# 30 Proteoform Catcher: The Power of 2D SDS-PAGE, Western Blotting, and Mass Spectrometry in Protein Characterization

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**Abstract**

Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) is a method that separates proteins according to their isoelectric point in the first dimension and molecular mass in the second dimension. Evidence is provided that 2D SDS-PAGE is reproducible, robust, and compatible with SDS in both dimensions, including isoelectric focusing in tube gels, the first dimension. The 2D gel pattern of rat liver microsomes shows more detail and sharper spot outlines when dissolved in SDS buffer with heating than in urea buffer, and is better yet when dissolved in a mixture of both buffers. Quantification of 60 proteins in rat liver cytosol over a wide range of pI and MW gave linear plots of spot density versus total protein for loads of 200, 400, and 600 µg protein dissolved in SDS buffer and run in triplicate on 2D gels (Average $R^2 = 0.987$). Examples are provided in which proteins found by 2D gel western blotting, cut from stained gels, and identified by mass spectrometry (MS) are proteoforms with unique post-translational modifications. Such proteins would not have been found by either method alone.

**Keywords**

Protein analysis · Electrophoresis · 2D electrophoresis · Western blotting ·

## 30.1 Abbreviations

| | |
|---|---|
| PTM | Post-translational modification |
| α-syn | Alpha-synuclein |
| 1D | One-dimensional |
| 2D | Two-dimensional |
| 2D SDS-PAGE | 2-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| IEF | Isoelectric focusing |
| pI | Isoelectric point |
| MS | Mass spectrometry |
| NP-40 | Nonidet-P-40 aka IGEPAL |
| nanoLC–MS/MS | Nanoliquid chromatography tandem MS |
| OSA | Obstructive sleep apnea |
| STK16 | Serine/threonine kinase 16 |

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A. G. Woods and C. C. Darie (eds.), *Advancements of Mass Spectrometry in Biomedical Research*, Advances in Experimental Medicine and Biology 1359, https://doi.org/10.1007/978-3-032-29437-1_30

## 30.2 Introduction

Two-dimensional electrophoresis polyacrylamide gel electrophoresis (2D PAGE), in place since 1975 [1], is a biochemical method for separating complex mixtures of proteins into individual species. Proteins are separated by charge in the first dimension using isoelectric focusing (IEF), then by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The resulting starburst pattern of protein spots is usually visualized on final 2D gels by protein stains such as silver, Coomassie, and Sypro Ruby, or by western blotting (WB). The gel patterns may be compared by eye or with commercial 2D software such as Progenesis SameSpots from TotalLab. Purified proteins of interest may be cut from stained gels and identified by MS. There are numerous applications for this approach.

Two major variations of 2D PAGE are in place that differ in the IEF protocol. There are advantages and disadvantages to each. In the most common variant, the pH gradient is fixed in place on commercially available solid supports called immobilized pH gradient (IPG) strips [2]. IPG strips are technically convenient to run but are incompatible with the detergent SDS, by far the best reagent for dissolving membrane proteins [3–5]. Sample preparation for IPG strips utilizes nonionic detergents and urea that work well for soluble proteins, less so for membrane proteins that comprise about 30% of the human genome [6]. For membranous samples, whole cell lysates, for example, a centrifugation step is often included to remove insoluble "cell debris," causing unknown losses. Protein losses occur at different stages of the IPG strip procedure as well [7,8].

The 2D classic method, first described by O'Farrell in 1975, uses acrylamide tube gels polymerized with commercial carrier ampholines for IEF. A pH gradient forms when voltage is applied; ampholines and proteins migrate to a steady-state position during the overnight IEF step. The tube gel is extruded, incubated with SDS buffer, and fixed on top of a slab gel prior to SDS-PAGE. The classic method is technically difficult but has the huge advantage of being compatible with SDS, as clearly demonstrated by the Andersons [9]. Tissue samples are homogenized in SDS buffer with heating until the solution clarifies; centrifugation is usually unnecessary.

Our laboratory (Kendrick Labs) has validated 2D SDS-PAGE for samples prepared in SDS buffer, with regard to reproducibility and linearity of response [10]. Furthermore, all aspects of 1D and 2D SDS-PAGE have been standardized according to good manufacturing practices (GMP) with written standard operating procedures (SOPs) subject to change control. Electrophoresis testing at Kendrick Labs is performed by trained biochemists using validated equipment and software. The final critical step, identifying proteins of interest using MS analysis, is performed at a reliable outside core facility.

