New Protein Extraction/Solubilization Protocol for Gel-based Proteomics of Rat (Female) Whole Brain and Brain Regions

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The rat is an accepted model for studying human psychiatric/neurological disorders. We provide a protocol for total soluble protein extraction using trichloroacetic acid/acetone (TCA/A) from rat (female) whole brain, 10 brain regions and the pituitary gland, and show that two-dimensional gel electrophoresis (2-DGE) using pre-cast immobilized pH (4–7) gradient (IPG) strip gels (13 cm) in the first dimension yields clean silver nitrate stained protein profiles. Though TCA/A precipitation may not be “ideal”, the important choice here is the selection of an appropriate lysis buffer (LB) for solubilizing precipitated proteins. Our results reveal enrichment of protein spots by use of individual brain regions rather than whole brain, as well as the presence of differentially expressed spots in their proteomes. Thus individual brain regions provide improved protein coverage and are better suited for differential protein detection. Moreover, using a phosphoprotein-specific dye, in-gel detection of phosphoproteins was demonstrated. Representative high-resolution silver nitrate stained proteome profiles of rat whole brain total soluble protein are presented. Shortcomings apart (failure to separate membrane proteins), gel-based proteomics remains a viable option, and 2-DGE is the method of choice for generating high-resolution proteome maps of rat brain and brain regions.

Keywords: Gel-based Proteomics; IPG; Lysis Buffer; Peptide Mass Fingerprinting; Rat Brain; TCA/Acetone.

Introduction

Proteomics, the systematic study of proteins present in a cell, tissue, organ, or organism at a particular time, is a key research area in the functional genomics era. Although DNA-based microarray technology (DeRisi et al., 1997; Schena et al., 1995; Shalon et al., 1996) has become increasingly popular for profiling genome-wide expression of genes at the mRNA level, it is “proteins not genes” that are directly responsible for cell function and phenotype. Moreover this powerful genomic tool does not provide any direct information on protein levels and their state of modification (Anderson and Anderson, 1998) mainly due to post-translational regulation, which results in a lack of correlation between mRNA and protein abundance (Fiehn et al., 2000; Gygi et al., 1999). This is just

Abbreviations: 2-DGE, two-dimensional gel electrophoresis; ADHD, attention-deficit hyperactivity disorder; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl] dimethylamino]-1-propanesulphonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 6-OHDA, 6-hydroxydopamine; IEF, isoelectric focusing; LB-TT, lysis buffer containing Thiouracil and Tris; MALDI-TOF-MS, matrix-assisted laser desorption/ionization - time of flight mass spectrometer; MQ, Milli-Q water; NCBI, National Center for Biotechnology Information; nESI-LC-MS/MS, nano electrospray ionization-liquid chromatography-tandem mass spectrometry; Pro-Q DPS, Pro-Q diamond phosphoprotein staining; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SHR, spontaneous hypertensive rat; TEMED, N,N,N',N'-tetramethylethylenediamine.

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one example of the many types of information, such as the total number of genes in a given genome, protein function, localization and compartmentalization, and protein-protein interactions that one cannot obtain from the study of transcripts alone. Other than the vast wealth of genomic data available to the proteomics researcher, a series of tremendous technological developments in immobileized pH gradient (IPG)-based two-dimensional gel electrophoresis (2-DGE), mass spectrometry (MS), staining and scanning methods, chromatography, bioinformatics, and protein chips, have all fuelled a proteomics revolution in yeast, humans and even plants (for review, Agrawal and Rakwal, 2006; Hanash, 2004; Hancock, 2004; Phizicky et al., 2003).

