).

Principles of proteomic analysis

In an ideal world, a proteomic analysis would describe each member of a complex mixture of proteins that can contain up to ۸۰۰۰ different proteins. In the real world proteomic analysis is limited by the sensitivity of separation techniques, technical difficulties in protein solubility, capabilities of mass spectrometry instrumentation, and time constraints. In proteomic analysis, one or more protein separation steps using electrophoresis or high performance liquid chromatography (HPLC) usually are required prior to analysis by mass spectrometry to identify individual proteins or peptides (Prenni et al., ۲۰۰۷).

Separation of complex protein mixtures

Protein electrophoresis

Electrophoresis the separation of charge particles through a porous membrane via an electric field. The most commonly used electrophoretic methods of protein separation are ۱-dimensional (۱D) and ۲-dimensional (۲D) polyacrylamide gel electrophoresis (PAGE).۴ Protein profiling by ۲D-GE is characterized by a first dimension separation of proteins by charge (isoelectric point), followed by a second dimension separation by molecular weight. Advances in ۲D-GE technology including gel strips with immobilized pH gradients have dramatically increased the resolving power of ۲D-GE. Additionally, the development of radioactive and fluorescent labeling has improved the ability to visualize proteins in a ۲D gel, as well as the detection of low-abundance and post-translationally modified proteins (Boehmer and Olumee-Shabon, ۲۰۱۲).

Chromatography

High-performance liquid chromatography (HPLC) is a method to separate molecules in the liquid phase. The type of HPLC most commonly used in proteomics research is reverse-phase HPLC separation of peptides (after digestion of whole proteins with trypsin) based on their hydrophobicity. A mixture of peptides is prepared in an aqueous solution and injected onto the column, where the individual molecules interact with the hydrophobic packing material. The mixture is separated by elution with a gradient solution of increasing concentration of organic solvent. Thus, hydrophilic peptides will be eluted off the column early in the gradient (low organic), and hydrophobic peptide will be eluted toward the end of the gradient (high organic). The other HPLC separation method is strong cation exchange (SCX), which separates peptides based on their charge by elution of a SCX column using isocratic salt injections or an increasing salt gradient. There are two basic approaches to a ۲D-HPLC separation. In the first, the peptides are separated off-line by SCX and each of the fractions is injected onto a reverse-phase column coupled to a mass spectrometer. In the second completely

automated approach, multiple HPLC pumps and switching valves are used or a custom column which created that contains ۲ chromatographic phases, SCX and reverse phase (Eng et al., ۱۹۹۴; Le Bihan, et al., ۲۰۰۳; McCormack, et al., ۱۹۹۷).

Protein Identification: Mass Spectrometry

The next step in a proteomics experiment is identification of peptides by measuring their mass and matching this mass with that of a peptide in a protein database obtained from the theoretical digestion of trypsin (which proteolyzes proteins at a predictable amino acid sequence).

Mass spectrometry measures the mass–to–charge ratio (m/z) of peptides that have been ionized by the addition or subtraction of a proton. The charged peptides then move through an electric or magnetic field in a manner dependent on their molecular weight (Westermeier and Naven, ۲۰۰۲). All mass spectrometers consist of an ion source to ionize the sample, a mass analyzer to measure the m/z of each ionized peptide, and a detector to record the number of ions at each m/z value. The detector in most types of mass spectrometers is an electron multiplier, however, a variety of different ion sources and mass analyzers are available. A mass spectrometer operating in "MS" (a single mass spectrometric measurement) mode measures the mass of a molecule, whereas mass spectrometer operating in "MS/MS" mode (tandem mass spectrometry) gives structural information (eg, amino acid composition) about the molecule (Prenni et al., ۲۰۰۷). The strengths of common mass spectrometers are listed in Table ۱.

