

Proteomics for the identification of specifically oxidized proteins in brain: Technology and application to the study of neurodegenerative disorders

Review Article

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Summary. Proteomics offers the opportunity elucidate the complex protein interactions of cellular systems by studying the products of genes, i.e., proteins, and their structure, function and localization. The purpose of proteomics is to explain the information contained in the genome sequences in order to provide clues on cellular events, especially related to disease.

Our proteomic approach has made possible the identification of specifically oxidized proteins in Alzheimer's disease (AD) brain, providing for the first time evidence on how oxidative stress plays a crucial role in AD-related neurodegeneration. This represents an example of the use of proteomics to solve biological problems related to disease. The field, which is still in its infancy, represents a very promising way to elucidate mechanism of disease at a protein level. However, the techniques that support its development present several limitations and require introduction of new tools and innovation in order to achieve a fast, reliable and sensitive method to understand normal biological processes and their regulation as well as these cellular properties in disease.

Keywords: Proteomics – Alzheimer's disease – Oxidized proteins – Neurodegeneration

Introduction

With the completion of the human genome sequence, science is trying now to solve the problems related to biological information that cannot be obtained from the study of genes. Proteomics is the science of elucidating the dynamics of cellular systems by studying the products of genes, proteins, and their structure, function and localization. The focus on proteins rather than genes represents an important step to understand the genome itself, by providing accurate information about the existence and function of a given gene. Proteomics also applies to protein expres-

sion studies, given that the analysis of mRNA does not necessarily reflect the actual cellular protein content (Anderson et al., 1997; Abbot, 1999). Additionally, clues on protein function and modification, together with its compartmentalization and interaction with other biomolecules, can also be retrieved from proteomic studies. Collection of this information offers the opportunity to study mechanisms of disease, conditions in which proteins are often involved in pathological events, and the identification of new potential therapeutic targets.

The technology that supports proteomic studies is the key for its development and advancement. The main technique that today is still the most effective way of separating complex mixtures of proteins, which is crucial for proteomic analysis, is two-dimensional (2D) protein electrophoresis. Despite the fact that the electrophoresis procedure is slow and non-automated, it is largely used to resolve complex biological mixtures. However, without the advances in mass spectrometry to meet the proteomic demand, the growth of proteomics to the current level would not have been possible. Softer ionization techniques (MALDI and ESI, for review see Aebersold and Goodlett, 2000) together with suitable mass analyzers, which resolve the molecular ions according to their mass and charge, have enabled studies of peptide and protein sequence to take place. Once separated in single detectable spots through electrophoresis, single proteins are digested in-gel with a proteolytic enzyme and the result-

ing peptides are then extracted and submitted for mass spectrometry analysis. The obtained mass values are then used to perform a database search in order to identify the protein corresponding to the given spots.

Proteomics and disease

One of the tasks of current proteomics research is to produce maps of protein expression that allow comparison between normal and treated, injured, or disease tissue, or to build comprehensive protein databases that are useful to provide information about the diversity of protein expression in different species or different organs or systems within a species. Recently, proteomics techniques have been applied to human brain samples from people affected by neurodegenerative diseases or brain from transgenic animals used as model of neurodegenerative diseases. Several publications have shown how the protein pattern is modified in disease (Korolainen et al., 2002; Tilleman et al., 2002a, b), providing clues on the effects of neurodegeneration at a protein level.

We recently have combined proteomics techniques such as 2D gel electrophoresis and mass spectrometry analysis together with immunochemical detection of protein carbonyls, a marker of protein oxidation (Butterfield and Stadtman, 1997), to identify specifically oxidized proteins in Alzheimer's disease brain (Castegna et al., 2002a, b). Our approach in brain to identify specifically oxidized proteins in AD has been complimented by the work of others (Choi et al., 2002), who identified oxidized proteins in AD plasma. In addition, a method for identification of detection of oxidized proteins in AD brain has been recently described, although actual identification of oxidized proteins was not provided (Korolainen et al., 2002).

Protein oxidation is indexed by increased levels of protein carbonyls (Butterfield and Stadtman, 1997), 3-nitrotyrosine (Beckman, 1996), and methionine sulfoxide (Moskovitz et al., 2001). Protein carbonyls arise in proteins in one of three principal ways: (a) peptide backbone scission with subsequent carbon-centered radicals that bind paramagnetic molecular oxygen; (b) oxidation of specific amino acids to introduce aldehydes or ketones, e.g., oxohistidine; and (c) by oxidative modifications of histidine, lysine, or cysteine residues following Michael addition of the reactive lipid peroxidation products, 4-hydroxy-2-trans-nonenal [HNE] or 2-propene-1-al [acrolein] (Esterbauer et al., 1991; Butterfield et al., 2001, 2002).