Our present research involves rats treated with neurotoxins as animal models for psychiatric and neurological disorders. The ideal animal models should be similar to clinical cases in terms of etiology, biochemistry, symptoms and treatment. Why use animal models? Animal models are genetically homogenous, and their environment can be easily controlled allowing for more interventions than in clinical cases. In addition, since the models usually have simpler nervous systems, their behaviors can be more easily interpreted (McKinney and Bunney, 1969; Sagvolden, 2005). Why not mice? We use rats because of their larger brain size. Moreover, rats treated with various drugs have long been used as animal models for important psychiatric disorders such as attention-deficit hyperactivity disorder (ADHD), and the spontaneous hypertensive rat (SHR) has been frequently used as a genetic model of ADHD (Kamimura et al., 2001). The use of rats as models of human psychiatric disorders by our team (at HSS) currently involves studying the mechanisms underlying behavioral hyperactivity in model ADHD rats with 6-hydroxydopamine (6-OHDA)-treated brain lesions (Masuo, 2004a; Masuo, 2004b), as part of a multi-parallel high-throughput functional genomic approach. Along with genomics, we have now undertaken a systematic proteomic approach to the analysis of rat brains. Proteomic analysis of brain/brain regions may help to understand their complexity, to investigate disorders of the central nervous system, and to search for corresponding early markers.

To date, proteomics has mainly studied the identity and amounts of the abundant rat and mouse brain proteins, and modifications resulting from various neurological disorders (for review, see Fountoulakis, 2004). For example regions of the rat brain, such as the thalamus (Paulson et al., 2004), hippocampus (Fountoulakis et al., 2005), striatum (Yeom et al., 2005) and frontal cortex (Kim et al., 2005), were homogenized or sonicated directly in chloroform/methanol/water (Paulson et al., 2004), isoelectric focusing (IEF) resuspension buffer (Fountoulakis et al., 2005; Yeom et al., 2005), or Tris-HCl buffer (Kim et al., 2005) to extract proteins, and the subsequent 2-D proteomic profiles obtained were presented. Different research groups use quite different protein extraction protocols for rat brains and their sub-regions, and it would be extremely desirable, if possible, to have a standardized protein extraction protocol to achieve better reproducibility between laboratories. It must be emphasized that we do not criticize any particular method but aim to improve the resolution of 2-DGE by employing an as-yet-unused trichloroacetic acid (TCA)/acetone extraction buffer (AEB), termed TCAEAB, for precipitating proteins coupled with a modified resolubilization lysis buffer (LB). We present a protocol from start (sampling/sample preparation) to finish (IPG and SDS-PAGE and staining) for achieving this goal for rat brain proteomes. Moreover, as good sample preparation - “extraction of a maximum number of proteins from a given cell, tissue, organ or organism” - is the first and most crucial step in protein separation and identification, we focused on establishing a new protein extraction protocol for rat brain proteomics that could theoretically be applied to any organ/tissue of the rat. This protocol, along with subsequent separation of proteins by 2-DGE using pre-cast IPG strip gels, standardization for IEF of rat brain proteins in the first dimension, followed by visualization of proteins by staining with silver, and image analysis, is described, using the female rat whole brain and three regions - frontal cortex, striatum, and the midbrain - as examples of our ongoing research program.

### Materials and Methods

**Growth conditions and brain dissection** Adult female Wistar rats were purchased from Clea Japan (Japan). The animals were housed in acrylic cages at 22°C with tap water and laboratory chow *ad libitum*. The breeding rooms were illuminated from 07:00 to 19:00 h in 12 h cycles. The rats were decapitated at around 10 weeks of age and whole brains were rapidly removed, and, when required, the frontal cortex, striatum, and midbrain were separated on ice (Masuo, 2004a; 2004b). The experiments were carried out in accordance with regional legal regulations. For the midbrain, we made a frontal section between the anterior and posterior edges of the substantia nigra in order to investigate alterations in this region rich in dopaminergic neurons. The 10 brain regions were dissected following the method of Glowinski and Iversen (1966) with some modifications. Each sample (whole brain or regions) was immediately weighed, flash frozen in liquid nitrogen, and stored at –80°C.