Evidence is presented here that using SDS for sample preparation imparts robustness to the classic 2D PAGE method. Furthermore, in combination with WB and MS, this method allows detection of protein post-translational modifications (PTMs) that serve as proteoform biomarkers for clinical diagnosis [11].

## 30.3 Evidence of IEF Compatibility with SDS

**2D SDS-PAGE (carrier ampholine 2D)** is compatible with sodium dodecyl sulfate (SDS), the best reagent by far for solubilizing proteins [4]. The method, originally developed by O'Farrell [1], was shown to have SDS compatibility by the Andersons [9], and refined at Kendrick Labs [12].

As demonstrated by the Andersons early on, SDS is stripped off proteins by the nonionic detergent NP-40 included in the tube gel polymerization mixture. During overnight IEF, micelles containing NP-40 and SDS migrate to the extreme acid end of the tube gel where they form a visible bulb that is cut off and discarded [9].

## 30.4 SDS Versus Urea Buffer for a Purified pI Standard

While it is difficult to demonstrate that SDS is stripped off every protein, 2D SDS-PAGE (abbreviated from here on as 2D) patterns are quite

reproducible. Figure 30.1 shows the 2D pattern of carbamylated creatine phosphokinase (Carb-CPK), an IEF pI standard prepared by urea carbamylation to generate a collection of isoforms differing by one charge [13]. Isoelectric points of the charge isoforms range from pH 5.0 on the left to 7.5 on the right. The 2D pattern of Carb-CPK standard dissolved in urea is virtually identical to that dissolved in SDS buffer.

## 30.5 SDS Versus Urea Buffer for a Membrane Fraction

The carb-CPK protein standard shown in Fig. 30.1 is water soluble, dissolving easily in both SDS and urea buffer. Roughly 30% of protein-coding genes in the human genome code for membrane proteins that have hydrophobic domains and are often difficult to dissolve without SDS [6]. Figure 30.2 shows 2D patterns of a rat liver microsome sample, derived from the endoplasmic reticulum of hepatic cells prepared with and without SDS buffer. The same sample was: (A) dissolved in SDS sample buffer and the tube heated in a boiling water bath for 5 min, (B) dissolved in 9 M urea plus 2% NP-40 without heating, and (C) dissolved in both, i.e., an aliquot of the sample from A was diluted 1:1 with urea buffer from B. The final concentration was 2.5% SDS, 4.5 M urea, and 1% NP-40.

The expanded sections show that the 2D pattern from microsomes dissolved in SDS buffer shows more detail and sharper spots than those dissolved in urea buffer. The best pattern, however, was obtained by combining reagents, i.e., adding urea buffer after prior preparation with SDS buffer and heating. Note that heating proteins in the presence of urea causes carbamylation artifacts and should be avoided.

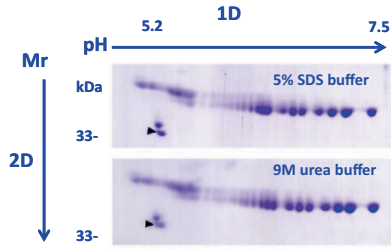



**Fig. 30.1** Carbamylated CPK standard dissolved in either SDS or 9 M urea buffer before IEF. The arrow marks the lower isoform of tropomyosin, a 2D marker of MW 33 kDa and pI 5.2. Preparation in SDS buffer does not affect separation of the single-charge Carb-CPK isoforms

## 30.6 Protein Quantification Using 2D SDS-PAGE

Using SDS for 2D sample preparation improves method robustness as shown in the following experiment. A complex mammalian sample, rat liver cytosol, was diluted with buffer containing 2.5% SDS + 4.5 M urea before running on large format 2D gels (20 × 20 cm) in triplicate at loads of 200, 400, and 600 µg. The gels were Coomassie blue-stained and scanned with a laser densitometer verified to be linear over 0–3 OD. Sixty polypeptide spots, chosen over a wide range of pI and MW, were quantified on each gel with Progenesis SameSpots software from TotalLab.

Results were tabulated in two ways: (1) as integrated spot density above background within the outline and (2) as spot percentage of total density of all spots combined. Figure 30.3 shows the 2D gel pattern for a 600 µg load along with spot numbering.

Figure 30.4 shows outlines for a single spot along with the corresponding plot of average spot volume ($n = 3$ gels) versus total protein load. Similar plots were determined for each of the 60 proteins.

