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Separation, Identification and characterization of proteins by Novel techniques and application

<Aboueladab, Ehab>[1,2*], < Shehata, Faten >[3], < albahari, Shawgi >[4]

[1]Damietta University, Faculty of Specific Education,
New Damietta City, Damietta 34517, Egypt.
[2]Alfarabi faculty of dentistry and nursing, Basic science Department, Jeddah, Saudia Arabia
Ehab10f@gmail.com

[3]Alfarabi faculty of dentistry and nursing, Jeddah, Saudia Arabia
Faten10f@gmail.com

[4]Alfarabi faculty of dentistry and nursing, Jeddah, Saudia Arabia
Dr.sha1979@hotmail.com

Abstract: Proteomic technologies will play a significant role in drug discovery, diagnostics and molecular medicine because it is the link between genes, proteins and disease. As researchers study defective proteins that cause particular diseases, their findings will help develop new drugs that either alters the shape of a defective protein or mimic a missing one. Advances in proteomics may help scientists eventually create medications that are "personalized" for different individuals to be more effective and have fewer side effects. Current research is looking at protein families linked to diseases, including cancer, diabetes and heart disease. Identifying unique patterns of protein expression, or biomarkers, associated with specific diseases are one of the most promising areas of clinical proteomics. Also, proteomics has been complemented by the analysis of modifications and techniques for the quantitative comparison of different proteins available proteomics techniques and their applications.

Keywords: proteomics techniques, classification of proteomics , Separation, Identification and characterization of proteins.

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1. Introduction

Proteomics is a branch of biological science that focuses on the study of proteins, their roles, their structures, their localizations, and their interactions. Proteomics are defined as a simultaneous analysis of all the proteins in a given cell at a given time point. It has complemented genomic research. More than fifteen years ago, considerable emphasis was given to genomic research and the dream of having completely sequenced genome of various organisms is now a reality. The complete sequence of several genomes, including the human is available now, however, the understanding of probably millions of human proteins encoded by thousands of genes is still a long way to go and the hard work is required to unravel the complexity of biological systems. The inability to identify functional protein targets by examining gene sequences has created a gap between genomics and drug discovery [1].

Marc Wilkins and colleagues first introduced the term "proteomes" in 1995. The major goal of proteomics is to make an inventory of all proteins encoded in the genome of an organism and analysis of interaction of these proteins. Proteomics is a combination of highly efficient technologies for separation and analysis of proteins in living organisms and to exhaustive information on biochemical properties of proteins in living systems [2].

2-Classification of Proteomics

A horizon of proteomics is very broad. Scope of proteomics is wide-ranging, so it can be divided into following categories.

2.1. Expression Proteomics:

The expression, proteomics deals with the profiling of proteins expressed from cells or tissues. Although there may be more than 100,000 proteins in humans, only a fraction of these are expressed and known. In order to discover and monitor the relevance of a protein to a diseaserelated process, it is important to determine that up to what extent a protein is expressed [3]. DNA microarray technology, which monitors the relative abundance of mRNA in a cell, has been used to measure the gene expression in the cell/tissue. However, since mRNA and protein levels do not always correlate in the cell and many regulatory processes occur after transcription, a direct measure of relative protein abundance is more

Corresponding Author: Dr.ehab Aboueladab,Biochemistry/Home economics Department, College of Specific Education, Damietta University, Egypt & Basic Science Department , Alfarabi College of Dentistry and nursing, P.O.Box:21512 - Jeddah 45107, Saudi Arabia.Email :ehab10f@gmail.com,Telephone:00966596335254

desirable. The mRNA splicing and covalent modifications generate protein isoforms that might be important in defining the protein composition and regulatory processes in the cell and modifications in proteins during disease can provide strategies for therapeutic intercession for that particular disease [4].

2.2. Functional proteomics:

Genome sequencing contributed a number of the protein components of cells, however, the functional significance of most of these proteins is not known. Determining what these components do is the task of functional proteomics. Proteome-scale screens for functional activities of proteins (protease, phosphatase) should be implemented in order to link new proteins with known activities [5]. In one application of this concept, a fusion of 1000 of yeast genes to the coding sequence of glutathionyl Stransferase and the expression of fusion proteins in the yeast was done. The fusion proteins were then tested for several catalytic activities (cyclic phosphodiesterase, cytochrome c methyltransferase).