**Extraction of total protein** Extraction of total protein was performed using a two-step protocol. Whole brain samples from three individual female rats were analyzed separately. The frozen brains were each placed in liquid nitrogen, and ground thoroughly to a very fine powder with a mortar and pestle. The tissue powder (50 to 100 mg) was transferred to sterile tubes containing cold TCAEAB [acetone containing 10% (w/v) TCA, and...
0.07% mercaptoethanol], and the proteins were precipitated for 1 h at –20°C, followed by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was decanted, and the pellet washed twice with chilled wash buffer (acetone containing 0.07% mercaptoethanol, 2 mM EDTA, and EDTA-free proteinase inhibitor cocktail tablets (Roche Diagnostics GmbH, Germany) in a final volume of 100 μl buffer), followed by removal of all the acetone. The pellet was subsequently solubilized in LB-TT [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 18 mM Tris-HCl (pH 8.0), 14 mM trizma base, two EDTA-free proteinase inhibitor cocktail tablets in a final volume of 100 μl buffer, 0.2% (v/v) Triton X-100 (R), containing 50 mM dithiothreitol (DTT)], and incubated for 20 min at 4°C with occasional vortexing (a total of 5 times for 10 s each), and centrifuged at 15,000 rpm for 15 min at 10°C. At this point some impurities may be visible as particles in solution; these can be removed either by filtration through a 0.45 μm syringe-driven filter unit or/and by another round of acetone (4 volumes) precipitation and resolubilization in LB-TT. Supernatant protein was determined using a Coomassie Plus™ (Pierce, USA) protein assay kit, and stored in aliquots at –80°C.

Two-dimensional gel electrophoresis 2-DGE was carried out using IPG strip gels (GE Healthcare Bio-Sciences AB, Sweden) on an IPGphor unit (GE Healthcare Bio-Sciences AB) followed by the second dimension using hand-cast polyacrylamide gels on a Nihon Eido (Japan) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) vertical electrophoresis unit. A sample containing 100 μg of total soluble protein was mixed with LB-TT containing 0.5% (v/v) pH 4–7 IPG buffer in a final volume of 250 μl. A trace of bromophenol blue (BPB) was added and the whole mixture was kept at room temperature (RT) for 5 min, vortexed, and centrifuged at 15,000 rpm for 15 min at 10°C followed by pipetting into a 13 cm strip holder tray. IPG strips (pH 4–7; 13 cm) were carefully placed onto the protein samples, covered with a lid, and placed into the IPGphor unit. The IPG strips were placed gel-face down onto the protein samples avoiding air bubbles and allowed to passively rehydrate with the protein samples for 90 min, followed by overlaying with 700 μl cover fluid, and this was directly linked to a five-step active rehydration and focusing protocol (13 cm strip). The whole procedure was carried out at 20°C, and a total of 60599 Vh (volt hour) was used for the 13 cm strips. For 18 cm IPG strips (pH 4–7), a final volume of 350 μl (containing 125 μg total soluble protein) was applied, followed by overlaying with 1000 μl cover fluid, and IEF (68902 Vh). Following IEF, the IPG strips were removed from the strip holder and the cover fluid adsorbed on filter papers. The strips were then immediately used for the second dimension or stored at –20°C.

The strip gels were incubated in equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS] containing 2% (w/v) DTT for 10 min × 2 times) with gentle agitation, followed by incubation in the same equilibration buffer supplemented with 2.5% (w/v) iodoacetamide for the same time periods as above at RT. For the second dimension, the IPG strips were rinsed with cathode running buffer [0.025 M Tris, 0.192 M glycine and 0.2% (w/v) SDS], placed onto polyacrylamide gels and overlaid with overlay agarose solution [60 mM Tris-HCl, pH 6.8, 60 mM SDS, 0.5% (w/v) agarose, 0.01% (w/v) BPB]. The lower anode buffer contained 0.05 M diethanolamine and 0.05 M acetic acid. SDS-PAGE (4% T, 2.6% C stacking gels, pH 6.8 and 12.5% T, 2.6% C separating gels, pH 8.8) was carried out at a constant current of 30/40 mA per 13/18 cm gels for ca. 2.5/4.5 h. The % T is the total monomer concentration expressed in grams per 100 ml and the % C is the percentage of cross-linker. The stacking and separating gel buffer concentrations were 0.125 M Tris-HCl, pH 6.8, and 0.375 M Tris-HCl, pH 8.8, respectively. Molecular masses were determined by running standard protein markers (DualColor PrecisionPlus Protein™ Standard; Bio-Rad). For each sample, duplicate IPG strips and polyacrylamide gels were run in the same conditions.

