Enriching Basic and Acidic Rodent Brain Proteins With Ion Exchange Spin Columns for Two-Dimensional Gel Electrophoresis

Ning Liu, Rose Gomez, Steve Freeby, Katrina Academia, Tim Wehr, Yuan Yan, Julie Hey, and Aran Paulus, Bio-Rad Laboratories, Hercules, CA 94547 USA

Introduction

A cell is estimated to contain anywhere from a few hundred thousand to over one million individual proteins, and the expression level for a given protein can range from as little as one copy to as many as one million copies per cell. Owing to this complexity and diversity, as well as to protein instability, proteome-wide analysis poses a challenge for current bioanalytical methods. Resolution of such a mixture of proteins requires a dynamic range effectively covering at least 6 orders of magnitude, a demand no analytical technique available today can meet.

Two-dimensional gel electrophoresis (2-DGE) can resolve up to a few thousand proteins in a single experiment with a dynamic range of detection typically within 2–4 orders of magnitude. The complexity of a protein sample can be reduced prior to 2-DGE to compensate for the limits of coverage and dynamic range. This can be accomplished by applying protein fractionation tools to the sample to allow analysis of one protein subset at a time, thereby increasing the load of proteins of interest and enriching low-abundance proteins. Ideally, such fractionation methods should be simple, fast, and efficient.

Here we discuss the chromatographic fractionation of proteins using Aurum™ ion exchange (IEX) mini spin columns containing either anion exchange (AEX) or cation exchange (CEX) media. We explore the compatibility of Aurum IEX columns with a 2-DGE proteomics workflow for analysis of rat and mouse brain proteins. By incorporating IEX columns into the sample preparation workflow, more low-abundance proteins were detected in both the acidic and basic regions of the gels, allowing more effective probing for differential expression.

A proteomic method was developed using rat brain proteins and then applied to a biomarker study in a transgenic mouse model of Alzheimer's disease. The neurons of APP23 transgenic mice overexpress a mutant form of human amyloid precursor protein (APP), a key molecule implicated in this age-related, progressive neurological disorder (Sturchler-Pierrat et al. 1997). APP23 transgenic mice exhibit Alzheimer's-like symptoms at 9 months but not at 3 months of age. We used brain tissues from 3-month-old control and APP23 transgenic mice for our study, with the aim of finding potential biomarkers associated with the early stages of the disease. We show that Aurum IEX mini spin columns effectively fractionate complex protein mixtures and that their use can improve detection of differentially expressed low-abundance proteins.

Methods

Lysis Buffer Compatibility With Aurum IEX Spin Columns

Aurum IEX spin columns were first tested for their compatibility with various components of the lysis buffers commonly used for isoelectric focusing (IEF) (Figure 1). A variety of buffers were made to test the effects of different combinations of urea, thiourea, detergent, and Tris on protein fractionation (Table 1). The pH of these buffers was adjusted to 7.0 with 20 mM Bis-Tris. Ovalbumin, conalbumin, and cytochrome c were solubilized in these buffers and applied to AEX columns using the protocol recommended in the Aurum IEX mini kit manual. The resulting fractions were analyzed by SDS-PAGE to determine whether the proteins were separated correctly. Finally, buffers were tested on CEX columns after the pH was adjusted to 6.5 using 20 mM Na_2HPO_4. The elution buffer for both the AEX and CEX columns consisted of 7 M urea, 2 M thiourea, 2% CHAPS, 1 mM Tris, and 1 M NaCl.

Fractionation of Rat Brain Proteins for 2-DGE

Figure 2 summarizes the general workflow and products used for the analysis of rat brain proteins. Rat brain proteins were extracted with the ReadyPrep™ total protein extraction kit using a modified lysis buffer containing 2% CHAPS instead of 1% ASB-14. Prior to fractionation, the Tris concentration in the lysate was adjusted to 1 mM by diluting the lysate 1:40 in a buffer containing 7 M urea, 2 M thiourea, and 2% CHAPS.

