Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 793–799, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

A computer-enhanced comparative study of brain region polypeptides and proteins by two-dimensional gel electrophoresis*

V. G. DWYER,†§ B. BENSON,† K. H. KWAN,‡ R. C. HUMPHREYS,‡ W. J. KO,‡ N. H. LIN‡ and D. W. SAMMONS‡

† *Department of Anatomy, AHSC, Tucson, AZ 85724, USA*
‡ *Center for Separation Sciences, Membrane Research Laboratory, University of Arizona, Tucson, AZ 85724, USA*

Abstract: A reproducible and quantitative strategy for identifying tissue-specific proteins of the central nervous system is described. The methods include a simple extraction procedure, two-dimensional polyacrylamide gel electrophoresis (2-DGE), silver staining, and computerized analysis. Acetic acid protein extractions of brain regions from three groups of male Sprague–Dawley rats were compared by computer analysis using 2-DGE with GELCODE silver staining. Protein spot mapping and characterizations of molecular weight and pI were compiled for the pineal gland, retina, hypothalamus and cerebral cortex. Regionally specific protein spots were identified using the Visage System (BioImage) for data acquisition and a new set of algorithms (University of Arizona) for assigning isoelectric point (pI) and molecular weight determinations, spot matching and selection of unique spots. Seventeen newly identified acidic proteins are unique to the pineal gland. Some others are also common to the retina but not in other regions examined. Further study of these and other regionally specific proteins are of particular interest under conditions which alter biological or disease mechanisms.

Keywords: *2-DGE; computer-enhanced; brain regions; proteins.*

Introduction

Improved two-dimensional gel electrophoresis (2-DGE) and computer analysis technologies are critical to protein database construction since the approach allows for the simultaneous separation of proteins in complex biological samples. Two-DGE offers the following strategic advantages: (1) selected proteins can be visualized and detected for each cell type, tissue or organism relevant to a question or problem; (2) a broad spectrum of different proteins can be analyzed in each biological system (i.e. representatives of genes specific to the system under investigation), and (3) both quantitative and qualitative protein changes may be revealed. In this study, computer-enhanced comparisons of gel patterns greatly strengthened the ability to determine and validate the

*Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

§To whom correspondence should be addressed.

presence of specific proteins. One of us (D.W.S.) has developed algorithms which provide for analyses of molecular weight, isoelectric point (pI) and integrated intensity of each protein spot, thereby providing information for inter-sample comparison. Through the use of these algorithmic computer programs, separate gel regions were analyzed and tissue-specific proteins identified relative to extrinsic and/or intrinsic mw and pI markers. These methods also illustrate the reproducibility between samples and gel patterns.

Morphological and physiological data indicate that the pineal gland is active in synthesis and secretion of a large number of proteinaceous neurochemicals which may be relevant to disorders of growth, metabolism, fertility and sexual maturation. Heydorn and others [1, 2, 3] have quantitated proteins from discrete nuclei of the rat brain by 2-DGE and demonstrated significant protein synthesis in the pineal gland [4]. Data from this latter study demonstrated a high activity of [35-S]-methionine-labelled protein synthesis within this region when compared with the hypothalamus and subfornical organ; however, no evaluation was made of the physiological significance of identified regional specific protein spots. Our approach, utilizing GELCODE colour silver staining [5], is directed at this time towards the identification of pineal gland-specific proteins. This investigation characterized 17 specific proteins present in acidic extracts of the rat pineal gland through comparisons with parietal cortex, retina and hypothalamus. Our methods establish the power and utility of this approach, and validates the use of algorithms and their application to these aims.

Experimental

Tissue preparation

Three groups of six male Sprague–Dawley rats (250–300 g) were housed for two weeks prior to sacrifice in animal facilities under standard photoperiod (14L:10D). Laboratory chow and water were available *ad libitum*. The number of animals in each group was defined by the small size of the pineal gland and the need for multiple gel electrophoresis runs for comparison. One group per day was sacrificed at mid-morning for three consecutive days. Rats were anesthetized with a lethal dose of Innovar (IM) for vascular perfusion of the brain and upper trunk with an excess of warmed normal saline. When the capillaries of the forepaw toes, ears and retinas had cleared (from pink to white) the body was decapitated and the brain rapidly removed.