Ion sources

Mass spectrometry analysis is based on the use of magnetic and electric fields to separate and detect sample molecules. Therefore, it is necessary that the sample molecules be charged (ionized). The ion source in a mass spectrometer converts the sample molecules to ions and is the sample inlet to the instrument. The ۲ ion sources primarily used in proteomic applications are matrix–assisted laser desorption/ionization (MALDI) (Karas & Hillenkamp, ۱۹۸۸) and electrospray ionization (ESI) (Fenn et al., ۱۹۸۹). Both of these are "soft" ionization techniques that do not cause significant fragmentation of the molecules and thus are most amenable to the ionization of fragile biomolecules such as proteins, peptides, and sugars.

In a MALDI–TOF/MS experiment, the protein or peptide(s) of interest is mixed with a suitable energy–absorbing matrix and allowed to co–crystallize by air–drying on a stainless steel plate. Matrices used in MALDI–TOF/MS are typically small aromatic molecules capable of absorbing high levels of UV light at a specific wavelength, such as sinapinic acid (SPA) or alpha–cyano–۴–hydroxycinnamic acid (CHCA). The generation of ions in MALDITOF/MS is initiated by short pulse irradiation with a laser, typically nitrogen or neodymium–doped yttrium aluminum garnet (Nd:YAG), and occurs when the matrix becomes electronically excited following absorption of photons from the UV laser.

Table 1
Strengths of common mass spectrometers used in proteomics.

Instrument	Ion source	Identification	Quantification	Throughput	Detection of modifications
Ion-trap	ESI	+	+++	++	+++
TOF-TOF	MALDI	++	++	+++	+
qTOF	ESI/MALDI	++	+++	++	+
FTICR	ESI/MALDI	+++	++	++	+
Orbitrap	ESI	+++	++	++	++

The strengths of common mass spectrometers used in proteomic experiments are listed. The mass spectrometers are graded as follows: excellent (+++), good (++), and fair (+).

In LC–MS/MS experiments, ESI is the dominant method of ionization. Ionization occurs in ESI after the peptide solution is dispersed as a fine spray of charged droplets after passage through a heated metal capillary tube to which voltage is applied. The charged droplets get desolvated by a dry inert gas, and multiply charged ions are produced. Nanospray ionization (NSI) functions in essentially the same manner as ESI, but flow rates from the LC instrument into the ionization source of the mass spectrometer are much lower with nano–spray than those used for ESI, and ionization efficiency is greatly improved. Ions resulting from either ESI or NSI are then directed into the vacuum chamber of the MS instrument, and are resolved according to their m/z ratio to produce the first MS spectrum (Boehmer and Olumee–Shabon, ۲۰۱۲).

Recently, the combination of two LC–based separation techniques coupled with MS was introduced, and has profoundly increased the ability to resolve and detect a greater number of peptides in LC–MS/MS–based proteomic experiments.

Multidimensional protein identification technology (MudPIT), utilizes the combination of strong cation–exchange chromatography and reverse–phase chromatography followed by ESI–MS/MS for the characterization of proteins in a complex mixture. Using the MudPIT approach, proteins are typically digested into peptides using a protease such as trypsin, which cleaves at every arginine and lysine residue, and separated online by –۲dimensional LC prior to introduction into the mass spectrometer for mass analysis (Boehmer and Olumee–Shabon, ۲۰۱۲).

Mass analyzers

A mass analyzer is the part of a mass spectrometer that separates ionized sample molecules based on their m/z. the ۳ main types of mass analyzers used in proteomic applications are time–of–flight (TOF), ion trap, and quadrupole. A TOF mass analyzer measures the m/z of an ion based on the time it takes for the ion to travel down a flight tube. All ions are initially accelerated to the same kinetic energy so that velocity (ie, the time it takes the ion to travel known

distance of the flight tube) is directly related to ion mass. TOF analyzers are instruments with high mass accuracy and high resolution, ۲ characteristics that are important in the analysis of peptides using peptide mass fingerprinting.