For enrichment of acidic proteins using AEX columns, 3.6 mg of rat brain total protein extract was treated with the ReadyPrep 2-D cleanup kit prior to fractionation. Cleanup was required to to help maintain the flow rate through the AEX column. The AEX binding buffer was also adjusted to pH 5.0 with 20 mM pyridine. For enrichment of basic proteins, the pH of the protein samples was adjusted to 7.0 with 20 mM Bis-Tris for AEX columns or with 20 mM Na_2HPO_4 for CEX columns; 1–2 mg was processed through the columns.

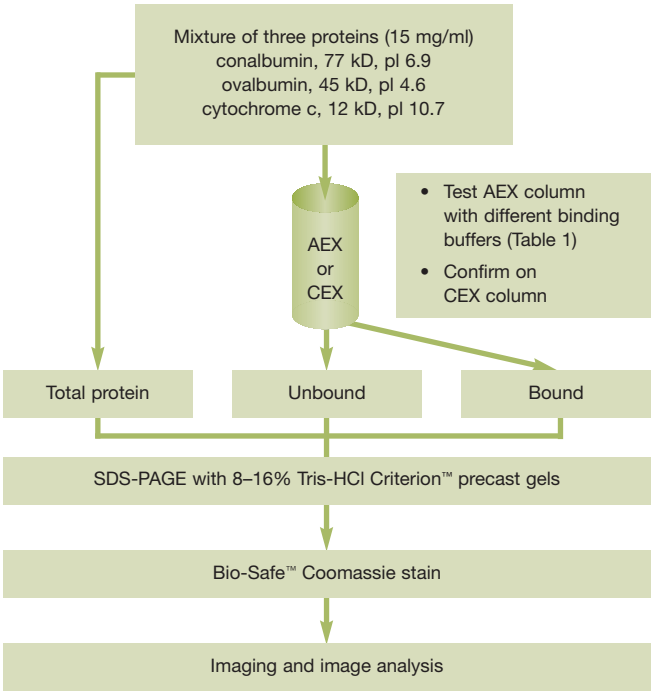


Fig. 1. Workflow for compatibility testing with lysis buffers.

Following 2-DGE, spots were excised from the gels, digested with trypsin, and analyzed by reverse-phase liquid chromatography-tandem mass spectrometry (LC-MS-MS) using an Agilent 1100 series capillary HPLC coupled to a Finnigan LTQ linear ion-trap mass spectrometer equipped with a nanoelectrospray ionization source (Thermo Electron Corp.). Proteins were identified by SEQUEST search of the rat.fasta database.

Comparison of Acidic Protein Profiles From Brains of Control and APP23 Transgenic Mice

Whole brain tissues from 3-month-old control and APP23 transgenic mice (kindly provided by Dr Rena Li, Sun Health Research Institute, Tucson, AZ) were homogenized in a buffer of 7 M urea, 2 M thiourea, 2% CHAPS, and 40 mM Tris. A 5 mg aliquot of each sample was treated with the ReadyPrep 2-D cleanup kit and separated on AEX columns at pH 5.0. The bound fraction was treated with the ReadyPrep reduction-alkylation and 2-D cleanup kits. IEF was performed in duplicate with 100 µg of each protein sample using 24 cm ReadyStrip IPG strips, pH 3–10, and was followed by SDS-PAGE separation on 12.5% Tris-HCl PROTEAN® Plus precast gels (200 V, 1 hr). Unfractionated total protein samples (500 µg per gel) were also examined by 2-DGE and served as controls to examine the efficiency of sample fractionation. Proteins were identified by SEQUEST search of the mouse.fasta database.