Protein visualization To visualize the protein spots, the polyacrylamide gels were stained with silver. Staining with silver nitrate was performed as described in the instructions provided with the Plus One Silver Staining Kit (GE Healthcare). Protein patterns in the gels were recorded as digitalized images using a digital scanner (Canon CanoScan 8000F, resolution 300 dpi), and saved as TIFF files. The gels were quantitated in profile mode as instructed in the operating manual of the ImageMaster 2D Platinum software (GE Healthcare). The phosphoproteins on
Matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-MS (MALDI-TOF-MS) The excised gel spots were destained with 100 μl of destain solution [30 mM potassium ferricyanide (Sigma), in 100 mM sodium thiosulfate (Merck)], with shaking for 5 min. After the solution was removed, the gel spots were incubated with 200 mM ammonium bicarbonate (Sigma) for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min and rehydrated with 20 μl of 50 mM ammonium bicarbonate containing 0.2 μg modified trypsin (Promega, USA) for 45 min on ice. After removal of solution, 30 μl of 50 mM ammonium bicarbonate was added and digestion was performed overnight at 37°C. The peptides were desalted and concentrated using a C18 nanoscale (porous C18) column (home-made). For the analysis of MALDI-TOF MS by the peptide-mass fingerprinting (PMF) method (Jensen et al., 1997), the peptides were eluted with 0.8 μl of matrix solution [70% acetonitrile (Merck), 0.1% TFA (Merck), 10 mg/ml alpha-cyano-4-hydroxycinnamic acid (Sigma)] and spotted onto a stainless steel target plate. Masses of peptides were determined by MALDI-TOF MS (Model M@LDI-R; Micromass, UK). Calibration was performed using the internal mass of a trypsin auto-digestion product (m/z 2211.105). To identify the protein, the peptide masses from MALDI-TOF MS were matched with the theoretical molecular weights of peptides for proteins in the NCBI database using MASCOT software (www.matrixscience.com).

Results and Discussion

2-D gels were detected with Pro-Q Diamond phosphoprotein gel stain (Pro-Q DPS; Molecular Probes, Inc., USA) following the manufacturer’s protocol and a published report (Steinberg et al., 2003). The SYPRO ruby stain for staining total protein as reference for Pro-Q DPS, and the PeppermintStick phosphoprotein molecular weight standards, were obtained from Molecular Probes. The 2-D gels were also stained using the optimized conditions presented in a recent study by Agrawal and Thelen (2005), which provides a simplified and cheaper protocol for detecting phosphoproteins on 1- and 2-D gels. For visualizing the phosphoproteins we used a UV-transilluminator (ATTO, Japan). The spots were excised from the 2-D gels with a gel picker (One Touch Spot Picker, P2D1.5 and 3.0, The Gel Company, USA), transferred to sterilized Eppendorf tubes (1.5 ml), and stored at –30°C.

Rat brain proteins after finely grinding the tissues in liquid nitrogen since this method removes several compounds (salts, pigments, etc.) that interfere with IEF, a very important criteria for high resolution separation of protein spots. An additional benefit is that using the finely ground powder, complementary techniques can be applied simultaneously for integrative functional genomics. However, we emphasize that protein precipitation usually results in protein losses and also causes difficulties in resolubilization of the proteins. We used LB-TT for solubilization of the precipitated brain proteins, and obtained reasonably good colloidal Coomassie brilliant blue-stained protein profiles from high to low molecular weight regions on SDS-PAGE in the second dimension (data not shown). The yield of soluble protein from the brain samples was on average ca. 3% of the total fresh weight brain tissue. Using the modified LB-TT we examined in detail the IEF protocols provided by GE Healthcare for the IPG gels, to obtain the best resolution of proteins in the first dimension. After experimenting with various IEF protocols, we developed a modified protocol for rehydration and IEF of brain proteins that gave very clean protein separation in the second dimension.