Absolute spot volume is useful for determining linearity of the method within a run but is seldom used for comparing samples prepared and run on different days. For the latter, measurements are normalized to correct for gel-to-gel loading and staining differences by expression as spot percentages. The latter is defined as spot volume taken as a percentage of total volume of all spots combined. Spot percentages are largely independent of sample load.

Table 30.1 shows results taken from both ways of expressing data. $R^2$ values for each plot are provided as a measurement of linearity of spot volume versus load. The Coefficient of Variation (CV = SD/

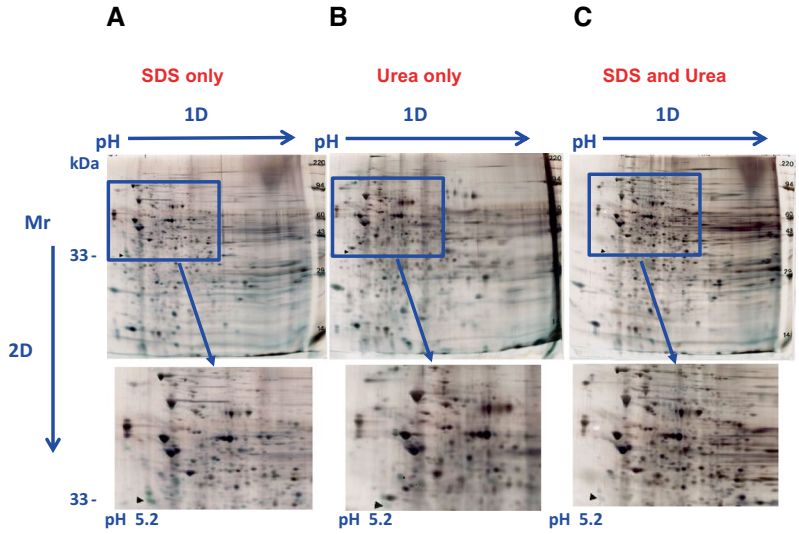



**Fig. 30.2** Rat liver microsomes prepared with and without SDS. For this figure, microsomes (pellet obtained after homogenization, low-speed spin to remove cell debris, and then 100,000 × *g* spin) were purchased and dissolved in (**A**) SDS buffer with heating, (**B**) urea buffer without heating, or (**C**) SDS buffer with heating followed by 1:1 dilution with urea buffer. The 13 × 15 cm silver-stained 2D gels were loaded with 50 µg protein. Patterns from microsomes prepared with both SDS and urea show the best resolution

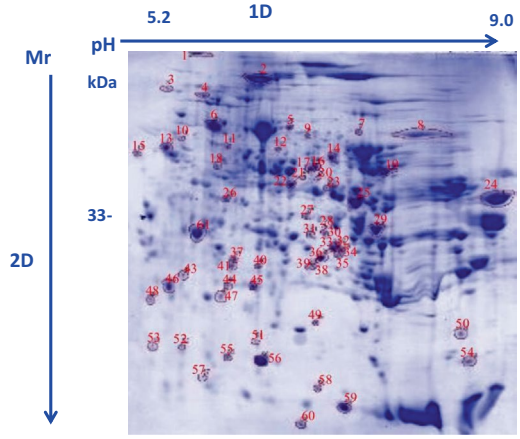



**Fig. 30.3** Rat liver cytosol (600 µg) prepared with SDS buffer plus urea showing outlines of 60 quantified protein spots

mean × 100) shows variability of spot percentage values between the nine gels.

The average $R^2$ value for the 60 spots was 0.987, indicating good correspondence between individual proteins and total sample load. When the latter is doubled, the individual spot volumes double, as well for Coomassie blue, a quantitative stain. A few spots were outliers for varying reasons. Spot 4 showed irreproducibility that we speculate was due to protein aggregation. It gave about the same spot volume at the 400 and 600 µg loads. Spots 17, 23, and 35 showed spot splitting problems. Isoforms of