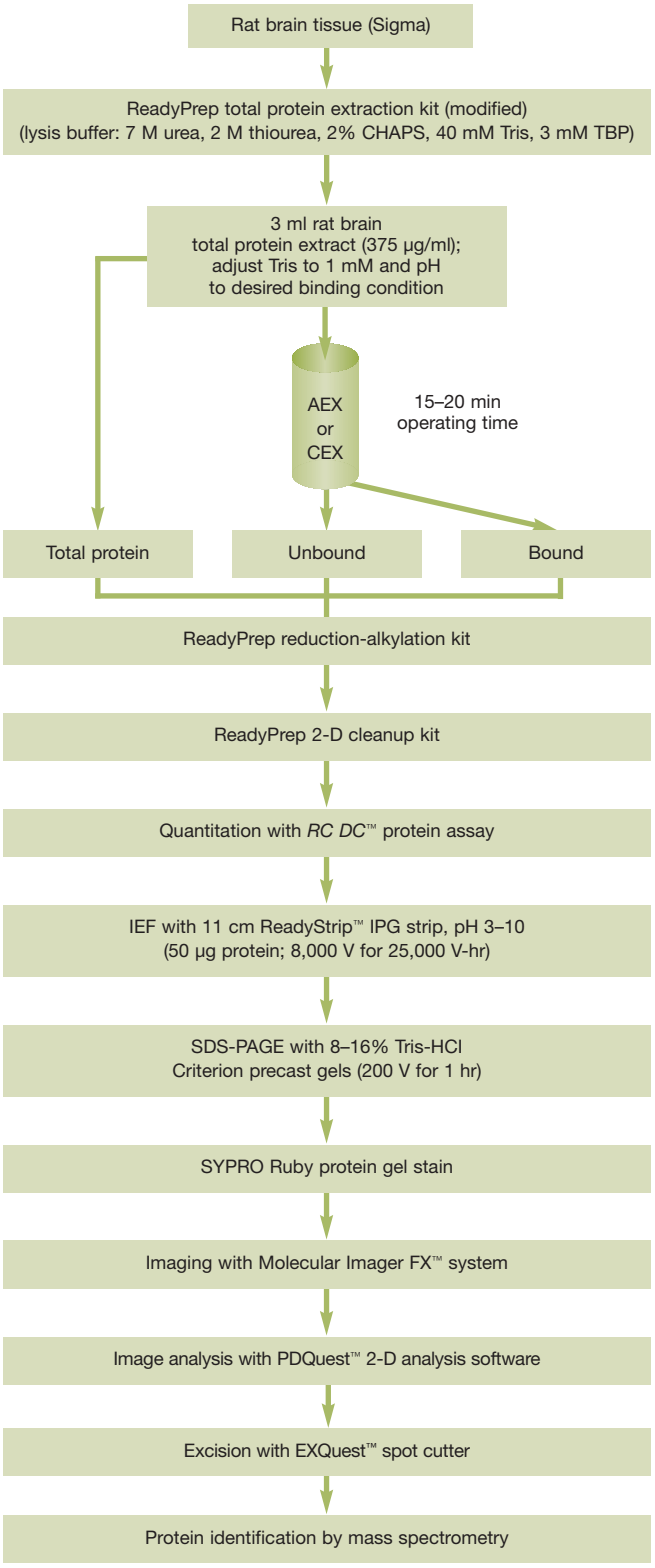


Fig. 2. General 2-D analysis workflow using Aurum AEX or CEX columns for fractionation prior to 2-DGE. Samples separated on AEX columns were treated with the ReadyPrep 2-D cleanup kit prior to fractionation (not shown).

Results and Discussion

Buffer Compatibility Testing

2-DGE requires high amounts of urea, thiourea, and detergents in sample lysis buffers to achieve optimal protein solubilization. In addition, Tris is often included to inhibit protease activity. To determine whether Aurum IEX mini spin columns could be used for sample fractionation prior to 2-DGE, we examined the ability of the columns to separate three proteins with distinctive pIs in various buffers containing urea, thiourea, detergents (CHAPS or Triton X-100), and Tris (Table 1).