The perfused pineal and other brain regions were carefully dissected, weighed and placed into 0.5 ml polyethylene centrifuge tubes containing ice-cold 0.2 M acetic acid at concentrations of 10.0 μl mg^{-1} for pineal and retina and 10.0 μl 3.0 mg^{-1} for parietal cortex and hypothalamus. Samples were kept on ice and sonicated (at minimal setting and time required for disruption of specific tissue) and centrifuged at 4°C (12,000 **g** for 30 min). All supernates were transferred into new vials with a small aliquot reserved for determination of total protein. Samples were stored at −90°C until aliquoted into new tubes at 100 μg total protein, lyophilized and held at −90°C until assayed.

Two-dimensional gel electrophoresis (2-DGE)

Equipment used for 2-DGE was of the design distributed by Health Products, Rockford, IL. The procedures for 2-DGE followed a modified version furnished by Health Products. Briefly, the dried samples for isoelectric focusing (iso-samples) were mixed with 30.0 μl of the standard buffer and 10.0 μl of a 5.0 μg l^{-1} mixture of carbamylated glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) pI standard. The

buffer consisted of 9.0 M urea, 4.0% NP-40, 0.8% ampholytes and 5.0% beta-mercaptoethanol, and the pI standards were prepared by the method of Anderson and Hickman [6]. The isoelectric gel, a 1.5 mm i.d. × 13 cm L "worm", was focused at 700 V for 17 h. In order to make room for the molecular weight markers, approximately 1.5 cm was trimmed from the basic end of the iso-gel prior to application to the SDS gel. A 2.0 mm portion of molecular weight standard (pre-cast within 2% agarose into a 1.5 mm i.d. capillary tube) was applied to the top of the SDS slab at each end of the iso-gel "worm". Liquified 0.5% agarose was then placed overtop. Electrophoresis was performed at 250 V for 5 h. The colour silver staining was done by the GELCODE method.

The isoelectric gradient of the iso-gel "worm" was calculated using tube gels on which 30 μl iso sample buffer +75 μg carbamylated G-3-PDH standard had been focused, as described. Three gels were cut into 1.0 cm segments and equilibrated for 2 h in tubes containing 250 μl of 10.0 mM potassium chloride. An average of three determinations was taken to derive a final pH value.

Data analysis

The scanning of the 2-D gels was performed with the BioImage Corporation VISAGE. The spot location and segmentation were manually performed using the BioImage EQ software. The spot lists were generated by the EQ Global program, thereby giving the maximum accuracy possible for the x,y coordinates and integrated/interrelated intensities.

The EQ generated spot lists were filtered into the program GETPIMW (Copyright, University of Arizona) and the x,y coordinates were converted via algorithm to determine relative pI and mw.

The files of the GETPIMW were loaded into a SPOTMATCH program (Copyright, University of Arizona) and all spots of the separate gels within each group matched for molecular weight (300 Da) and pI (0.2 of relative value) tolerance. These data were filtered into the MATCHBASE program (Copyright, University of Arizona) and spot lists from each region compared to determine regional-specific proteins.

The matched spots from SPOTMATCH and MATCHBASE were then filtered into a SPOTPLOT program and mapped according to their pI and molecular weight and clustered according to their uniqueness within a given region.

Results

Of the four gels from the three separate animal sample groups, maps were created to represent matched spots of each tissue region in Fig. 1. These computerized maps indicate x,y coordinates of mw and pI, respectively, as well as unique spots (●) for each tissue region. Data are based upon a computer calculated/derived reproducibility of that spot existing at that coordinate in each gel. Each spot represents 100% consistency of detection between different 2-DE runs (technique) and animal groups (sample) by the analysis scheme illustrated in Fig. 3.

