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Application of Proteomics in Chinese Medicine Research

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Abstract: Proteomics technologies can be applied to simultaneously study the function, organization, diversity, and dynamic variety of a cell or a whole tissue. The integrative approach of proteomics is in line with the holistic concept and practices of traditional Chinese medicine (TCM). In this review, the technologies of proteomics, their adoption leverages the depth and breadth of TCM research are introduced. This article presents some examples to illustrate the use of proteomics technologies in the study of pharmacological effects and their action mechanisms relevant to TCM. Proteomics technologies could be used to screen the target molecules of the TCM actions, identify new bioactive components, and elucidate the underlying mechanisms of their effects. With proteomics approaches, it was found that the Siwu decoction could regulate the protein expression of the bone marrow of blood (*Xue*) deficient mice, including some proteins and enzymes involved in the hemopoiesis system. *Ganoderma lucidum* spores might promote the survival and axon regeneration of injured spinal motor neurons in rats by regulating the expression levels of proteins involved in the energy and tissue regeneration system. *Polygonatum zanlanscianense* Pamp exhibited cytotoxicity towards human myeloblast leukemia HL-60 cells through multiple apoptosis-including pathways. *Panax ginseng* might be beneficial to patients suffering from diabetes mellitus and its complications by alleviating inflammation. Taken together with a discussion on the challenges and perspectives, this paper provides an overview of the recent developments of proteomics technologies in TCM research, and contends that proteomics will play an important role in the modernization and internationalization of TCM.

Keywords: Proteomics; Traditional Chinese Medicine; Herbal Medicine; Proteinchip.

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Introduction

A new age in the development of life sciences, the post-genome era, has arrived after the accomplishment of the Human Genome Project. Researches on proteomics in this epoch further reveal the biological functions of proteins determined by genes, and it will promote the accuracy and efficiency in the designation and application of biotechnology. Proteomics technologies may be applied to simultaneously study the function, organization, diversity and dynamic variety of a cell or a whole tissue. The integrative approach of proteomics is in line with the holistic concept and practices of traditional Chinese medicine (TCM). Proteomics technologies can be used to screen the target molecules of the action of TCM, isolate and characterize new active components, as well as analyze toxic substances from TCM. Coupling recent advances in multidimensional liquid chromatography, free-flow electrophoresis and capillary electrophoresis-based separation techniques, it is now possible to separate the hundreds or even thousands of components in TCM (Gao *et al.*, 2007). Since proteomics aids in understanding the complex mechanisms of TCM at the cellular and molecular levels, it has great meanings to the modernization and internationalization of TCM (Wen and Han, 2004).

In this article, the technologies of proteomics are introduced, and the application of proteomics technologies in TCM research and development is expounded, highlighting the application in TCM drug mechanistic investigation. Based on recent achievements in the protein fingerprints developed for TCM, this review summarizes some research results of protein fingerprints for TCM evaluation and drug discovery. The findings from the author's experiences with proteomics application in TCM research are also shared in this article. Finally, the paper is closed with a discussion of the challenges and perspectives for the application of proteomics in TCM research.

Proteomics Technologies

The Nobel Prize in Chemistry for 2002 was shared between scientists in two important fields: mass spectrometry (MS) and nuclear magnetic resonance. This revolutionary breakthrough, has allowed chemical biology to become one of the most significant scientific fields of study of our time. Scientists can now rapidly and reliably identify what proteins a sample contains, and it is easier than ever to produce three-dimensional images of protein molecules in solution. We can now approach a better understanding of proteins and discern how they function in the cells (Cho, 2007; Cho and Cheng, 2007).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) is a key tool for comparative proteomics research. Possessing the ability to separate complex protein mixtures with high resolution, 2-DE is a technique commonly employed for protein profiling studies since the mid-70's. Mixtures of proteins are separated by charge (isoelectric point, pl) in the first dimension and they are separated by mass in the second dimension on 2-D gels (O'Farrell, 1975;

Klose, 1975). Coupling 2-DE with immobilized pH gradients, IPG-Dalt, has provided higher resolution, improved reproducibility, and higher loading capacity for preparative purposes. The 2-DE can achieve the separation of several thousand different proteins in one gel. Stains such as Coomassie blue, silver, SYPRO Ruby and Deep Purple can be employed to visualize the proteins (Lauber *et al.*, 2001).