High Tris concentrations in the binding buffer interfered with column performance, but other commonly used reagents, such as urea, thiourea, and CHAPS, were compatible with the columns (Table 1 and Figure 3). In binding conditions of pH 7.0, it is expected that an AEX column will separate cytochrome c (pI 10.7) from ovalbumin (pI 4.6) and conalbumin (pI 6.9) (Figure 3). However, adequate separation on the column was only achieved when the Tris concentration was decreased from 40 mM to 1 mM; similarly, the CEX column was expected to separate ovalbumin from conalbumin and cytochrome c with binding conditions at pH 6.5, but only worked properly with binding buffers containing 1 mM Tris (Table 1). For the rest of our study, we included 7 M urea, 2 M thiourea, and 2% CHAPS in the column binding and elution buffers, and Tris was reduced to 1 mM (1:40 dilution with binding buffer) in the protein samples prior to loading onto the Aurum IEX mini spin columns.

Table 1. Compatibility of various IEF buffer components with Aurum AEX columns. All buffers included 7 M urea and 20 mM Bis-Tris, pH 7.0.

Binding Buffer	2 M Thiourea	Tris	2% CHAPS	2% Triton X-100	Compatible?
1	+	40 mM	+	–	No
2	+	40 mM	–	+	No
3	–	40 mM	–	+	No
4	–	1 mM	–	+	Yes
5	+	1 mM	+	–	Yes
6	+	1 mM	–	+	Yes

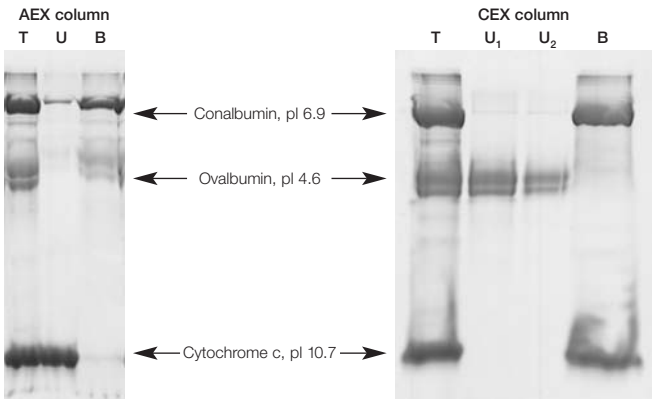


Fig. 3. Buffer compatibility and confirmation of fractionation. Coomassie Blue-stained gel showing separation of cytochrome c from ovalbumin and conalbumin using the AEX column, and ovalbumin from conalbumin and cytochrome c using the CEX column. Binding buffer #5 (Table 1) was used. T = total protein; U = unbound fraction; B = bound fraction.

Enrichment of Basic Proteins at pH 7.0

Once buffer compatibility was verified, rat brain total protein extracts were fractionated by both IEX columns at pH 7.0 to enrich basic proteins. Both unbound and bound fractions were analyzed and compared to unfractionated samples by 2-DGE (Figure 4).

Proteins in the AEX unbound and CEX bound fractions focused mostly on the basic side of the 2-D gels; in contrast, proteins in the AEX bound and CEX unbound fractions focused mostly on the acidic side of the gels (Figure 4). The intensities of many of the proteins from fractionated samples were higher (Figure 4 insets, red and yellow circles) compared to the total protein samples. PDQuest analysis of the gels revealed that 14% and 28% of the protein spots on the 2-D gels of the AEX unbound and CEX bound fractions, respectively, showed at least a 2-fold increase in intensity compared to the total protein gels, while 27% and 15%, respectively, were not observed at all on the total protein gels (see Table 2 for total spot counts).

Using MS, the identities of 14 of the protein spots from the CEX bound fraction gel were determined. The pIs of these proteins ranged from 6 to 10.7 (Figure 5), demonstrating that the IEX mini spin columns can enrich basic proteins at pH 7.0 for 2-D gel analysis.