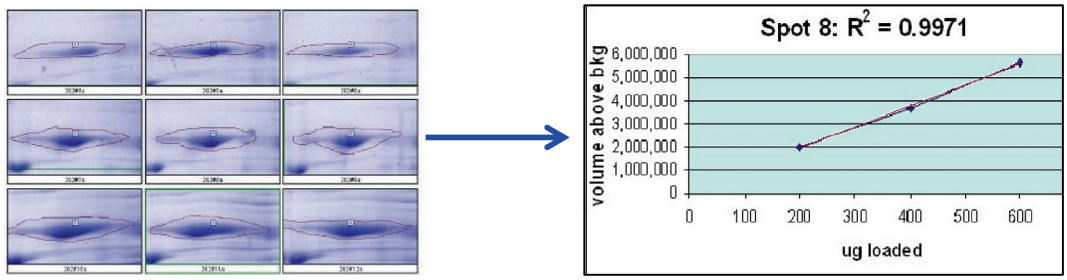



**Fig. 30.4** Quantification of protein 8 based on spot volume. Left: Montage of Spot 8 outlines taken from the nine 2D gels to show the 200 (top), 400 (middle), and 600 µg (bottom) patterns. Right: Graph showing a plot of average integrated density from triplicate gels versus load for spot 8

**Table 30.1** Spot data, linearity, and % CV of Spot percentages obtained from 60 polypeptide spots on nine gels. Spot outlines are shown in Fig. 30.3. Spots with high error (% CV for spot percentage ≥ 20 or $R^2$ values from the plots 0.95) are highlighted with formattings.

| Spot | **pI** | MW (kDa) | $R^2$ value for plot | % CV (n = 9) of Spot % | Spot | **pI** | MW (kDa) | $R^2$ value for plot | % CV (n = 9) of Spot % | Spot | pI | MW (kDa) | $R^2$ value for plot | % CV (n = 9) of Spot % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.7 | 300 | 0.990 | 12% | 21 | 6.8 | 47.7 | 0.999 | 17% | 41 | 6.0 | 28.7 | 0.993 | 14% |
| 2 | 6.3 | 177 | 0.999 | 16% | 22 | 6.7 | 44.4 | 0.991 | 15% | 61 | 5.7 | 33.8 | 0.997 | 6% |
| 3 | 5.4 | 131 | 0.974 | 13% | 23 | 7.1 | 43.6 | 0.971 | *24%* | 43 | 5.6 | 27.0 | 0.999 | 10% |
| 4 | 5.7 | 118 | **0.786** | **20%** | 24 | 9.4 | 41.0 | 0.989 | 8% | 44 | 6.0 | 26.2 | 0.995 | ***26%*** |
| 5 | 6.7 | 79.9 | 0.998 | 12% | 25 | 7.4 | 40.9 | 1.000 | 4% | 45 | 6.2 | 26.2 | 0.991 | 17% |
| 6 | 5.8 | 78.4 | 0.989 | 9% | 26 | 6.0 | 40.5 | 0.994 | 8% | 46 | 5.4 | 25.7 | 0.997 | 5% |
| 7 | 7.4 | 78.3 | 0.998 | 14% | 27 | 6.8 | 37.6 | 1.000 | 13% | 47 | 5.9 | 24.8 | 0.999 | 9% |
| 8 | 8.1 | 76.1 | 0.997 | 4% | 28 | 7.1 | 32.3 | 0.984 | 23% | 48 | 5.3 | 24.4 | 0.998 | 12% |
| 9 | 6.9 | 73.9 | 0.984 | 16% | 29 | 7.5 | 35.9 | 0.985 | 12% | 49 | 6.9 | 22.0 | 0.996 | 16% |
| 10 | 5.5 | 67.9 | 0.982 | 8% | 30 | 7.1 | 35.0 | 0.992 | 8% | 50 | 8.9 | 21.2 | 0.992 | ***24%*** |
| 11 | 6.0 | 65.4 | 0.994 | 8% | 31 | 6.9 | 34.3 | 0.998 | 18% | 51 | 6.3 | 19.5 | 0.999 | ***28%*** |
| 12 | 6.5 | 64.0 | 0.998 | 15% | 32 | 7.2 | 32.8 | 0.996 | 15% | 52 | 5.6 | 18.6 | 0.999 | 22% |
| 13 | 5.4 | 60.8 | 0.990 | 4% | 33 | 7.1 | 32.6 | 0.977 | 11% | 53 | 5.3 | 18.3 | 0.994 | 15% |
| 14 | 7.1 | 60.3 | 0.985 | 10% | 34 | 7.2 | 31.9 | 1.000 | 9% | 54 | 9.0 | 17.9 | 0.967 | *60%* |
| 15 | 4.9 | 58.4 | 1.000 | 14% | 35 | 7.2 | 31.1 | 0.994 | *37%* | 55 | 6.0 | 17.5 | 1.000 | 12% |
| 16 | 7.0 | 54.1 | 0.984 | 16% | 36 | 7.0 | 30.5 | 0.998 | 7% | 56 | 6.3 | 17.0 | 1.000 | 7% |
| 17 | 6.9 | 52.8 | 0.978 | *24%* | 37 | 6.1 | 29.5 | 0.972 | 16% | 57 | 5.7 | 14.8 | 1.000 | ***36%*** |
| 18 | 5.9 | 52.6 | 0.969 | 11% | 38 | 6.9 | 29.4 | 0.981 | 10% | 58 | 6.9 | 14.5 | 0.998 | 6% |
| 19 | 7.6 | 52.0 | 0.979 | 8% | 39 | 6.9 | 28.8 | 1.000 | 22% | 59 | 7.2 | 10.1 | 0.997 | 13% |
| 20 | 7.0 | 48.2 | 0.958 | ***34%*** | 40 | 6.3 | 28.7 | 0.999 | 5% | 60 | 6.7 | 4.1 | 0.985 | 13% |
| | | | | | | | | | Average All Spots ($n = 60$) | | | | 0.987 | 15% |