The mechanism for 3-nitrotyrosine formation undoubtedly involves reaction with peroxynitrite or its reaction

products with CO₂ or SH-containing compounds (Beckman, 1996). Methionine sulfoxide formation, which is reversed by the action of methionine sulfoxide reductase (Moskovitz et al., 2001), usually involves reaction with H₂O₂, but possibly with sulfuranyl radicals and oxygen (Bobrowski et al., 1998).

In AD brain, protein oxidation is as indexed by protein carbonyls (Hensley et al., 1995; Butterfield et al., 2001; Butterfield and Lauderback, 2002), and 3-nitrotyrosine (Smith et al., 1997). Increased HNE in AD brain (Markesbery and Lovell, 1998) may account for excess HNE binding to important transporters or enzymes in AD brain. [The effect of which is increased protein carbonyls (Lauderback et al., 2001)]. For example, the glutamate transporter GLT L-1 is oxidatively modified by HNE in AD brain (Lauderback et al., 2001), and amyloid β -peptide (1-42) [A β (1-42)], which causes HNE formation (Mark et al., 1997), also leads to HNE binding to GLT-1 (Lauderback et al., 2001).

The detection of markers of oxidation in AD brain, which clearly shows the importance of oxidative stress in the progression of the disease, (Butterfield et al., 2001, 2002; Butterfield and Lauderback, 2002), still does not provide evidence on how oxidized proteins, lipids and DNA might affect cellular metabolism and ultimately lead to synaptic loss and neuronal death, known events in AD. Identification of specifically oxidized proteins in AD brain allows one to determine which proteins are more affected by oxidation in AD and, consequently, more prone to inactivation, and thus represents a significant step in linking well-established AD neurodegeneration with oxidative events at a protein level.

Proteomics offers a means of identifying such oxidized proteins. The key steps in our proteomics approach to identify specifically oxidized proteins are the separation of brain homogenate into single detectable proteins through 2D-gel electrophoresis, paralleled with immunochemical detection of protein carbonyls, followed by mass spectrometry analysis. The overall procedure is outlined in Fig. 1 and described in detail in the method section.

Identification of proteins containing reactive carbonyl groups in AD and control brain samples is determined by 2D Oxyblot analysis (Fig. 2). Comparison of 2D Oxyblots with images of Coomassie Blue-stained 2D gels from the same samples revealed that many, but not all, individual protein spots in brain extracts from the inferior parietal lobule (IPL) exhibit anti-protein carbonyl immunoreactivity (Fig. 2A and B). 2D Oxyblots and the subsequent 2D gel images are matched and the anti-DNP immunoreactivity of individual proteins separated by 2D PAGE are