Enrichment of Acidic Proteins at pH 5.0

To enrich acidic proteins, rat brain total protein extracts were fractionated through AEX columns at pH 5.0. Again, both unbound and bound fractions were analyzed on 2-D gels and compared to the unfractionated sample (Figure 6). Protein spots on the AEX bound fraction focused on the acidic side (pH <5.0) of the 2-D gels. In contrast, proteins in the AEX unbound fraction focused above pH 5.0. There was little overlap between the 2-D gels of the proteins in the bound and unbound fractions, indicating a clear separation between these two groups of proteins. PDQuest analysis of the gels revealed 86 protein spots on the 2-D gel of the AEX bound fraction; in contrast, only 59 spots were found in the same pH range on the total protein gel. In the AEX bound fraction gel, 21 spots showed at least a 2-fold increase in intensity compared to the total protein gel, and 27 spots were only observed in the AEX bound fraction gel.

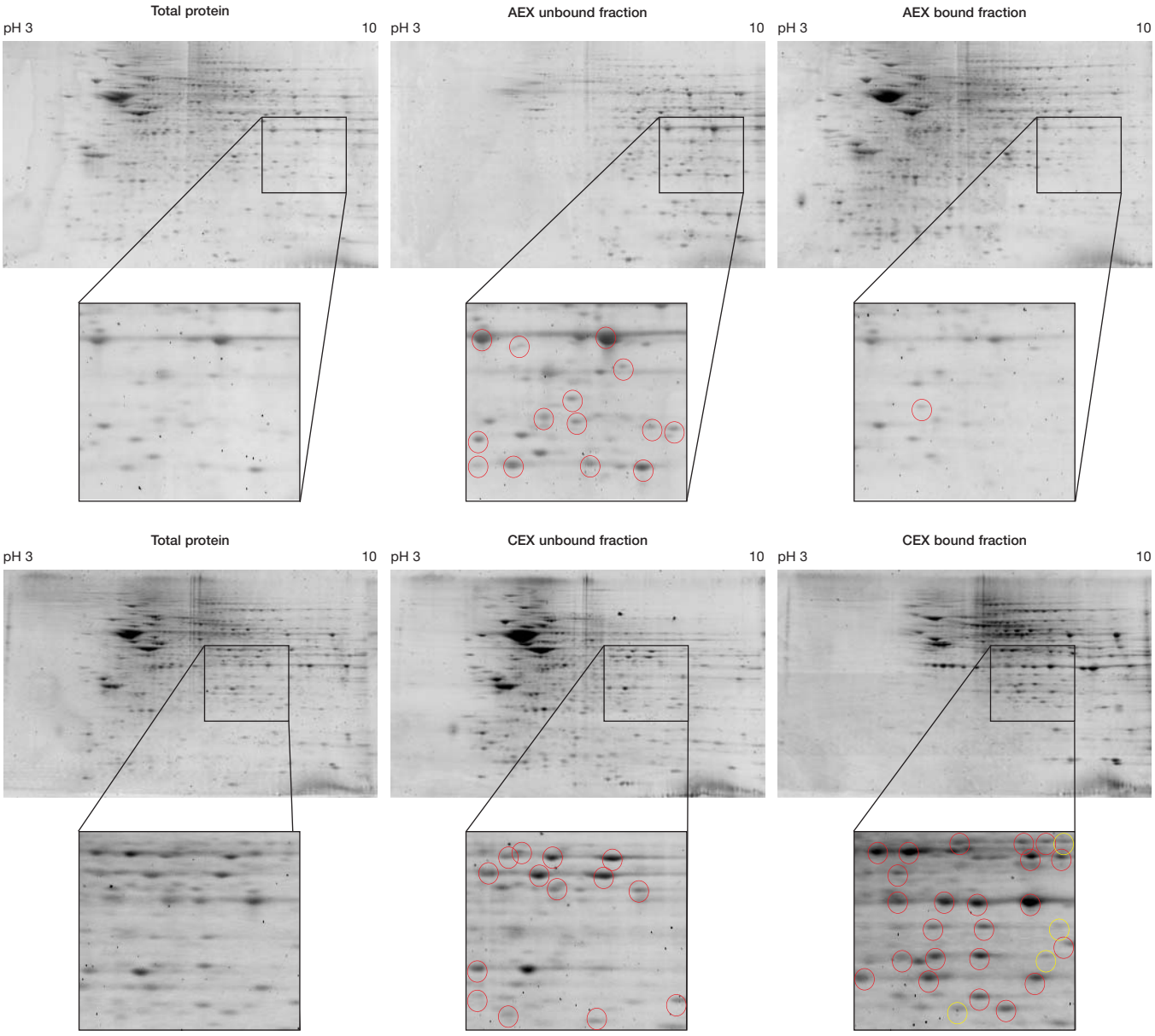
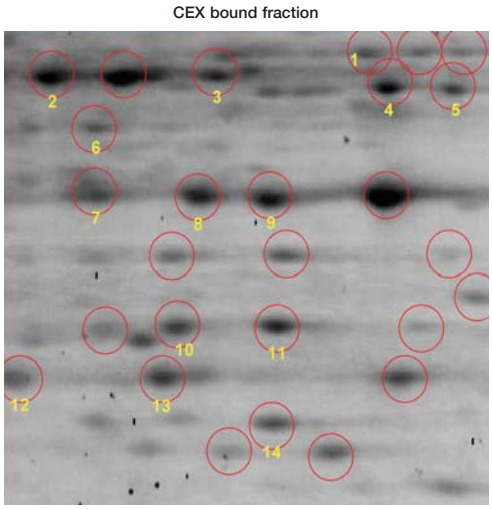