1. 3. Chemical Proteomics:

It is an alternative term for chemical genomics when proteomics approaches are used to study the interaction of small molecules with cells. Although it is impossible to screen for chemical reactions that are unknown, in theory, identifying small molecules (ligands) that bind to the new proteins may elucidate clues to novel activities. These ligands might be found by screening the new proteins against diverse chemical libraries, using existing methods such as NMR spectroscopy, microarrays, may result in the identification of small molecule ligands [6].

1. 4. Structural Proteomics:

The main aim of structural proteomics is to provide 3-D information for all proteins [7]. The primary sequence of a protein determines its three-dimensional structure, which in turn determines its function. Often, proteins of similar function share structural homology in the complete absence of sequence homology.

As a result, many of the newly sequenced proteins share unrecognized structural and functional homology with known proteins [8].

1. 5. Interactive Proteomics:

It refers to the localization of proteins and protein, protein interactions [9]. The protein, protein interactions are the part of most cellular processes, including cell-cycle regulation, signal transduction, certain metabolic processes as well as cellular architecture. A complete understanding of cellular function depends on a full characterization of the complex network of cellular protein-protein interactions. More importantly, many human diseases such as cancer, autoimmune disorders, and viral infections occur because of failure or aberrations in protein- protein interactions [10].

3-Separation of Proteins

2-1 Two-dimensional Gel Electrophoresis (2-DGE):

2- DGE is usually used for the separation and isolation of proteins for further characterization by mass spectroscopy. This technique can be used for two main purposes, firstly the separation of proteins from cells/tissues and secondly, for the large scale identification of all proteins in a sample [11]. This is undertaken for investigating the global protein expression of organisms whose genomes have been fully sequenced. In this way the individual proteins can be more readily identified from the mass spectrometry data [12]. Other uses of this technique are differential expression that is to compare the protein expression of two or more samples [13]. The 2-DGE technique is a high resolution separation technique in which protein samples were separated by isoelectric focusing (IEF) in one dimension and then on an SDSPAGE for size-determined separation in the second dimension [14]. It can resolve hundreds of components on a single gel. In the classical 2-DGE technique, ampholytes were used in the first dimension. The replacement of the ampholytes by immobilized pH gradients (IPG) resulted in significant improvements in the technique [15]. Staining with silver, Coomassiecolloid solution, special fluorescent dye are popular and widely used methods of gel staining [13].

2.2. Identification and characterization of proteins

A number of techniques allow to test and identify proteins produced during a particular disease, which helps to diagnose the disease quickly. Techniques include western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA) or mass spectrometry (MS) [16]. MS is a high-throughput and reliable method for protein identification. It provides the ability to accurately measure the mass of almost any molecule that can be ionized to the gas phase. A mass spectrometer consists of three essential components an ion source, a mass analyzer and a detector. An ion source converts molecules into gas-phase ions. Once these ions are created, they are separated in the mass analyzer by their mass (m) to charge (z) ratio and detected by an electron multiplier. MS data are recorded as spectra' which display ion intensities versus their m/z value. MS allows ionization of macromolecules without destroying their chemical entity. There are two main modifications of ionization of native proteins and peptides viz. Matrix-assisted laser desorptionionization (MALDI) and electrospray ionization (ESI). MALDI- MS are used for protein identification through treatment of the sample with trypsin or any other proteases by means of peptide fingerprinting (PMF). Tandem MS are equipped with ion trap, quadrupole chamber for fragmentation or hybrid quadrupole-time of flight (Q-TOF) apparatus. They are used for protein sequencing during mass spectrometry of products of fragmentation of primary molecular ions of peptides or proteins within the format of chromatography tandem mass-spectrometry (LC-MS/MS using the principle of ESI).