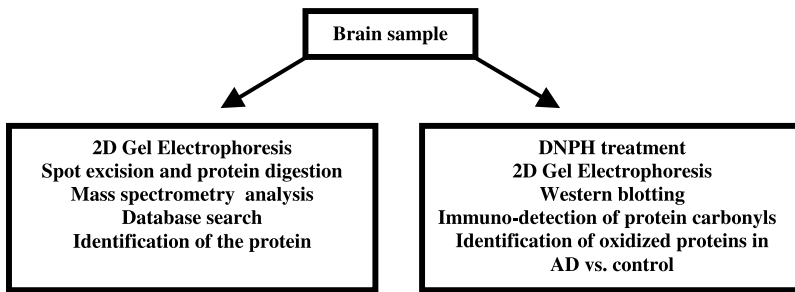


Fig. 1. Outline for identification of oxidized proteins in AD

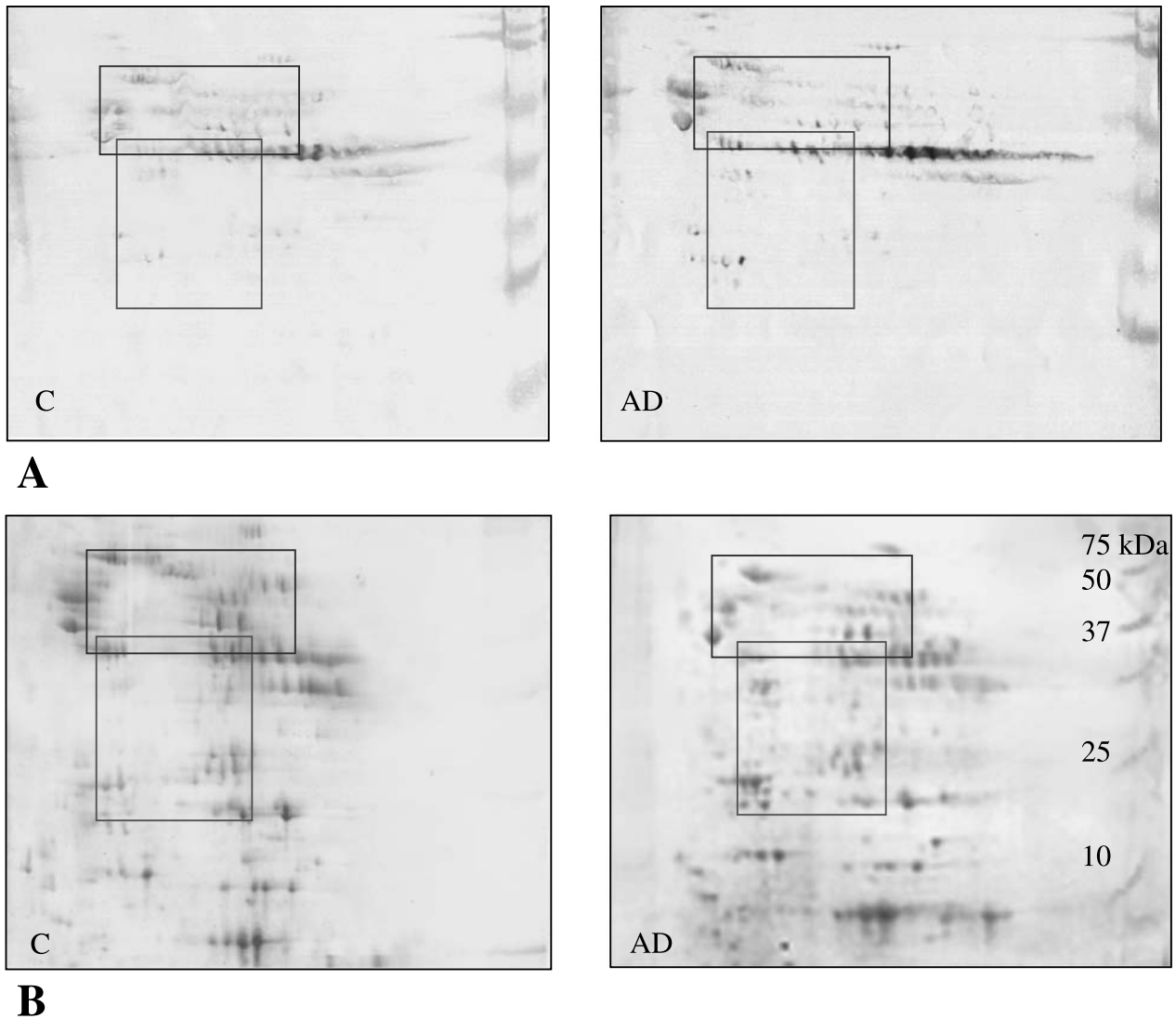


Fig. 2. 2D Oxyblots (A) and Coomassie Blue-stained gels (B) from control and AD brain extracts. Boxes mark image areas that are enlarged in Fig. 3

normalized to their content, obtained by measuring the intensity of colloidal Coomassie Blue staining. This procedure allows comparison of oxidation levels of brain proteins in AD versus control subjects. The first use of

proteomics to identify specifically oxidized proteins in AD brain led to the identification of several specifically oxidized proteins: creatine kinase BB, glutamine synthase, ubiquitin carboxy-terminal hydrolase L-1, and