Unfortunately, 2-DE technique is a time-consuming and labor-intensive process. Conventional 2-DE is restricted to the detection of denatured proteins in the size range of 10~200 kDa at pH 3.5~11.5. Traditionally, vertical and horizontal streaking of proteins can obscure analysis, and membrane proteins are usually under-represented due to extraction and insolubility problems (Nilsson *et al.*, 2000). Furthermore, 2-DE is ineffective at distinguishing low abundant proteins and small molecular weight proteins (<10 kDa). In recent years, some modified 2-DE platforms have been developed to detect non-denatured proteins in extreme size and pI (Giometti *et al.*, 2003; Li and Giometti, 2007). Moreover, significant improvements have been made in 2-DE technology with the development of two-dimensional fluorescence difference gel electrophoresis, which can be used to reduce gel to gel variations. Proteins are first labeled with one of three spectrally resolvable fluorescent cyanine dyes before being separated over the first and second dimensions according to their charge and size respectively. It builds on 2-DE by adding a highly accurate quantitative dimension, which enables multiple protein extracts to be separated on the same 2-D gel. When used in conjunction with automated analysis packages, this multiplexing approach can accurately and reproducibly quantify protein expression for control and experimental groups. Differentially expressed proteins can be subsequently identified by mass spectrometric methods (Marouga *et al.*, 2005).

Electrospray Ionization

Electrospray ionization (ESI) involves the release of ions achieved by spraying the sample using an electrical field, so that charged droplets are formed. As the solvent gradually evaporates from these droplets, freely hovering stark naked protein molecules remain. Because the molecules take on strong positive charges, the mass/charge ratio becomes small enough to allow the substances to be analyzed in ordinary mass spectrometers. Another advantage is that the same molecule causes a series of peaks since each can take up a varying number of charges, which gives information that makes identification easier. In recent years, a novel linear ion trap (LIT) mass spectrometer with ESI and matrix-assisted laser desorption/ionization (MALDI) has been built in the MALDI-LIT-ESI configuration. The design features two independent ion source/ion optical channels connected to opposite ends of a single mass analyzer (Smith *et al.*, 2007).

Matrix-Assisted Laser Desorption/Ionization

Ionization by MALDI involves a laser pulse striking the sample which, unlike in the spray method, is in a solid or viscous phase. When the sample takes up the energy from the

laser pulse, it is blasted into small bits. The molecules let go of one another, released as intact hovering ions with low charge which are then accelerated by an electrical field and detected as described above by recording their time-of-flight (TOF). The technology is able to analyze proteins down to attomole quantities. It can tolerate small amounts of contaminants. The information obtained from MALDI analysis can be automatically submitted to a database search for further examination (Andersson *et al.*, 2007). Currently, there is a development of direct analysis and MALDI imaging of formalin-fixed, paraffin-embedded tissue sections using the strategy based on *in situ* enzymatic digestion of the tissue section after paraffin removal. This approach provides access to massive amounts of archived samples in the clinical pathology setting (Lemaire *et al.*, 2007).

Surface-Enhanced Laser Desorption/Ionization

The surface-enhanced laser desorption/ionization (SELDI)-TOF MS is a technological breakthrough combining chromatographic active surfaces with an interface chips for MALDI. Using as little as one microliter of sample, a high resolution mass spectrum following a complete chromatographic separation can be performed. The development of SELDI technology holds much promise for future protein analysis. It can be used for protein purification, expression profiling, or protein interaction profiling. There are many types of substances bound to the protein arrays, including antibodies, receptors, ligands, nucleic acids, carbohydrates, or chromatographic surfaces (e.g. cationic, anionic, hydrophobic, or hydrophilic). Some surfaces have broad specificity that bind the whole classes of proteins, while others are highly specific that only a few proteins from a complex sample are bound. After the capture step, the array is washed to reduce nonspecific binding. When subjected to short bursts of laser beam, the retained proteins are uncoupled from the array surface and analyzed by laser desorption/ionization TOF MS. Some protein arrays contain antibodies covalently immobilized onto the array surface that capture corresponding antigens from a complex mixture. Many analyses can be followed, e.g. analysis of proteolytic digests of the proteins bound to the array can disclose the antigenic determinant, other proteins of interest can be immobilized on the array, bound receptors can reveal ligands, and binding domains for protein-protein interactions can be detected. It should be concerned that proteins must often remain folded in the correct conformation during the preparation and incubation with the array for protein-protein interactions to occur (Cho, 2006a).