Bold indicates a protein aggregation problem. Italic indicates a spot-splitting problem. Bold and italic indicates that the spot was faint on the 200 µg load gels. The fainter the spot, the higher the error

the same or adjacent proteins are merging at higher loads; small shifts in the splitting line increase error. Five remaining spots were faint on the 200 µg gels; fainter spots have higher error. Overall, we have great correspondence between individual proteins and total sample load.

## 30.7 2D SDS-PAGE Followed By MS Is Useful for Identification of Specific Changing Proteoforms in Complex Mixtures

The human genome is an invariant blueprint present in all cells and tissues of each person. In contrast, there is no constant human "proteome," rather the protein composition of cells and tissues varies with function and developmental stage. Furthermore, post-translational modifications (PTMs) and genetic polymorphisms may generate multiple proteoforms for a given gene [11]. Deciphering how transcription translates into proteoforms that vary dramatically between tissues and are altered in disease states is quite difficult. Using a concerted approach of several orthogonal techniques rather than one alone holds promise for giving the best outcome [14].

## 30.8 Proteoform Changes Between Samples May Be Found by Comparing 2D Stained Patterns for Differences and Identified by MS, as Shown in Two Examples Below

**Sleep apnea:** Lux et. al. studied protein dysregulation in obstructive sleep apnea (OSA) by comparing 2D patterns from rat atrial tissue induced to have OSA via a surgical implant versus normal control tissue. Over 2000 protein spots were compared for differences, of which 208 differing spots were identified by nanoLC-MS/MS. Figure 30.5 shows a silver-stained 2D gel atrial pattern from a severe apnea sample. Spots increased in averaged severe apnea vs averaged control are outlined in blue; decreased spots are outlined in red.

In total, the data show that enzymes involved in glycolysis, beta-oxidation, electron transport chain, and the Krebs cycle were downregulated in apnea. This model adds to understand of OSA and may serve to elucidate cardiac effects of OSA as well [15].

**MPS 1:** Ou et al. used 2D SDS-PAGE to investigate mucopolysaccharidosis type 1 (MPS 1), a disease where alpha-L-iduronidase is deficient, causing an accumulation of undegraded glycosaminoglycans (GAG). To identify potential biomarkers and further characterize neuropathology, brains from MPS 1 and control mice were analyzed by 2D SDS-PAGE. A total of 2055 protein spots were compared. Of these, 25 spots corresponding to 50 different proteins with a fold change of > 3.5-fold and a $p$ value <0.05 were identified by nanoLC–MS/MS. The MS spectrum of protein 1351, Sirtuin SIR2L2, is shown as an example in Fig. 30.6 along with a 2D silver-stained gel image.

The identified proteins were involved in metabolism, neurotransmission, and the cytoskeleton. STXBP1, a regulator of synaptic vesicle fusion and docking, was downregulated, suggesting impaired synaptic transmission. Notably, alterations in proteins associated with cell death, ubiquitin, or inflammation were not detected [16].

## 30.9 Proteoforms with PTMs involved in Disease Processes May Be Found by Comparing 2D Western Blots and Identified by MS

Over 680 types of PTMs have been discovered to date [17]. Trying to identify which proteins have which PTMs in whole cell lysates using MS alone is difficult. Large numbers and amounts of interfering proteins cause problems. Using 2D WB with high-affinity antibodies is a way to focus in on PTM subsets. Proteins found by WB may be cut from heavily loaded 2D and subsequently identified by MS. For example, Kendrick et al. used phosphotyrosine (pTyr) WB to search for putative Src substrates in pancreatic cancer. They found an abundant pTyr-protein, which was identified from heavily loaded 2D gels as pTyr-mutant desmin D300Y [18]. This novel phosphorylation would not be identifiable from a whole cell lysate.