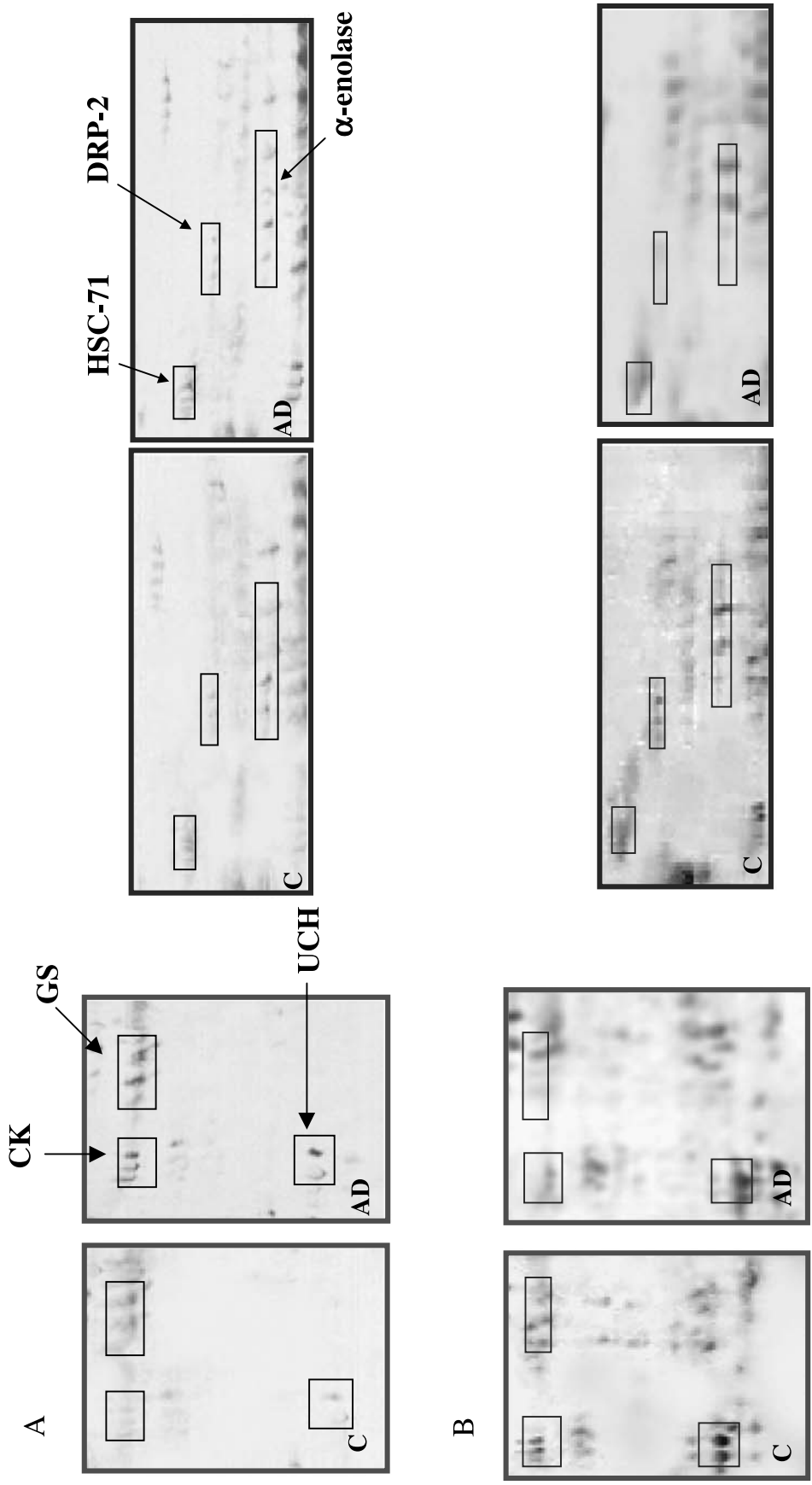


Fig. 3. A Expanded Oxyblot images (boxed areas in Fig. 2A) show protein carbonyl immunoreactivity in creatine kinase (CK BB), glutamine synthetase (GS), ubiquitin carboxy-terminal hydrolase L-1 (UCH L-1), dihydropyrimidinase related protein 2 (DRP-2), also known as collapsin response mediator protein 2, CRMP-2), α-enolase and heat shock cognate-71 (HSC 71). **B** Expanded 2D gel images (boxed in Fig. 2B) show the position of protein spots identified by mass spectrometry. Relative changes in carbonyl immunoreactivity, after normalization of the carbonyl intensities to the protein content, were significant for all the proteins identified, except HSC-71. In the latter case, significance was nearly reached. See Castegna et al. (2002a, b) for details

dihydropyrimidinase related protein 2 (Castegna et al., 2002a, b). Plausible mechanisms of neurodegeneration in AD brain can be generated based on each of these specifically oxidized proteins. Moreover, some of the pathological characteristics of AD brain, such as accumulation of aggregated, damaged proteins, excess ubiquitination, and shortened dendritic lengths are consistent with putative dysfunction of some of these specifically oxidized proteins. [See Butterfield et al., 2003 and Castegna et al., 2003 for additional discussion of these points.]