The dashed line in Fig. 2 represents the analysis 'windows' for spots matched by the computer. Windows a were compared by an algorithmic program using externally (extrinsic) applied pI (G-3-PDH) markers and a combination of intrinsic and extrinsic molecular weight markers. Windows b (Fig. 2b) were compared using intrinsic gel pattern markers which were of known molecular weight and/or pI and consistently

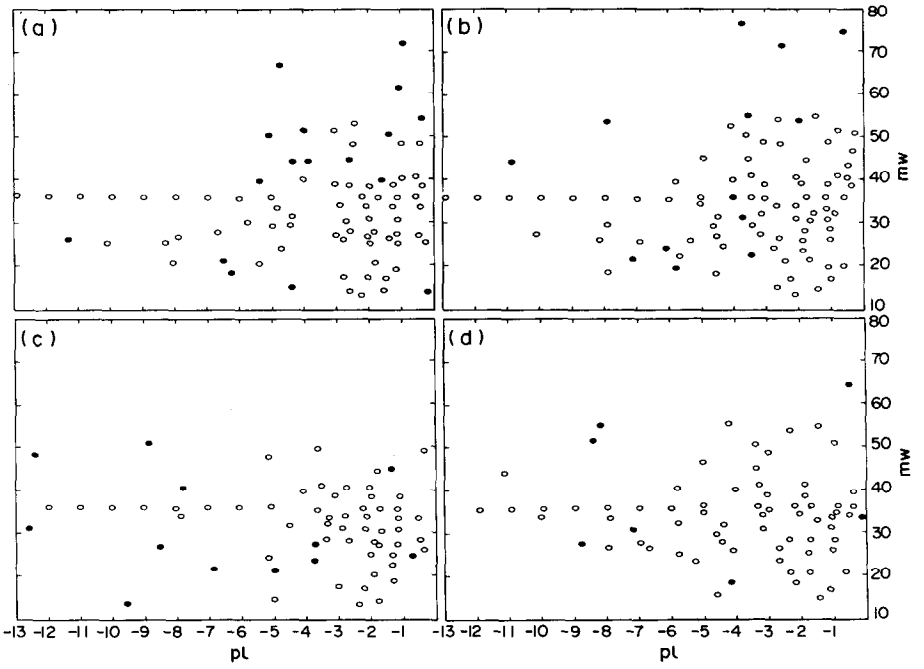

Figure 1
Matched 2-DGE protein maps from inter-sample and -group comparisons of (a) pineal gland (PN), (b) parietal cortex (CX), (c) retina (RT), and (d) hypothalamus (HT). Each spot represents 100% detection by the computer when four gels were compared. Solid spots indicate those proteins found to be tissue-specific by computer analysis. Symbols are defined in Fig. 2.

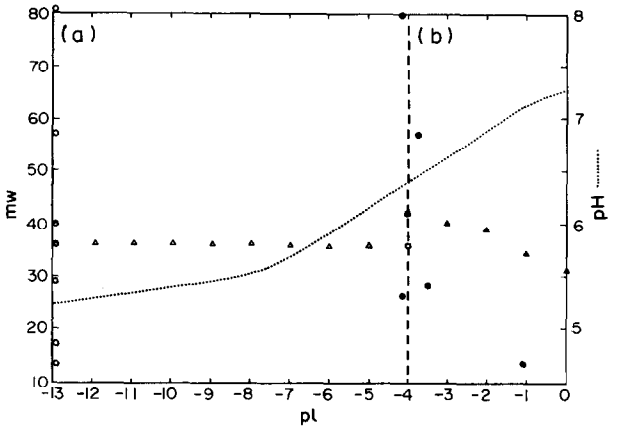

Figure 2
Reference markers used for computer comparison of 2-DGE samples. Windows (a) (delineated by dashed line) were compared by an algorithmic program using extrinsic pI markers –△–, –□–, and extrinsic mw markers, –○–. Windows (b) were compared using both intrinsic pI markers, –▲–, –■– and molecular weight markers, –●–, –■–, and extrinsic molecular weight marker, –□–. Relative pI ranges were from 0 to –4.0 (5.2–6.1 pH) for Window (a), and –4.0 to –13.0 (6.1–7.25 pH) for Window (b).