Fig. 4. Enrichment of basic proteins in rat brain after fractionation with Aurum AEX and CEX mini columns at pH 7.0. Red circles indicate protein spots with increased intensity compared to the total protein gels. Yellow circles indicate spots observed only in that gel.

Table 2. Summary of numbers of protein spots detected. Data were derived from PDQuest analysis of the gels in Figure 4, and revealed that 14–28% of the protein spots in the 2-D gels of the IEX bound and unbound fractions showed at least a 2-fold increase in intensity compared to total protein gels; 15–27% of the spots were not observed on the total protein gels (unique).

	AEX Fractionation			CEX Fractionation		
	Total Protein	Unbound	Bound	Total Protein	Unbound	Bound
Total detected	557	247	550	428	418	391
≥2x enrichment	—	34	66	—	61	109
≥3x enrichment	—	11	22	—	19	46
Unique	—	66	112	—	69	59



Spot	Protein ID	MW	pI	Probability	US*	% AA**
1	Citrate synthase	52	8.3	1.1×10^{-10}	100.3	18
2	Septin 2	42	6.0	2.8×10^{-9}	38.2	7.2
3	Glutamine synthetase	42	6.0	7.3×10^{-5}	10.2	4.3
4	Creatine kinase	43	8.3	3.6×10^{-6}	78.3	21
5	Creatine kinase II	45	7.2	5.0×10^{-12}	40.3	13
6	MAPK 1	41	6.0	4.5×10^{-13}	68.3	21
7	GAPDH	36	8.3	1.9×10^{-7}	54.2	19
8	GAPDH	36	8.3	4.8×10^{-7}	40.2	11
9	GAPDH	36	8.3	1.1×10^{-7}	40.2	15
10	Carbonic anhydrase 2	29	6.0	1.1×10^{-11}	20.2	9
11	Brain Y-box binding protein 1	36	10.7	8.9×10^{-4}	8.1	6
12	Similar to triosephosphate isomerase	27	6.0	5.9×10^{-11}	60.3	42
13	Triosephosphate isomerase	27	7.2	4.6×10^{-10}	20.2	10
14	GTPase Ran	24	6.0	5.2×10^{-6}	20.2	10

* US = SEQUEST unified score.
** % AA = % sequence coverage by amino acids.

Fig. 5. Identification by MS of the proteins enriched in the CEX bound fraction.