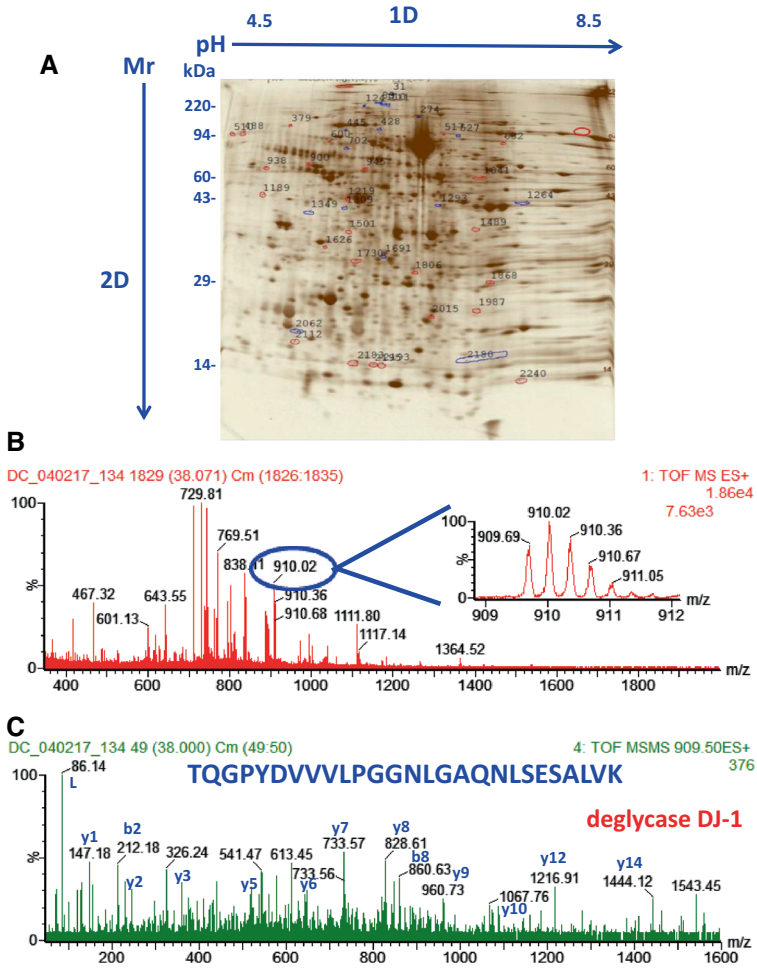



**Fig. 30.5** (**A**) Image of a 2D SDS-PAGE gel run with atrial tissue from a severe OSA animal showing difference versus control. Spots increased in averaged severe OSA vs averaged control are outlined in blue; decreased spots are outlined in red. (**B**) The MS spectrum that contains the precursor ion with *m*/*z* 909.69(3+) corresponds to peptide TQGPYDVVVLPGGNLGAQNLSESALVK, which is part of protein deglycase DJ-1 isoform 2. (**C**) The MS/MS fragmentation of precursor ion with *m*/*z* 909.69(3+) produced a series of b and y ions, whose analysis confirmed the sequence of peptide TQGPYDVVVLPGGNLGAQNLSESALVK and, therefore, the identity of protein deglycase DJ-1 isoform 2

### 30.9.1 Protein Serine, Threonine, and Tyrosine Phosphorylation

Olsen et. al. using MS to study synchronized cultured cells, concluded that as much as 70% of cell proteins (5192/6695 proteins analyzed) are phosphorylated during the cell division cycle [19]. The human genome contains over 500 kinases [20]. Some are well understood, but the role of many kinases in cellular differentiation and disease processes remains to be worked out. Direct identification of phosphoserine (pSer) and phosphothreonine (pThr) by MS is certainly possible, as shown in Fig. 30.7.