With these optimized conditions in place, we used whole brain and various brain regions to test the efficiency of the standardized methodology for proteomic analysis of each of the samples presented in Fig. 1. Gel-to-gel reproducibility, an extremely important factor especially when dealing with 2-D gels, was confirmed using two IPG strips and SDS-PAGE gels, respectively, per sample, after pooling protein extracts from three independent samples (brain). Moreover, to reduce variation between gels, we maintained consistency with respect to buffer conditions, sample preparation, and silver staining. Using IPG strips, especially in the range of pH 4–7, where most of the protein spots are concentrated, it was possible to obtain highly reproducible protein profiles in the second dimension, where high-resolution separation of spots is evident from the gel images of the four brain samples (Fig. 1). A total of 100 μg protein was separated. Ca. 454, 532, 639, and 695 spots were detected on the 2-D gels of whole brain, frontal cortex, striatum, and midbrain, respectively. These numbers are significantly higher than those obtained with hand-cast IEF tube gels (non linear, pH 3.5–10) taking into account the differences in protein loading volumes (data not shown). The increase in the number of spots in the frontal cortex, striatum and midbrain compared to whole brain is most probably due to enrichment of proteins by selecting specific regions for sample preparation and extraction of total soluble protein. 12, 24 and 26 spots were differentially expressed in these three regions, respectively. This result not only demonstrates the importance of IPG strips and silver staining in creating high-resolution proteome maps of the brain, but also stresses the importance of sample selection. To fur-
Fig. 2. Rat whole brain and its regions. The whole brain (1) was removed from female rats, and each region (2–11) was dissected out along with the pituitary gland (12), and photographed in anatomical order, i.e. from anterior to posterior: whole brain (1.7 g), olfactory bulb (81 mg), cerebral cortex (553 mg, frontal and other cortices), septum (38 mg), striatum (90 mg), hippocampus (65 mg), thalamus (97 mg), hypothalamus (100 mg), midbrain (95 mg), cerebellum (249 mg), medulla-oblongata (219 mg), and pituitary gland (11 mg).

Fig. 3. Representative 2-D gel images of rat brain proteins (whole brain, 10 regions, and pituitary gland as shown in Fig. 2) separated on IPG strips (13 cm, pH 4–7) in the first dimension; ca. 100 μg soluble protein was loaded. IEF and SDS-PAGE were performed as described in Fig. 1. Proteins were visualized by staining with silver, and spot numbers in each gel are given at the top right-hand corners. The pI points and molecular mass standards are marked. The figures in parenthesis beside the region names below each gel image (A and B) are the numbers of differentially expressed spots (marked by arrows) as compared to the whole brain.

ther demonstrate our methodology and its suitability for rat brain proteomics, we dissected a further 10 regions of the female brain, namely, olfactory bulb, cerebral cortex, septum, striatum, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, and medulla-oblongata, and the pituitary gland (Fig. 2). The results in Fig. 3 show the proteomes of these 12 regions, including that of the whole brain, as well as the pituitary gland of the female rats. A total of 476, 801, 628, 873, 564, 490, 677, 626, 452, 467, 467, and 588 protein spots were detected (Figs. 3A and 3B). Again, there was an increase in the number of detected spots (given in parenthesis in Fig. 3) over the whole brain. The pituitary gland also had a unique proteome map. It is important to mention here that, 1) the whole brain and 4 regions (hippocampus, midbrain, cerebellum and medulla-oblongata) contained similar numbers of protein spots, and 2) the difference in spot number between the midbrains shown in Figs. 1 and 3B was due to the procedure employed to dissect the midbrains shown in Fig. 2. As the volume (weight) of the midbrain used in Fig. 1 was significantly less than that of the midbrain used in Fig. 3B, we would expect enrichment (more spots on the gel) with the smaller midbrain. A similar experiment using the brains of male rats resulted in reproducible 2-D gel protein profiles similar to those observed for the female rat brains (data not shown).