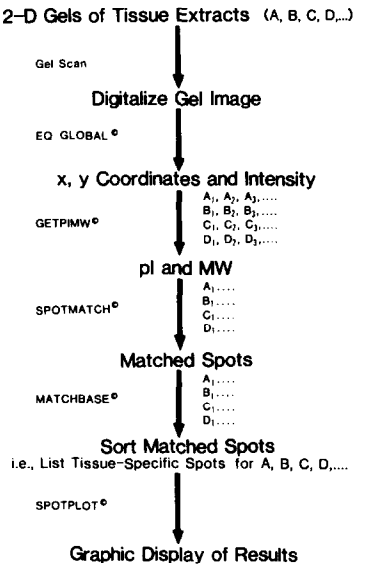

Figure 3
Computerized data analysis. Spot coordinate lists were acquired with the VISAGE (BioImage) system and processed by the sequence of algorithmic programs. Symbols A–D represent different brain regions compared in this study; however, this scheme is generalized to include other combinations of tissues.

observed within the tissues. The boundaries for the windows a were set to include six molecular weight markers from 81–12 kDa and extended from the 4th to 13th G-3-PDH external pI marker (5.20–6.10 pH). The boundaries of window b included 7 molecular weight markers ranging from 60–13 kDa and included four pI markers (6.10–7.25 pH).

Table 1 defines the molecular weight (kDa), relative pI and estimated pH values for the tissue-specific spots indicated in Fig. 2a–b. In addition, spots are listed that were found to be common to the pineal and retina but not found in the hypothalamus and cortex (see Discussion).

Discussion

These results represent an enhancement of 2-DGE techniques for the systematic identification and organization of visible proteins from neuroanatomical samples. Preparation of tissues by thorough vascular perfusion aided in the elimination of interference from blood proteins. In addition, colour silver staining assisted in manual inter-gel spot location in cases where the position of an individual protein varied slightly from gel to gel.

In this analysis, the computer was able to match intrinsic markers in four gels of a particular tissue group to 4–11% matching error. The matching errors may be contributed to, in part, by pattern differences due to experimental variation. Dissociation, phosphorylation, reduction or other process reaction, may distort the natural proportion of proteins in the visualized pattern of a tissue type. Also, a random tissue sample may not necessarily be representative of a population. However, generalizations of protein content should be consistently evident and visibly characteristic of that region when these 2-DGE methods are applied. Spots listed in Fig. 1 represent those which were matched in all 4 gels (100%). The resolution of spot matching was within ±300 Da and 0.2 relative pI units. Thus, it is possible to have spots which appear to overlap in these plots. Since only matched spots were indicated in this figure, any matching error

Table 1
Characters of tissue-specific protein spots. Values for pI are estimated relative to the intrinsic and extrinsic pI markers. Estimated pH values (±0.03) are derived from an average of readings taken from three gels (see Methods)

Tissue	Molecular weight (kDa)	−pI	pH	Molecular weight	−pI	pH
PN	13.9	0.1	7.25	50.7	5.1	6.12
	14.8	4.3	6.31	51.2	1.3	7.12
	18.0	6.2	5.83	52.0	4.0	6.43
	20.9	6.5	5.76	54.9	0.3	7.23
	25.9	11.4	5.31	62.2	1.0	7.13
	39.7	5.4	6.04	67.4	4.8	6.11
	40.1	1.5	7.03	72.7	0.9	7.17
	44.4	4.3	6.32			
	44.7	3.8	6.47			
	45.0	2.5	6.7			
RT	14.1	9.6	5.42	27.6	8.6	5.50
	21.6	4.9	6.19	30.5	12.5	5.25
	22.0	6.8	5.68	41.3	7.8	5.56
	23.4	3.7	6.49	45.3	1.2	7.08
	24.7	0.6	7.23	48.3	12.5	5.25
	27.3	3.6	6.50	52.0	8.8	5.49
HT	17.9	4.2	6.37	52.0	8.4	5.52
	27.6	8.8	5.49	55.5	8.2	5.51
	30.9	7.2	5.63	84.4	0.4	7.23
	33.0	0.0	7.25			
CX	19.1	5.8	5.95	54.0	7.9	5.56
	21.3	7.2	5.63	54.1	1.9	6.93
	22.2	3.4	6.56	55.3	3.5	6.53
	24.0	6.1	5.88	72.0	2.4	6.77
	31.2	3.7	6.49	75.2	0.5	7.23
	35.9	4.0	6.43	77.1	3.7	6.49
	44.4	10.9	5.38			
PN/RT*	25.7	0.1	7.25	29.2	4.9	6.19
	26.6	1.3	7.12	38.8	2.5	6.77
	27.3	0.4	7.23			

*Indicates protein spots common to both the PN and RT but not found in the CX or HT.

was therefore insignificant to this study. These limitations not withstanding, the combined consistency of these techniques provides for a dependable separation and reproduction of protein patterns.