2.2.1. Electrospray Ionization (ESI):

ESI, first described in 1984, produces gaseous ionized molecules directly from a liquid solution of the analysts at atmospheric pressure. The sample solution is sprayed from the tip of a thin capillary and a strong electric field is applied between the capillary and a counter electrode. A characteristic feature of ESI is the multiple charging of analysts, which increases proportionally with molecular mass. This multiple charging allows for mass determination of proteins within the limited m/z range of quadrupole Analysers (Figure 1).

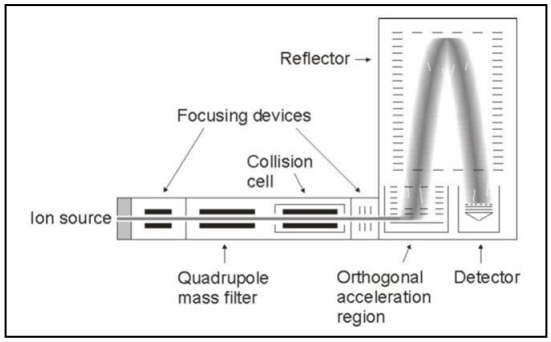

Figure1: Schematic illustration of the principal components of ESI-MS

The use of ESI results in dispersion of the analyte solution which is supplied via a thin capillary under high voltage. Modern MS used for analytical proteomics studies usually contain special equipment, so called nano-electrospray allowing sample administration at the flow rate of 0.01-0.5 Ill/min. This makes possible direct connection of ESI-MS detectors with multidimensional chromatography.

2.2.2. Matrix-assisted Laser Desorptionionization (MALDI)

The MALDI-TOF/MS instrument combines a MALDI- an ion source and a TOF analyzer. MALDI is a soft ionization technique, initially described in 1988, which results in most intact peptides/proteins in the gas phase with little fragmentation MALDI the protein sample is first mixed with a special matrix and spotted on an Anchor Chip target or a stainless steel target. Upon drying, the matrix molecules crystallize and solid sample/matrix co-crystals are finally formed. The MALDI- target is then inserted into the ion source of the mass spectrometer, which is under a high vacuum. A strong electrical field is applied between the target and the extraction plate(s). A laser is fired onto the sample, resulting in desorption event due to absorbance of the laser energy by the matrix molecules. Energy deposition into the matrix molecules results in the modulation of the absorbed energy into heat. This rapid heating causes sublimation of the matrix crystals and subsequent expansion of matrix molecules and the co-crystallized analytic molecules into the gas phase. The ions are repelled from the target surface and accelerated into the mass analyzer. In the such ionization process, on the surface of a chip singly-charged ions are mainly formed by protonation of the basic residues such as the side chains of arginine, lysine, histidine and the free alpha-amino group. Usually, MALDI is coupled to a time-of-flight (TOF) tube for mass analysis (Figure 2).

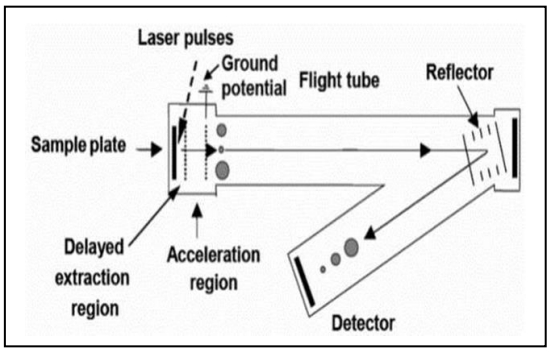

Figure 2: Schematic illustration of a MALDI-TOF/MS

The TOF tube is under a high vacuum and is a field-free drift region. All ions entering the TOF tube have a fixed kinetic energy and the ions pass the field-free drift tube with acertain velocity. The higher the mass of the ion, the lower its velocity and vice versa and their m/z ratio can be calculated from their flight time after calibration of the analyzer using compounds with known mass.

3. Bioinformatics study for analysis and comparison of the data

One of the aims of proteomics is to move from an experimental to an in silicon science, in which changes in cellular physiology and pharmacology can be predicted using computational methods. The integrated databases should meet the following criteria like: each entry in the database should be available during search by key words; the database should be connected to other databases via active links; an entry in the database should contain the main index. Software for image analysis developed for the work with the integrated database should provide direct access of individual entries into any integrated database [2].