Application of Proteomics in Traditional Chinese Medicine Research

Proteomics technologies can be used to screen the target molecules and explore the mechanisms of the actions of TCM. Several examples are presented below to illustrate the use of proteomics technologies in TCM research.

Panax ginseng and *Panax quinquefolius* are two widely used valuable TCMs. However, conventional separation methods cannot distinguish different parts (main root, lateral roots, rhizome head and skin) of the two species. The 2-DE maps have been applied to

identify different ginseng samples containing distinct and common protein spots to permit easy discrimination. The use of these potential biomarkers might help to speed up the identification process of TCM (Lum *et al.*, 2002).

Ancient TCM prescriptions have been used for centuries by Asians to treat disease and maintain health (Shen *et al.*, 2005). However, the molecular mechanisms underlying their efficacies remain largely unclear. Siwu decoction is an ancient composite formula of four popular herbal medicines (*Rehmannia glutinosa*, *Angelica sinensis*, *Paeonia lacitflora*, *Ligusticum chuanxiong*) used to replenish blood (*Xue*), stimulate the hemopoiesis of the bone marrow for blood (*Xue*) deficient subject, as well as increase the peripheral blood count. With proteomics technologies including 2-DE, image analysis, in-gel digestion, MALDI-TOF MS, and bioinformatics, Guo *et al.* (2004) found that Siwu decoction could regulate the protein expression of the bone marrow of blood (*Xue*) deficient mice, including lymphocyte specific protein 1, proteasome 26S ATPase subunit 4, hematopoietic cell protein-tyrosine phosphatase, glyceraldehyde-3-phosphate dehydrogenase, growth factor receptor binding protein 14, and lgals12. Their analysis of the ancient TCM prescription provided a possible explanation of the mechanism underlying TCM drug-promoted hemopoiesis.

New knowledge about circuit function and formation must be brought to bear on the urgent need to repair the injured nervous system. This is particularly important for spinal cord trauma, brain injury, and stroke. TCM has long been used to treat neural symptoms. Increasing evidence indicates that neuroglia-derived chronic inflammatory responses play a pathological role in the central nervous system, anti-inflammatory herbal medicine and its constituents are being proved to be a potent neuroprotector against various brain pathologies (Hsieh *et al.*, 2005; Suk, 2005). *Ganoderma lucidum* spores are deemed to promote the injured motoneuron and spinal cord survival, as well as promote the generation of injured sciatic nerve. However, the precise mechanisms of the neural regeneration action of *Ganoderma lucidum* spores have yet to be determined. Using 2-DE and MALDI-TOF MS, Zhang *et al.* (2006) found that *Ganoderma lucidum* spores might promote the survival and axon regeneration of injured spinal motor neurons in rats by regulating the expression levels of collapsin response mediator protein 2, F-actin capping protein beta subunit, isocitrate dehydrogenase subunit beta, ATPase, glutamate oxaloacetate transaminase-1, and M2 pyruvate kinase. *Ganoderma lucidum* spores might promote the survival and axon regeneration of injured spinal motor neurons in rats by virtue of regulating the expression levels of the above mentioned proteins. This study documented the validity and usefulness of proteomics approach in unraveling the action mechanisms of TCM as a neural regeneration drug.