The greatest strength of 2-DGE lie not only in its affordability, ease of use and establishment of reference maps, but also in exploring post-translational modifications (PTMs) because post-translationally modified proteins differ in charge as well as mass. Reversible protein phosphorylation, a ubiquitous PTM mechanism, controls a myriad of biological processes. (Pawson and Scott, 2005) This new field of systems biology is loosely referred to as phosphoproteomics (Reinders and Sickmann, 2005). Advances in proteomics technologies, including enrichment, detection, phosphorylation site mapping, and
quantification of phosphoproteins, have made feasible the large-scale study of phosphoproteins (Loyet et al., 2005; Reinders and Sickmann, 2005). A major advance in this field has been the development of a unique fluorescent dye, Pro-Q DPS, with high sensitivity and linearity, for detecting phosphoproteins (Steinberg et al., 2003). Therefore, we next examined its potential for detecting phosphoproteins in-gel after separating a rat whole brain total protein extract by 2-DGE. Figure 4 reveals ca. 35 to 40 proteins stained with Pro-Q DPS using the original protocol recommended by Molecular Probes (Steinberg et al., 2003), and a modified protocol that uses 3-fold-diluted Pro-Q DPS, developed by Agrawal and Thelen (2005). As reported recently, the 3-fold diluted Pro-Q DPS offers the same sensitivity and linearity of signal as that obtained with undiluted Pro-Q DPS (Agrawal and Thelen, 2005). We selected some protein spots at random for excision and identification. Three major spots stained by Pro-Q DPS were identified by PMF using the Mascot Search Engine; these were, 1) tubulin alpha-2 chain (Alpha-tubulin 2) isoform 8 [Bos taurus], 2) tubulin, beta 4 [Canis familiaris], and 3) a putative beta-actin (aa 27–375) [Mus musculus]. Two of these proteins have been previously reported from the rat brain (Fountoulakis et al., 1999; 2005), while the tubulin beta 4 is a new finding. However, to the best of our knowledge, no previous report has detected these three proteins as phosphoproteins. It should be noted that we still have to confirm them as phosphoproteins by determining their phosphorylation sites.

Fig. 5. Representative 2-D gel images of rat whole brain proteins separated on large 18 cm IPG strips (pH 4–7 and 6–9) in the first dimension. Ca. 125 μg total soluble protein was loaded for IEF, and SDS-PAGE was performed as described in Figs. 1 and 3. The pI points and molecular mass standards are marked. Proteins were visualized by staining the gels with silver, and spot numbers in each gel are indicated at the bottom-center of the gel image.

Conclusions In conclusion, we present a simple and efficient proteomics methodology for protein extraction and subsequent 2-DGE. The novelty of the study lies in the use of dissected brain regions for revealing protein profiles by computerized image analysis. Precise dissection of the 10 brain regions resulted in an increased yield of proteins for some of the brain regions, including the detection of differentially expressed spots. The better spot focusing and resolution obtained by using the IPG strips (18 cm or longer, and covering the pH range from 4–7, and 6–9) versus hand-cast IEF tube gels tilts the balance strongly in favor of IPG for generating high-quality 2-D gel proteome maps. Using the 18 cm IPG strip and large hand-cast 12.5% polyacrylamide gels, ca. 892 (pH 4–7) and 475 (pH 6–9) high-quality silver stained spots were visualized (Fig. 5). Due to limitations of sample (protein) loading, we recommend the use of either 18 or 24 cm IPG strips. The (IEF/IPG) standardized protocol provides a basis for reproducibly investigating specific or altered rat brain proteins (tissues), which can be applied from lab to lab and used to investigate disorders of the central nervous system. We believe that despite its limitations, a gel-
based proteomics approach will serve as a powerful tool for identifying biochemical disturbances in complex mental or developmental disorders such as ADHD. We have now embarked on the comparative proteomic analysis of brains (and selected regions) from rats with neonatal 6-OHDA lesions as animal models of ADHD.

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