In the case of whole cell lysates, direct detection of pSer and pThr residues becomes difficult because of the vast number of interfering proteins. For example, Lopez-Corel et. al. directly detected

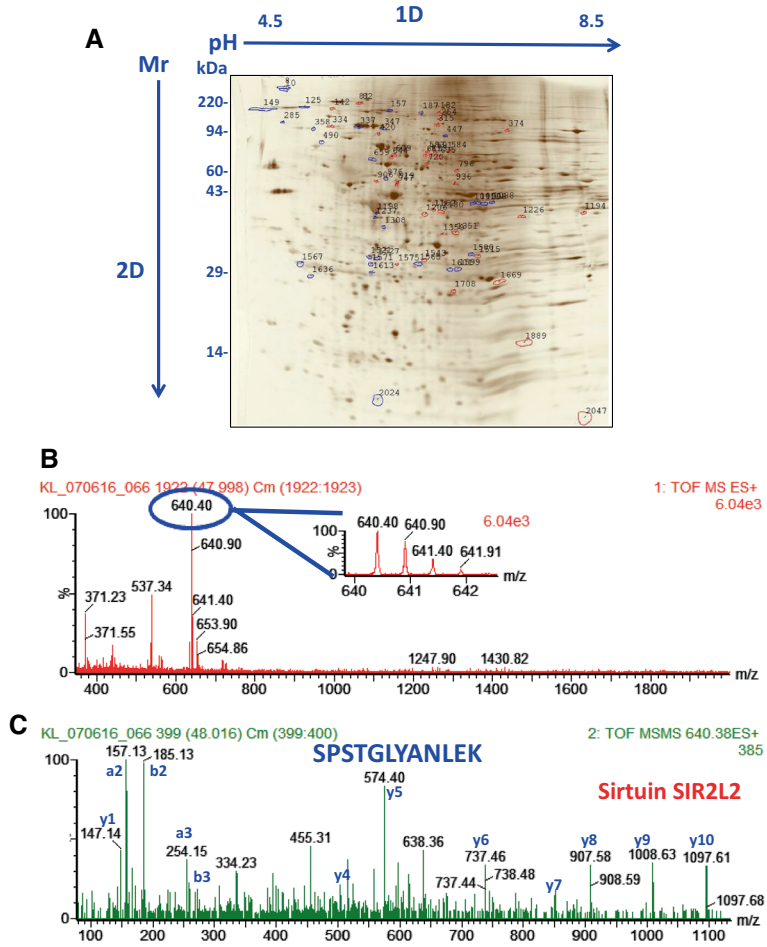



**Fig. 30.6** (**A**) The 2D silver-stained difference pattern from an MPS 1 brain sample. Spots increased in MPS 1 versus control are outlined in blue; those increased in the control are outlined in red [12]. Spot 1351, MW 78 kDa, which increased by 4.1-fold in the MPS 1 samples, was analyzed by LC–MS/MS. (**B**) The MS spectrum that contains the precursor ion with *m/z* 640.38(2+) corresponds to peptide SPSTGLYANLEK, which is part of protein Sirtuin SIR2L2. (**C**) The MS/MS fragmentation of precursor ion with *m/z* 640.38(2+) produced a series of b, a, and y ions, whose analysis confirmed the sequence of peptide SPSTGLYANLEK and, therefore, the identity of protein Sirtuin SIR2L2

these PTMs by pSer/pThr WB with follow-up identification of the proteins by MS. This group's focus was on the interesting problem of how the plasma membranes of hepatocytes become polarized. Hepatocyte apical membranes, which face the bile, have a different contingent of proteins than the basolateral membranes, which face the blood. This group had been using WIF-B cells, a well-characterized hepatic cell model, to study the role of MAL2 (myelin and lymphocyte protein 2) in regulating hepatic polarized protein sorting. They learned that serine/threonine kinase 16 (STK16) was a binding partner of MAL2.

In order to find STK16 substrates, Lopez-Coral et al. compared post-nuclear supernatants of WT WIF-B cells versus a kinase-dead strain (E202A) for differences in pSer/pThr-proteins using 2D WB as shown in Fig. 30.8. Red outlines indicate pSer/pThr-proteins decreased in E202A, i.e., putative STK16 substrates. A total of 28 proteins identified