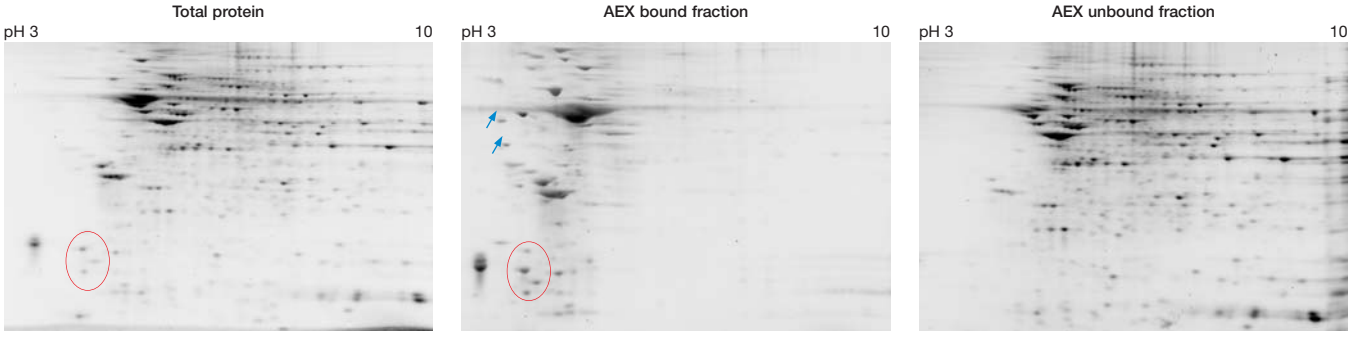
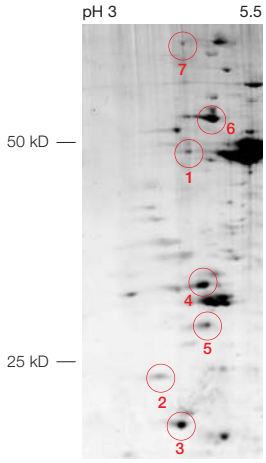


Fig. 6. Enrichment of acidic proteins in rat brain after fractionation with Aurum AEX mini spin columns at pH 5.0. Red circles indicate a group of protein spots with increased intensities after fractionation. Blue arrows show two representative spots detected only in the gels of the AEX bound fraction.



Spot	Protein ID	MW	pI	Probability	US*	% AA**
1	Tubulin β-2	34	4.2	1.9×10^{-19}	22.2	14.7
2	Similar to Sid3177p	24	3.8	5.8×10^{-12}	26.2	19.2
3	Synuclein	14	4.2	1.6×10^{-9}	10.2	10.5
4	Tryosine-3-oxygenase/tryptophan-5-monogenase activation protein	29	4.2	1.9×10^{-8}	48.2	21.0
5	S41476 calretinin	22	4.2	1.1×10^{-6}	10.1	5.6
6	Neurofilament light polypeptide	61	4.2	2.2×10^{-11}	100.3	23.6
7	Ubiquitin protein ligase E3 component n-recognin 1	200	6.0	3.9×10^{-3}	18.1	1.08

* US = SEQUEST unified score.
** % AA = % sequence coverage by amino acids.

Fig. 7. Identification by MS of the proteins enriched in the AEX bound fraction.

In another study using AEX columns to fractionate mouse brain tissue, seven protein spots from the 2-D gel of the AEX bound fraction were picked for identification by MS. The pIs of these proteins vary from 3.8 to 6 (Figure 7), showing that the columns can also enrich acidic proteins.

Reproducibility

To examine the reproducibility of this protein fractionation procedure, 5 mg of rat brain extract treated with the ReadyPrep 2-D cleanup kit was fractionated on three AEX columns at pH 5.0. The same amount of protein from each bound fraction was analyzed by 2-DGE in triplicate, and

PDQuest software was used to match the protein spots among the gels. The correlation coefficients of these analyses between different groups of gels were very close to 1.0 (Table 3), indicating that the fractionation process was highly reproducible from column to column.

Table 3. Correlation coefficients from three AEX columns for a rat brain sample tested in triplicate by 2-DGE.