A number of TCM have been reported to have immunomodulatory and anti-tumor effects in cancer cells (Cho and Leung, 2007a; 2007b; Koo *et al.*, 2007). However, the detailed mechanisms of TCM-induced cancer cell death have not yet been fully elucidated. In some TCM herbal medicines, saponins are the major components that have long been used to treat various diseases such as cancer, lung illness, palpitation, upset stomach, and diabetes mellitus. Recent biological and pharmaceutical researches have shown that diosgenyl saponins exert a large variety of biological functions, with a potential for use in

cancer chemoprevention (Liu *et al.*, 2004). Employing 2-DE, tryptic in-gel digestion and MALDI-TOF MS analysis, Wang *et al.* (2006) suggested that dioscin, a saponin extracted from *Polygonatum zanlanscianense* Pamp., exhibited cytotoxicity towards human myeloblast leukemia HL-60 cells. Proteomics analysis revealed that the expression of mitochondrial associated proteins was substantially altered in HL-60 cells corresponding to the dioscin treatment, suggesting that mitochondria were the major cellular target of dioscin cytotoxicity. They also suggested that other pathways were involved in dioscin cytotoxicity, including phosphorylation cellular signaling, RNA-related protein synthesis, and oxidative stress processes. The study successfully demonstrated that proteomics approach could be used to study the cytotoxicity mechanism of a potential anticancer TCM drug.

The superiority of TCM in the prevention and treatment of chronic disease has been well recognized by Asians (Cho *et al.*, 2005). Diabetes mellitus is a chronic progressive disease with metabolic disorder of the endocrine system. *Panax ginseng* has been used to treat diabetes mellitus since ancient time (Xie *et al.*, 2005). Following the previous experiments performed to find potential biomarkers for the pathogenesis of diabetes mellitus, further study was carried out by Cho *et al.* to investigate the antidiabetic actions of ginsenoside Re, an active compound of *Panax ginseng*. Results indicated that ginsenoside Re demonstrated significant antidiabetic actions. Employing high-throughput SELDI-TOF MS and bioinformatics technologies to explore the possible proteins involved in the antidiabetic actions of ginsenoside Re, the author detected the profiling of a panel of potential biomarkers corresponding to ginsenoside Re treatment (Figs. 1 and 2). One of the biomarkers was found to be C-reactive protein, this finding was validated by ELISA, indicating that ginsenoside Re might be beneficial to patients suffering from diabetes mellitus and its complications by alleviating inflammation. This study has shed light on the possible therapeutic application of ginsenoside Re on the prophylaxis of diabetes mellitus. Connecting the exploration of TCM with powerful proteomics tools will be an ideal integration for discovering new treasure within herbal medicine and bring TCM research to a new horizon (Cho *et al.*, 2006a; 2006b; 2006c; 2006d).

Recent studies have shown that acupuncture can reduce neuropathic pain. Electroacupuncture (EA) treatment was applied to Zusanli (ST36) of neuropathic pain model to examine the analgesic effect of EA. The protein expression profile of the hypothalamus in both neuropathic pain and EA treatment models was analyzed using 2-DE-based proteomics. A number of potential biomarkers were identified and further exploration of the role of these proteins might be applied for the identification and characterization of signaling pathways involved in EA treatment (Sung *et al.*, 2004). Another study was conducted to investigate the differences of myocardial protein expression between median nerve stimulation (MNS) by EA and by local somatothermal stimulation (LSTS). The results showed that either MNS by EA or by LSTS had cardioprotective effect against ischemia-reperfusion injury. It was found that MNS by either EA or LSTS attenuated ischemia-reperfusion injury in rat hearts through different protective mechanisms and that EA and LSTS might provide an alternative treatment strategy for ischemic heart disease (Tsou *et al.*, 2004).