Methods

Sample preparation

Brain samples were minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4 , 0.6 mM MgSO_4 and proteinase inhibitors: leupeptin (0.5 $\mu\text{g}/\text{ml}$), pepstatin (0.7 $\mu\text{g}/\text{ml}$), type IIS soybean trypsin inhibitor (0.5 $\mu\text{g}/\text{ml}$) and phenylmethylsulfonyl fluoride (PMSF) (40 $\mu\text{g}/\text{ml}$). Homogenates were centrifuged at 14,000 g for 10 min to remove debris. Protein concentration in the supernatant was determined by the Pierce BCA method.

2D gel electrophoresis

2D PAGE was performed in a Multiphor II Electrophoresis system using 110-mm pH 3–10 Immobiline DryStrips and ExcelGel XL 12–14 gels (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Samples were incubated with agitation for 30 min at room temperature in four volumes of 10 mM 2,4-dinitrophenylhydrazide (DNPH)/2 M HCl for Oxyblot, or 2 M HCl for mass spectrometry analysis, and proteins were precipitated by addition of ice-cold 100% trichloroacetic acid to a final concentration of 15%. Precipitates were centrifuged at 14,000 g for 10 min following incubation on ice for 10 min and the residues were washed with 1 ml of 1:1 (vol/vol) ethanol/ethyl acetate. After centrifugation for 15 min at 15,000 g three times, the samples were resuspended in 8 M urea to a final protein concentration of 4 mg/ml, and were diluted to 2–4 mg/ml in 8 M urea and mixed 1:4 (vol/vol) with IEF sample buffer (8.7 M urea, 1.0% (wt/vol) dithiothreitol, 2.0% (vol/vol) Pharmalyte 3–10, 0.5% Triton X-100, and bromophenol blue. For the first-dimension, 100 μg of proteins were applied via a sample cup to a rehydrated Immobiline DryStrip, and the isoelectric focusing was carried out at room temperature. Isoelectric focusing was performed for 22,700 Vh. For the second-dimension separation, Immobiline Dry-Strips were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (wt/vol) SDS, 30% (vol/vol) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 10 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. All strips were placed on ExcelGel XL SDS 12–14 gels, unstained molecular standards were applied, and the electrophoresis was started. Second dimension gels were run at 1000 V/20 mA for 45 min, followed by 1000 V/40 mA, for 2.5 h.

SYPRO Ruby staining

The gel slabs were fixed in 10% methanol and 7% acetic acid for 30 min. The fixed solution was removed and 500 mL of SYPRO Ruby gel stain (Bio-Rad) was added to each gel and gently rocked at room temperature for 18 hrs.

Western blotting

For immunoblotting analysis, the electrophoresis was carried out in the same way as described above, and the gels were transferred to a nitrocellulose membrane. The membranes were incubated with rabbit anti-DNP

antibody (Oncor, Gaithersburg, MD, U.S.A.) diluted 1:150 in 0.3% BSA/PBST for 1 h at room temperature. After incubation with primary antibodies was complete, membranes were washed for 10 min in PBST three times. Secondary antibody (anti-rabbit alkaline phosphatase-conjugated IgG, Sigma), diluted 1:30,000 in 0.3% BSA/PBST, was then added to blots for 1 h at room temperature. Membranes were washed three times (10 min per wash) with PBST and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma-Fast tablets; Sigma).

In gel protein digestion

The protein spots were excised with a clean scalpel into 1-mm cubes. The gel pieces were transferred to clean 1.5-ml microcentrifuge tubes and wash with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was added to the gel pieces and incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in laminar flow hood. The gel pieces were rehydrated with 20 μl of 20 mM DTT in 0.1 M NH_4HCO_3 and incubated at 56°C for 45 min to reduce the protein. The tubes were chilled at room temperature, and the DTT solution was removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and incubated at room temperature in the dark for 30 min. The iodoacetamide was removed and replaced with 0.2 ml of 50 mM NH_4HCO_3 and incubated at room temperature for 15 min. Acetonitrile (0.2 ml) was added, and the samples were incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in laminar flow hood. The gel pieces were rehydrated with 20 ng/ μl modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 with the minimal volume to cover the gel pieces. The gel pieces were chopped into four to five smaller pieces and incubated at 37°C overnight in shaking incubator to enhance microcirculation of the digestive solution and to prevent drops formation under the cover of microcentrifuge tubes.