Spot Match Analysis	Correlation Coefficient
Columns 1 and 2	0.97
Columns 1 and 3	0.98
Columns 2 and 3	0.97

Application to Biomarker Discovery for Alzheimer's Disease

Control and APP23 transgenic mouse brain total protein lysates (5 mg each) were fractionated on AEX columns at pH 5.0, and the bound fractions from each sample were compared on 2-D gels (Figure 8). Again, the intensities of many of the protein spots on the 2-D gels of the AEX bound fractions were increased significantly compared to those on the total protein gels, and several low-abundance proteins were only detected on the gels of fractionated protein samples (Figure 8, red arrows).

One spot that had a significantly decreased intensity level in the APP23 transgenic mouse brain was identified by MS as tubulin β-2 (Figure 8, blue arrow). This form of tubulin β-2 focused at a position distinct from that of most tubulin β-2 proteins (Figure 8, black arrow), indicating that it may have different posttranslational modifications than the other tubulins in brain cells. The level of this uniquely modified tubulin was decreased in the APP23 transgenic mouse brain, suggesting that microtubules, the major cytoskeleton component and transport system in cells, are altered by age 3 months in APP23 mice, even though the mice are symptom-free. This hypothesis is suggestive because microtubule malfunction in brain cells is associated with another well-known progressive neurological disorder, Huntington's disease. Future studies will need to be carried out to confirm this change in APP23 mice and to examine its involvement in the Alzheimer's disease mechanism.

The differentially expressed tubulin β-2 protein was not apparent on the total protein gels of samples from either control or APP23 transgenic mice even though 500 µg was loaded, versus 100 µg on the gels of fractionated sample.

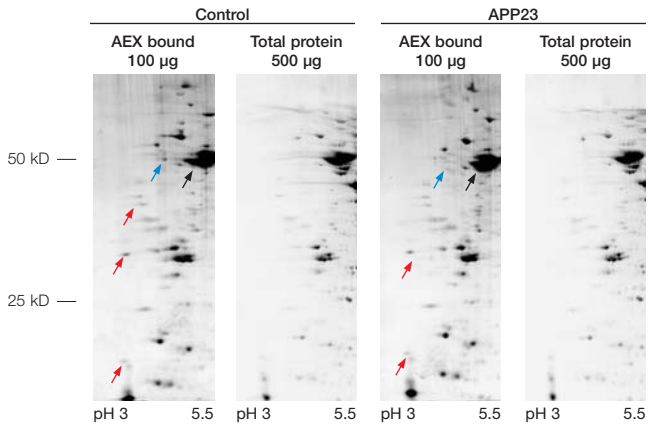


Fig. 8. Comparison of control and APP23 transgenic mouse brains with and without fractionation on AEX columns at pH 5.0. Red arrows, proteins detected only on gels of AEX fractionated sample; blue arrows, downregulated tubulin β-2 protein; black arrows, other tubulin β-2 proteins.

Conclusions

- Requiring only 15–20 min operating time, Aurum IEX mini spin columns provide a quick, convenient, and reproducible sample preparation tool for 2-DGE
- Aurum IEX mini spin columns are compatible with the lysis buffer components commonly used in 2-D gel electrophoresis; however, the concentration of Tris used with these columns should be ≤1 mM
- Fractionating rat and mouse brain total proteins with Aurum IEX columns improves detection of low-abundance proteins on 2-D gels and may facilitate the discovery of potential disease biomarkers in 2-D gel-based proteomics studies
- The identification of tubulin β-2 as a potential biomarker for Alzheimer's disease is promising. Future studies will need to be carried out to confirm the alteration of tubulin β-2 in APP23 transgenic mice and examine its involvement in the Alzheimer's disease mechanism

Reference

Sturchler-Pierrat C et al., Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology, Proc Natl Acad Sci USA 94, 13287–13292 (1997)

Information in this tech note was current as of the date of writing (2005) and not necessarily the date this version (rev A, 2006) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 712 2771* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300/5013478* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 5811 6270* ***Korea*** *82 2 3473 4460* ***Mexico*** *55 5200 05 20* ***The Netherlands*** *0318 540666* ***New Zealand*** *64 9415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 095 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 0861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*