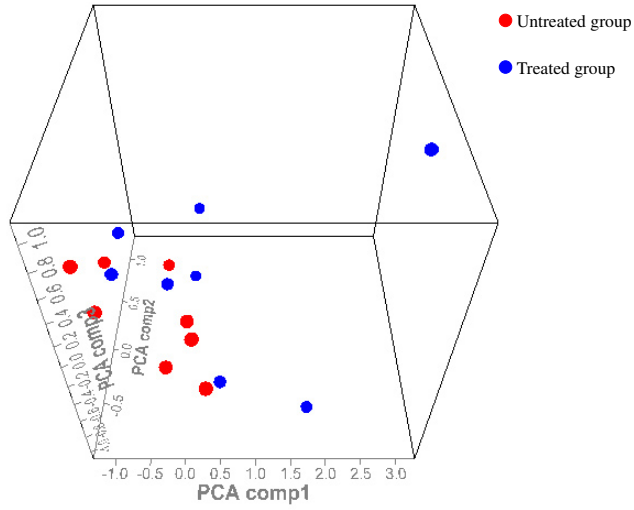

Figure 1. Differentiation of the treated diabetic rats from the untreated diabetic rats. Principal components analysis of the proteome data of sera from the ginsenoside Re-treated diabetic rats (in blue) and the untreated diabetic rats (in red) represented in a three-dimensional graph with components 1–3 displayed on the three axes.

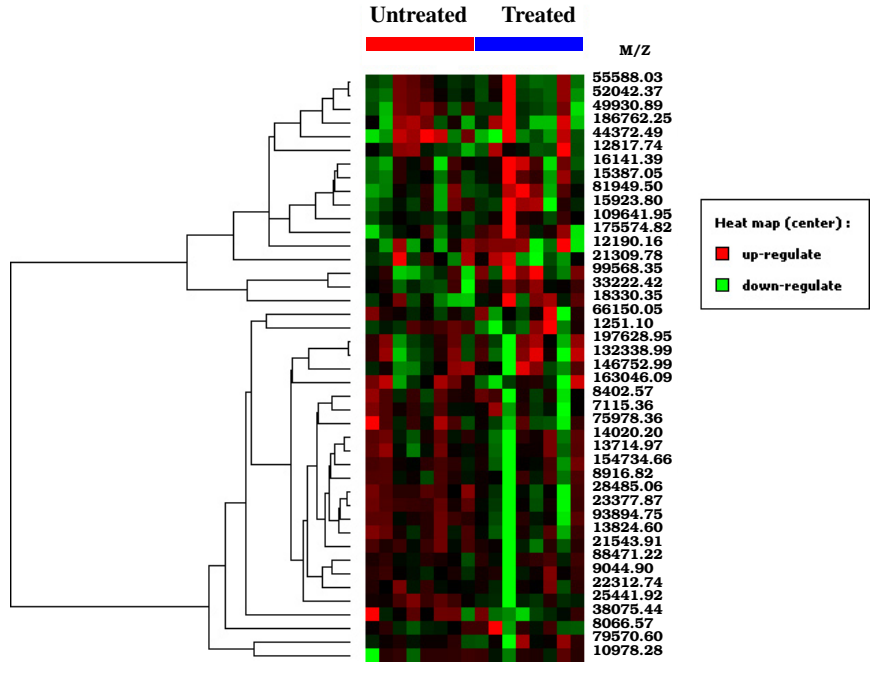

Figure 2. Heat map presentation. Hierarchical cluster representing similarities in the expression patterns between the sera from the ginsenoside Re-treated diabetic rats (in blue) and the sera from the untreated diabetic rats (in red). The intensity of the red or green colour indicates the relative protein concentration, that is, higher or lower than the median value respectively.

Challenges and Perspectives

There is a conceptual difference between the TCM systems and the proteomics approach. The TCM prescriptions are complex mixtures normally composed by numerous herbs whereby even a single herb may contain hundreds of chemical constituents. It is difficult for TCM prescriptions to illuminate their efficacies by analyzing a few chemical components because TCM treats diseases by the mutual effect of ingredients. Not all the TCM practitioners are in favor of fractionating and screening; some think that is missing the point as TCM remedies often depend on the cooperating action of herbs. Extracting only some of the ingredients from the herbs might impair the original effectiveness, so the modernization of TCM should conform to the principles of the TCM theories. On the other hand, although some TCM herbs belong to the same species and contain almost the same ingredients, they have considerably different functions if they are harvested in different places or times. So there is a long way to go to ensure the quality of TCM.