Ion trap analyzers trap ions using an electric field and subsequently eject specific ions by changing the voltage of the electric field. The detector measures the ejected ions. Although ion traps have excellent sensitivity, they do not have the high mass accuracy or resolution of the TOF analyzers. Ion traps are most commonly coupled with ESI to facilitate on–line HPLC separation, but they can also be coupled with a MALDI source.

The third type of mass analyzer is a quadrupole, which acts as a mass filter. A quadrupole consists of ۴ parallel metal rods with oscillating voltages. As the ions travel through the quadrupole rods, those ions with the correct m/z will reach the detector and all others will be ejected into the rods. In an extension of this technology the second quadrupole is replaced with a TOF analyzer (Q–TOF), which is one of the most powerful for proteomic applications because it combines the selectivity of a quadrupole with the excellent resolution and mass accuracy of a TOF instrument (Prenni et al., ۲۰۰۷).

Peptide mass fingerprint

The most straightforward protein identification experiment is peptide mass fingerprinting (PMF) (Scheler et al., ۱۹۹۸). In a typical PMF experiment, a mixture of proteins is purified by either ۱D– or ۲D–PAGE. The gel

The most straightforward protein identification experiment is peptide mass fingerprinting (PMF) (Scheler et al., ۱۹۹۸). In a typical PMF experiment, a mixture of proteins is purified by either ۱D– or ۲D–PAGE. The gel is stained for visualization and the band/spot of interest is excised from the gel. The protein is reduced, alkylated, and digested, followed by extraction of the resulting peptides into solution. The sample is usually desalted and concentrated using a reverse–phase material. A common method for this step uses micropipette tips containing a small amount of C۱۸ packing material. The mixture of peptides is mixed with an excess of matrix and spotted on a MALDI target for analysis (Prenni et al., ۲۰۰۷). The use of a MALDI source enables high throughput analysis of well isolated, single protein samples.

Tandem mass spectrometry

To identify a protein based on the actual sequence of the peptides it is necessary to generate fragmentation spectra (MS/MS) using a tandem mass spectrometer such as an ion trap, TOF-TOF, triple quadrupole, or Q-TOF (Prenni et al., 2007). In general, the fragmentation patterns of ions observed in a MS/MS spectrum are matched to theoretical fragmentation patterns in the protein database to identify the protein.

Database searching

The development of genomic and protein sequence databases and the tools to search these databases is

what has made protein identification by mass spectrometry a reality. A variety of public databases of proteins are maintained and updated. The most commonly used comprehensive database is NCBI–nr. Two most frequently used search engines are Mascot and Sequest. In both search engines a "score" is reported for each possible match (Prenni et al., ۲۰۰۷).

What will proteomics be able to do?

Despite the limitations that the field of proteomics currently has to deal with, it has become an extremely important tool in biological sciences. The first unique advantage of this technology is the fact that a fairly large number of proteins can be identified and quantitated at one time, without any prior knowledge that any specific protein might exist in a sample. Analyzing a proteomic dataset can often lead to surprising results, and the unexpected may be the most interesting observation. In fact, most shotgun proteomic experiments are not typical "hypothesis driven" experiments, but may be better considered experiments designed to find a hypothesis. In these experiments hundred of proteins can be identified whose expression is altered by a defined experimental condition. Some changes in protein expression may be expected and even well characterized. However, somemay be unexpected or unknown and lead to new hypothesis for the connections between protein expression and cellular processes (Lippolis & Reinhardt, 2010).

Application of proteomics in clinical pathology

Proteomics analysis of biological fluids, particularly serum and plasma, carries great promise for the sensitive detection of disease states.

The working principle of this type of analysis is that proteins or peptides in low abundance and produced in remote anatomical sites (eg, a tumor) can be detected in serum and used as markers for presence of pathologic conditions (Petricoin & Liotta, ۲۰۰۴).