The SWISS-2D page is the most popular database. Besides images of 2D-gels with identified proteins on them and such experimental data as isoelectric point, molecular mass, this database contains information obtained during comparison of gels, microsequencing, and immunoblotting, analysis of amino acid composition and MS analysis of peptide fingerprints [17].

Database searching using MS or MS/MS data is done with the MASCOT search engine. The digested fragments were searched against the SWISS-PROT and NCBInr databases. Monoisotopic mass were used to search the database and mass tolerance was allowed less than 40 ppm and one partial cleavage. Oxidation

Of methionine and acrylamide modification of cysteine was considered during the search. There are so many data protein bases such as; UniProt, PIR, Swiss- Prot, Pfam, PDB, NCBI. Although such databases are not yet comprehensive, they are already being developed and mined. In the proteomics sector, databases linking protein sequences with biophysical properties have been created, and rules that govern protein solubility and protein crystallization are being extracted. As an application of this knowledge-discovery

concept moves from individual proteins to protein pathways and then to cellular pathways, we will see a dramatic increase in the efficiency of the drug discovery process.

4. Mass spectrometry (MS)

Mass spectrometry, which is a technique for identifying proteins, is the most important technique in proteomics. MS based proteomics is the large-scale study of proteins, particularly related to their identification, structures and functions. This approach provides the information regarding proteins/genes that are expressed in the cell. MS based proteomics approaches using in help in functional interpretation of the sequence information contained in biological organisms.

5. Definitions of a biomarker and proteomics

Abiomarker isdefined byAtkinsonet al. The US Food and Drug Administration (FDA) Biomarker Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [18]. Those characteristics that are informative for clinical outcome can be categorized broadly as prognostic or predictive biomarkers. Prognostic biomarkers

Classify patients into subgroups with distinct expected clinical outcomes, such as progression or death, but they do not inform the choice of therapy. Conversely, predictive biomarkers should identify subgroups of patients whose tumors are likely to have therapeutic sensitivity or resistance based upon marker status [19,20].

6. Identification of differentially expressed biomarker candidates

Methods to identify biomarkers from clinical specimens can be categorized into two principle methodologies: mass spectrometer- based methods and antibody array-based methods. In cases where the nature of the biomarkers or biosignatures is not known, mass spectrometry is most commonly employed to both map differences in detectable protein expression, as well as to identify the proteins that are differentially expressed [21]. Different groups have employed different mass spectrometer-based relative consultations. Among the three most commonly employed methods are SELDI-TOF, iTRAQ coupled to liquid chromatography (LC)–MS/ MS, [22,23]. And different variations of spectral abundance or total ion current counting of peptides. [24]. SELDI-TOF uses different capture surfaces to separate proteins based on their biophysical properties. [25,26]. TOF/MS is then used to determine protein profiles, which are analyzed by software to detect and quantify differences in protein expression profiles. To identify differentially expressed proteins of interest, proteins have to be elected from SELDI chip surfaces and analyzed after trypsinisation, which adds to the time and cost of this approach. Since most clinical samples that are to be compared have similar composition, identically prepared samples, after sufficient protein and peptide separation (to reduce sample complexity), can be directly compared in sequential mass spectrometer runs without the use of labels. Some methods compare the total ion current for a given peptide at a given point in time during the separation, while the others compare the number of times a unique MS/MS spectrum has been sampled. Assuming that the samples have similar complexity and that the mass spectrometers and separation columns have been well calibrated with established standards, this approach has been successfully used in major biomarker discovery projects [24].