Sample preparation for MALDI-TOF mass spectrometry

Nitrocellulose solution was made by dissolving a nitrocellulose membrane in 1:1 acetone/isopropanol solvent. Alpha-cyano-4-hydroxycinnamic acid (α -CN) was washed with 50 μL of acetone and acetone phase was discarded. The α -CN was dissolved in acetone to a concentration of 10 mg/ml. and the nitrocellulose and α -CN solutions were mixed to 1:4 ratio and 1 μL of this mixture was deposited onto the 96-well MALDI target plate. The samples were prepared for addition to the plate by mixing 2 μL of sample with 2 μL of 10 mg/mL α -CN solution in 0.1% trifluoroacetic acid in 1:1 H_2O /Acetonitrile. The sample mixtures (1 μL) were loaded onto each thin film. After the sample mixtures were dried, 1.5 μL of 2% formic acid in 18 mega Ohm water was added to each spot. The formic solution was removed by gentle blotting. This washing step was performed twice. The samples were then dried at room temperature. Fragment size was determined by MALDI-TOF mass spectrometry.

Mass spectral data were obtained using a Micromass TOF-Spec 2E instrument with a 337 nm N_2 laser at 20–35% power in the positive ion reflectron mode. Spectral data were obtained by averaging 10 spectra, each of which was the composite of 10 laser firings. The mass axis was calibrated using known peaks from tryptic autolysis.

Analysis of gel images

The analysis of gels and membranes to compare protein and carbonyls content between control and AD samples were performed with HT Analyzer 2D PAGE software (Genomic Solutions). Images from Sypro Ruby stained gels for proteomic analyses were obtained using a high-resolution 12-bit camera with UV light box system (Genomic Solutions Inc.). Gels were exposed to UV light for five different exposure time-points (1, 2, 3, 4 and 5 sec). Coomassie Blue stained gels, used to measure protein content, were scanned with a Scanjet 3300C (Hewlett Packard).

Analysis of peptide sequences

Peptide mass fingerprinting was used for protein identification from tryptic fragment sizes by using the Mascot search engine (<http://www.matrixscience.com>) querying to entire theoretical human peptide masses in the NCBI and SwissProt protein databases using the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Up to 1 missed trypsin cleavage was allowed although most matches did not contain any missed cleavages. A mass tolerance of 150 ppm was allowed for matching the peptide mass values. Probability-based MOWSE scores were calculated by the software by comparison of search results against estimated random match population and were reported as $-10 * \text{LOG}_{10}(P)$, where P is the absolute probability. Scores greater than 71 were considered significant ($p < 0.05$). We also used the ProFound search engine (http://129.85.19.192/profound_bin/WebProFound.exe) as a complementary and confirmatory search tool to the former. Z scores were estimated by comparison of search results against estimated random match population and were the distances to the population mean in unit of standard deviation. Scores greater than 1.65 were considered significant ($p < 0.05$).

Future developments

The simultaneous challenge and opportunity to gain increased understanding of the mechanisms of the protein network in the cell will provide insight into biological events and disease, although this increased understanding has to face limitations related to the analytical techniques employed. Protein separation still relies on electrophoresis, limiting the possibilities of obtaining a more vast picture of the cellular status. Only cytosolic proteins can be “seen” on a gel map, whereas it is still difficult to reproduce a decent map of membrane-bound proteins. Additionally, more basic proteins tend to escape the focusing gradient, and low-abundant proteins, potentially very interesting for the study of disease, remain silent on a gel map. Recently, the introduction of isotope coded affinity tags (Gygi et al., 1999), and techniques of protein separation without 2D-electrophoresis (Link et al., 1997; Opiteck et al., 1997), show how proteomics is pointing to new tools in order to achieve the goal of “fast-reliable-sensitive”.

Our study of oxidized proteins in AD brain demonstrates how proteomics can give insights into the cellular state during development of disease and illustrates nicely an example of the intersection of analytical techniques with the most common biological tools for the detection and identification of protein modifications in neurodegenerative diseases.

Despite its current limitation, proteomics is now one of, if not the, most promising approaches for providing clues on the molecular basis of neurodegenerative diseases. One has the opportunity in favorable cases of obtaining answers that cannot be achieved by simply studying gene arrays or single proteins. In our view, the future is bright

for the applications of proteomics to neurodegenerative disorders, and our laboratory is actively employing this methodology to the study of a wide variety of neurodegenerative disorders and models thereof.

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