The most widely studied biological fluids in proteomic–based biomarker discovery analyses in swine and cattle have been serum, plasma, and milk. To date, protein profiles have been generated for serum and plasma of both healthy cattle and pigs, using ۲D–GE (Miller et al., ۲۰۰۹; Talamo et al., ۲۰۰۳). Comparative proteomic analyses have likewise been conducted using ۲D–GE to profile differentially expressed proteins in plasma from pigs with peritonitis induced sepsis, with findings that the APP inter–alpha trypsin inhibitor heavy chain۴– (ITIH۴–), HPT, hemopexin, alpha–۲–HS–glycoprotein, albumin, and apolipoprotein–A۱ all exhibited modulated expression levels as a result of disease (Thongboonkerd et al., ۲۰۰۹).

Of the bovine diseases studied, however, APP expression has been investigated most extensively during mastitis in lactating dairy animals (Safi et al., ۲۰۰۹; Suojala et al., ۲۰۰۸; Eckersall et al., ۲۰۰۱ ,۲۰۰۶; Grönlund et al., ۲۰۰۳; Hirvonen et al., ۱۹۹۹). The two most recent comparative proteomic analyses of

normal versus mastitic bovine milk, however, have not only identified several APPs in milk, but tracked changes in APPs over the course of infection, and quantified modulation in relative abundance of APPs during disease (Boehmer et al, ۲۰۱۰; Danielsen et al., ۲۰۱۰).

The earliest comparative proteomic analyses of normal versus mastitic bovine milk were accomplished using ۲D–GE followed by MALDI–TOF/MS (Smolenski et al., ۲۰۰۷; Hogarth et al., ۲۰۰۴). Previously, the analyses of APP expression in milk during bovine mastitis using more traditional quantitative strategies had only identified the APPs SAA, HPT, and LBP (Hiss et al., ۲۰۰۴; Bannerman et al., ۲۰۰۴; Eckersall et al., ۲۰۰۱). Similar to the ۲D–GE– MALDI–TOF/MS of bovine milk, the proteomic analysis of changes in the bovine BALF proteome induced by dexamethasone revealed increases in the APPs alpha–۲–HS–glycoprotein, alpha–۱–antichymotrypsin, alpha–۱–antitrypsin, and AGP in treated animals when compared to controls (Mitchell et al., ۲۰۰۷).

To avoid some of the limitations imposed by a ۲D–GE–MALDI–TOF/MS proteomic experiment, more recent proteomic analyses of bovine milk have been accomplished through the use of LC–MS/MS (Boehmer et al., ۲۰۱۰; Danielsen et al., ۲۰۱۰; Smolenski et al., ۲۰۰۷).

Modification of the glycosylation patterns of APPs has been implicated in a number of inflammatory diseases in humans and food animals (Gruys et al., 2005). Because posttranslational modifications often dictate the biological activity of certain proteins, the characterization of glycosylation patterns of APPs during disease may advance current knowledge of the mechanisms involved in food animal disease, and aid in the development of new therapeutics. Glycosylation is the most diverse PTM involved in the modulation of protein function (Ohtsubo et al., ۲۰۰۶). Glycosylation is a site specific enzymatic process which covalently binds sugar moieties to proteins in two ways, either through linkage of polysaccharides to the amide nitrogen of asparagine side chains, or to the oxygen atoms of serine, threonin, or tyrosine, which forms N–linked or O–linked glycans, respectively. N–linked glycosylation modulates protein folding and stability through a variety of mechanisms, whereas O–linked glycosylation plays important roles in protein localization, trafficking, and solubility (Spiro ۲۰۰۲). The study of proteomics and its applications have expanded greatly in recent years because of the many successful genome sequencing projects and recent advances in mass spectrometry. The canine genome was recently sequenced and those of a number of other domestic animal species are in progress (http://www.ncbi.nlm.nih.gov/genomes) (Prenni et al., ۲۰۰۷). Proteomics applications have been used extensively to address research problems. If properly addressed, these techniques hold great potential for use in drug toxicity testing, disease diagnosis, therapeutic assessment, and disease susceptibility screening.

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