Another approach that has been successfully used in the discovery of biomarkers from a variety of clinical specimens is the iTRAQ approach [23,24,27-30]. iTRAQ labels are isobaric labels that react with primary and secondary amines of peptides. Current marketed versions of iTRAQ labels contain four or eight labels as part of a labelling kit. Each label has an identical mass, but fractions differently during a collision- induced fragmentation while performing MS/MS analysis. For example, peptides labelled with the 114 reagents will generate a 114 m/z ion in an MS/MS spectrum, whose peak area is proportional to the relative abundance of the peptide- derived protein in the original biological sample. By mixing four or eight

Differently labelled samples followed by further separation and MS/MS analysis, one can then determine the ratio between the different isobaric reporter ions, which indicate the fragmented peptide's relative abundance level in different samples.

To perform differential labelling of clinical samples with iTRAQ, individual samples are trypsinised and each trypsinised sample is labelled with a different isobaric label (e.g. 114, 115, 116 and 117). Differently labelled peptide mixtures are then combined at equimolar concentrations and further separated by two- dimensional chromatography. As peptides enter the mass spectrometer, individual peptides are fragmented, and for a given peptide species the amino acid sequence is determined, as well as the ratio is determined between different iTRAQ reporter ions that represent a direct relative abundance comparison between the original samples. The advantage of the iTRAQ labelling method over other relative quantitation methods is that after trypsinisation of samples and iTRAQ labelling and mixing of samples, all subsequent separation conditions are identical, resulting in very low coefficients of variance and faster analysis. By including a pooled standard control against which all samples are compared, relative quantitation is possible across large patient cohorts. This approach has been successfully employed for the discovery of biomarkers from both plasma and tissue. [23,27,28, 31-36].

In 2008 McLerran and colleagues reported a 3-stage validation process of putative serum biomarkers identified via surface enhanced laser desorption ionization-time of flight (SELDI-TOF). This study was the result of a rigorous validation effort undertaken by the National Cancer Institute early detection research network (EDRN). The validation study was specifically targeted at the evaluation of a previously published EDRN study of

spectral peaks for the detection of prostate cancer [37- 39]. In the first stage of the study a group of six separate institutions first demonstrated that SELDI-TOF mass spectrometry instruments and protocols could be standardized and used to classify previously studied prostate cancer patient and control sera using known spectral features. A decision algorithm was then developed by analyzing serum samples from patients with prostate cancer.

Along with protein identification, protein quantification is another main component of the biomarker discovery process to determine changes in protein expression between disease and control state. Popular methods of proteomics quantification used in prostate cancer research are:

1) A combination of gel electrophoresis and mass spectrometry. In this approach 1- DE or 2-DE are used to distinguish differentially expressed proteins based on staining intensity of the gels and proteins identified by MS/MS. Protein identification may also be carried out via simple MALDI-TOF MS peptide mass fingerprinting;

2) Stable isotope labeling of proteins introduces pairs of chemically, metabolically, or enzymatically identical "mass" tags that may be separated by MS and then corresponding proteins identified by MS/MS of the labeled peptides. Popular methods for introducing the stable isotope mass tags are isotope coded affinity tags (ICAT) [40] isobaric stable isotope labeling (iTRAQ) [41] and stable isotope labeling with amino acids in cell culture (SILAC) [42] **(Fig. 3-6).**

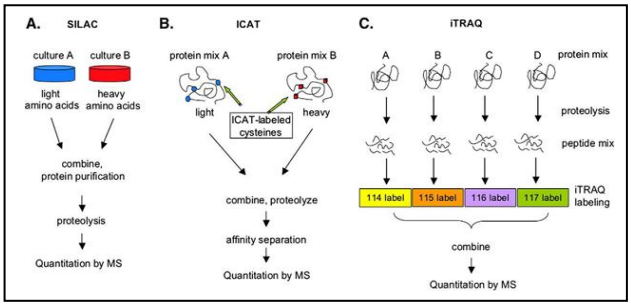

Figure 3: Stable isotope labeling for protein quantification currently used in prostate cancer research. A) SILAC (metabolic labeling). Cells grow in isotopically enriched culture media contain amino acids (15N or 13C) and metabolically incorporated during cell culture. Labeled cells are combined, purified and proteolyzed prior to MS and MS/MS to determine relative protein abundance and for protein identification. It is applicable only to cultured cells and cannot be used for tissues and other body fluids. B) ICAT (chemical labeling). Heavy and light affinity tags are labeled to cysteine residues of protein mixture. Labeled proteins are combined, proteolyzed, affinity separated, and relative protein abundance is determined. C) iTRAQ (chemical labeling). Labeling occurs at the peptide level after protein digestion. N-terminal and lysine residues of all peptides are labeled though acylation with reactive chemical compounds. It can multiplex up to 8 different samples. (4 labels shown here).