Nevertheless, the future perspectives of proteomics application in TCM research are still optimistic. Current concepts in Western drug therapy often attempt treatment of large patient populations as groups, irrespective of the potential for individual differences in drug response. In contrast, TCM may help focus effective therapy on smaller patient subpopulations which although demonstrating the same disease phenotype are characterized by distinct profiles. To exploit the opportunities in TCM individualized medicine, novel technologies are needed. Proteomics technologies are contributing to molecular diagnostics, the foundation of personalized medicine, which enable discovery and development of TCM drugs suitable for personalized therapy (Cho, 2004).

Proteomics technology is one of the fastest growing technologies in the world; it can be used to deepen our understanding of the biological phenomena. Using proteomics approaches to identify therapeutic targets, to evaluate the effects of new drugs, and to explore the functional mechanism of the effects of TCM, may meet the shortcomings of the conventional methodology being applied in the current studies. Proteomics technologies are also useful for the chemical and pharmacological standardization, as well as the proof of the toxicological potential of a plant extract. Over a long-term perspective, they may economize the proof of efficacy and the determination of the action mode of phytomedicines, and allow the investigation of herbal extracts without prominent active principles. Proteomics can be applied not only to the discovery of a single bioactive lead compound, but also to the development of active fractions, effective and safe herbal prescriptions as new medicines, and high quality dietary supplements. The application of the proteomics technologies may lead to a change of paradigms towards the application of complex mixtures in medicine and open the new field of phytoproteomics, such as speeding-up the integration of TCM and the use of modern science and technology, as well as promoting the internationalization of TCM (Cho, 2006b; Ulrich-Merzenich *et al.*, 2007).

The understanding of protein-protein interactions is of fundamental importance in biology. The process of cell growth, programmed cell death and the decision to proceed through the cell cycle are all regulated by signal transduction through protein complexes.

Functional proteomics combines proteomics tools and bioinformatics to explore and unravel the molecular machinery of the cell. It is a broad term for many specific and directed proteomics approaches. These approaches allow a selected group of proteins to be studied and characterized, which can provide important information about protein signaling, disease mechanisms, or protein-drug interactions. Using the functional proteomics approaches, protein-protein interaction networks can be unraveled, cells are explored for multi-enzyme complexes producing secondary metabolites, and protein complexes are studied in the context of the cell cycle. Functional proteomics combined with the comprehension of the cell signaling networks may substantially contribute to the development of a molecular evidence-based TCM research (Graves and Haystead, 2002; Ventura, 2005).

Numerous proteins may play a role in TCM drug response and toxicity, which introduce a daunting level of complexity into the search for candidate proteins. The high-throughput proteomics technologies enable the search for relevant proteins. They have essentially spawned a new discipline, coined as pharmacoproteomics, which seeks to identify the variant proteins affecting the response to drugs in individual patients. Pharmacoproteomics is a functional representation of patient-to-patient variation. Proteomics-based characterization of multifactorial diseases may help to match a particular target-based therapy to a particular marker in a subgroup of patients. Moreover, pharmacoproteomics analysis can identify disease susceptibility proteins representing potential new drug targets. All of these will pave the ways to novel approaches in TCM drug discovery, an individualized application of TCM drug therapy, and new insights into disease prevention.

On the other hand, many of the proteomics technologies are used in systems biology, and they have a profound interrelation between the integrity and systematic characteristics of TCM. High-throughput proteomics technologies can be used to study the disease-resistant mechanism of TCM at molecular level to bring clinical TCM and drug development into an evidence-based framework. Proteomics overcomes the limitations of genomics, which can reveal the mechanisms of more complicated life and disease processes, thus becoming a valuable new tool to study the mechanisms of TCM (Smith and Figeys, 2006).

Striding into the 21st century, modern technologies develop the scientific innovation in TCM. Proteomics greatly facilitates the progress in quality evaluation and standardization of TCM. It can be used to bridge TCM and life sciences, constitute international standard and canonical research on TCM, as well as promote the modernization and internationalization of TCM. Seeing a huge demand for herbal medicines in the international drug market, application of proteomics in TCM research is now faced with unprecedented development opportunity.

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