The central region of each gel presented the highest density of protein spots using broad-range ampholytes. Interference by other proteins in the region between 35–37 kDa molecular weight required use of internal markers intrinsic to each tissue observed. These points allowed for algorithmic correlation of spots in gels which lacked visibility of extrinsically-applied markers. The spot-match programs utilized the extrinsic molecular weight and pI markers in order to merge data from the two windows and to confirm spot coordinates and characterizations.

The PN was seen to have seventeen unique protein spots distinguished by these methods [7]. These determinations were made both by single and multiple gel computer comparisons, within and between animal groups. Manual inventory (in supplement to computer scanning) of tissue-specific spots confirmed their consistency between (a) the

animal groups sampled, and (b) replicate run conditions. This comparison analysis was taken a step further in order to determine those proteins shared by the PN and RT, but not by CTX and HT (see Table 1), as an indication of their evolutionary and functional inter-relationships. Compounds shared between these two regions include S-antigen, interphotoreceptor retinoid-binding protein [8], transducin [9] and possibly opsinoids [10, 11]. In addition, both the pineal gland and the retina are known to contain the indoleamine melatonin and the enzymes required for its synthesis from serotonin.

With this study, a reproducible strategy involving 2-DGE and computerized mapping was demonstrated for the identification of tissue-specific proteins of the central nervous system. Accurate quantitation of proteins within a tissue sample is an additional aspect of this technology to be applied in future investigations.

Acknowledgements — This work was supported by N.I.H. grant No. HD 19521 and NASA Grant NAGW-693.

References

[1] W. E. Heydorn, G. J. Creed, D. Goldman, D. Kanter, C. R. Merril and D. M. Jacobowitz, *J. Neurosci.* **3**, 2597–2606 (1983).
[2] W. E. Heydorn, G. J. Creed, C. R. Creveling and D. M. Jacobowitz, *Neurochem. Int.* **8**, 581–586 (1986).
[3] D. M. Jacobowitz and W. E. Heydorn, *Clin. Chem.* **30**, 1996–2002 (1984).
[4] M. A. Gold, W. E. Heydorn, G. J. Creed, J. L. Weller, D. C. Klein and D. M. Jacobowitz, *Electrophoresis 1984*, **5**, 116–121 (1984).
[5] D. W. Sammons, L. D. Adams, T. J. Vidmar, C. A. Hatfield, D. H. Jones, P. J. Chuba and S. W. Crooks, in *Two-dimensional Gel Electrophoresis of Proteins, Methods and Applications* (J. E. Celis and R. Bravo, Eds), Ch. 4. Academic Press, Orlando, FL, USA (1984).
[6] N. L. Anderson and B. J. Hickman, *Anal. Biochem.* **93**, 312–320 (1978).
[7] D. W. Sammons, R. C. Humphreys, N. H. Lin, K. H. Kwan, W. J. Ko, N. Egen, F. Markland and K. Chan, *Frontiers in BioProcessing Conference*, Boulder, CO (1988) (in press).
[8] H.-W. Korf, R. G. Foster, P. Ekstrom and J. J. Schalken, *Cell Tiss. Res.* **242**, 645–648 (1985).
[9] T. van Veen, T. Ostholm, P. Gierschik, A. Spiegel, R. Somers, H. W. Korf and D. C. Klein, *Proc. Natl. Acad. Sci. USA* **83**, 912–916 (1986).
[10] R. L. Somers and D. C. Klein, *Science* **226**, 182–184 (1985).
[11] B. Vigh and I. Vigh-Teichmann, *Cell Tiss. Res.* **221**, 451–463 (1981).

[*Received for review 23 September 1987; revised manuscript received 15 October 1987*]