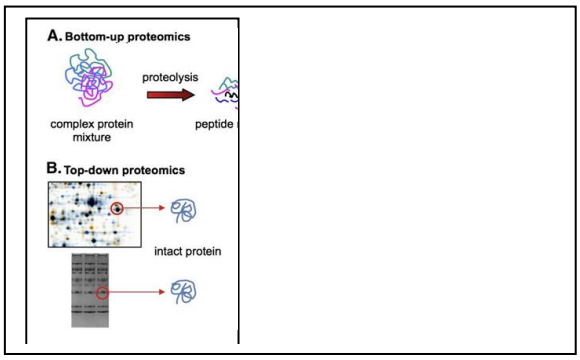

Figure 4: A) Bottom-up proteomics generally involve protease digestion of a complex protein sample to make peptide that can be analyzed by MS for protein identification following optional peptide fractionation to reduce complexity.

B) In the top-down approach, intact protein of interests is isolated from a 1-DE or 2-DE gel. A complex protein mixture is optionally fractionated by enrichment or depletion methods. Intact proteins are subsequently applied to MS-based analyses without proteolysis. Advantage of top-down proteomic is to increase ability of detecting protein isoforms, PTMs, and genetic insertion/deletion events. The two approaches can be used to complement each other.

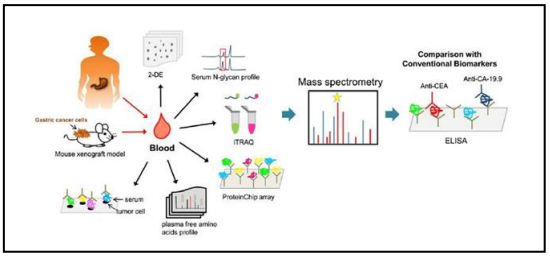

Figure 5: Schematic representation of blood biomarker discovery workflow. Serum or plasma specimens were collected from patients with different stages of gastric cancer, patients with different types of cancer, healthy individuals and different tumor The following profiles were used: 2-DE, serum N-glycan, and plasma-free amino acids. The protein reactivity between serum and tumor cells, iTRAQ and ProteinChip array, and MALDI-TOF-, LC-, SELDI-TOF-, and HPLC-ESI-MS analysis were used to identify target proteins. To confirm the efficacy of potential biomarkers, the sensitivity and specificity of the potential target proteins.

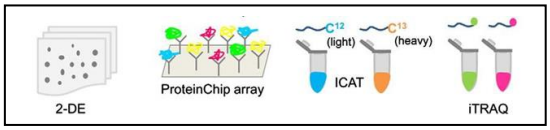

Figure 6: Proteomic technologies and instruments for optimal specimen pretreatment and preparation and to search for novel protein targets. The development in instruments and in predication strategies may increase the biomarker detection efficiency and enable innovative approaches in

future proteomic projects.

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Author Profile

Author Name received the B.Sc. in Chemistry, M.Sc. Degrees in Biochemistry from Faculty of Science, Mansoura University and Ph.D. in Biochemistry from Faculty of the science-Cairo university in 2006. During 2006 until now he was honors and awards from the following.

1-Third World Academy Award awarded by the International Academy of Chemical Sciences, Italy and work in India, Postdoctoral Prize, TWAS-CSIR, 1/10/2009

2-State Prize for Science Chemical 2010 Prize, Academy of Scientific Research and Technology. 28/6/2011

3- Office of the Vice President of Graduate Studies and Research and cultural relations. 25/6/2012

4-Incentive Award University of the Mansoura University Prize, 2011/2012

5-Misr El Kheir Award for best scientific research Prize, Misr El Kheir, 2012/2013