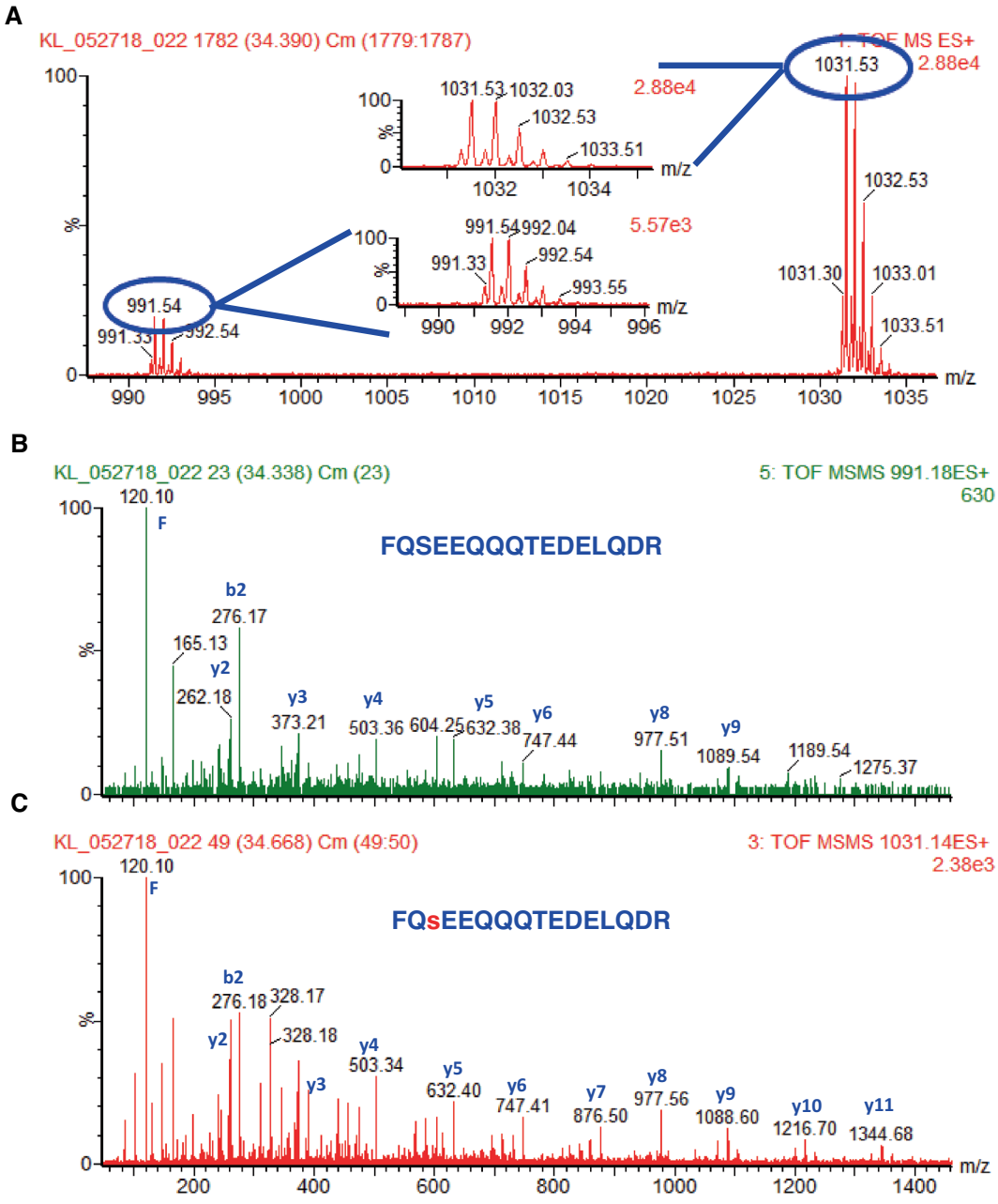



**Fig. 30.7** Identification of pSer residues by MS. (**A**) MS spectrum showing two peaks with *m/z* 991.54 (2+) and 1031.53 (2+), which correspond to peptide FQSEEQQQTEDELQDR (unmodified) and the same peptide phosphorylated at either serine or threonine. (**B**) MS/MS spectrum of the precursor ion with *m/z* 991.54 (2+) produced a series of b and y ions that led to identification of unmodified peptide FQSEEQQQTEDELQDR. (**C**) MS/MS spectrum of the precursor ion with *m/z* 1031.53 (2+) produced a series of b and y ions that led to identification of peptide FQSEEQQQTEDELQDR, phosphorylated at either Ser or Thr. Identification of y8, y9, y10, and y11 shows clearly that the Thr residues are not phosphorylated, leaving pSer the only possibility. Therefore, not only can the amino acid sequence of a peptide be identified, but also PTMs can be identified and the modified amino acid determined (Courtesy of Dr. Costel C. Darie, Clarkson University)

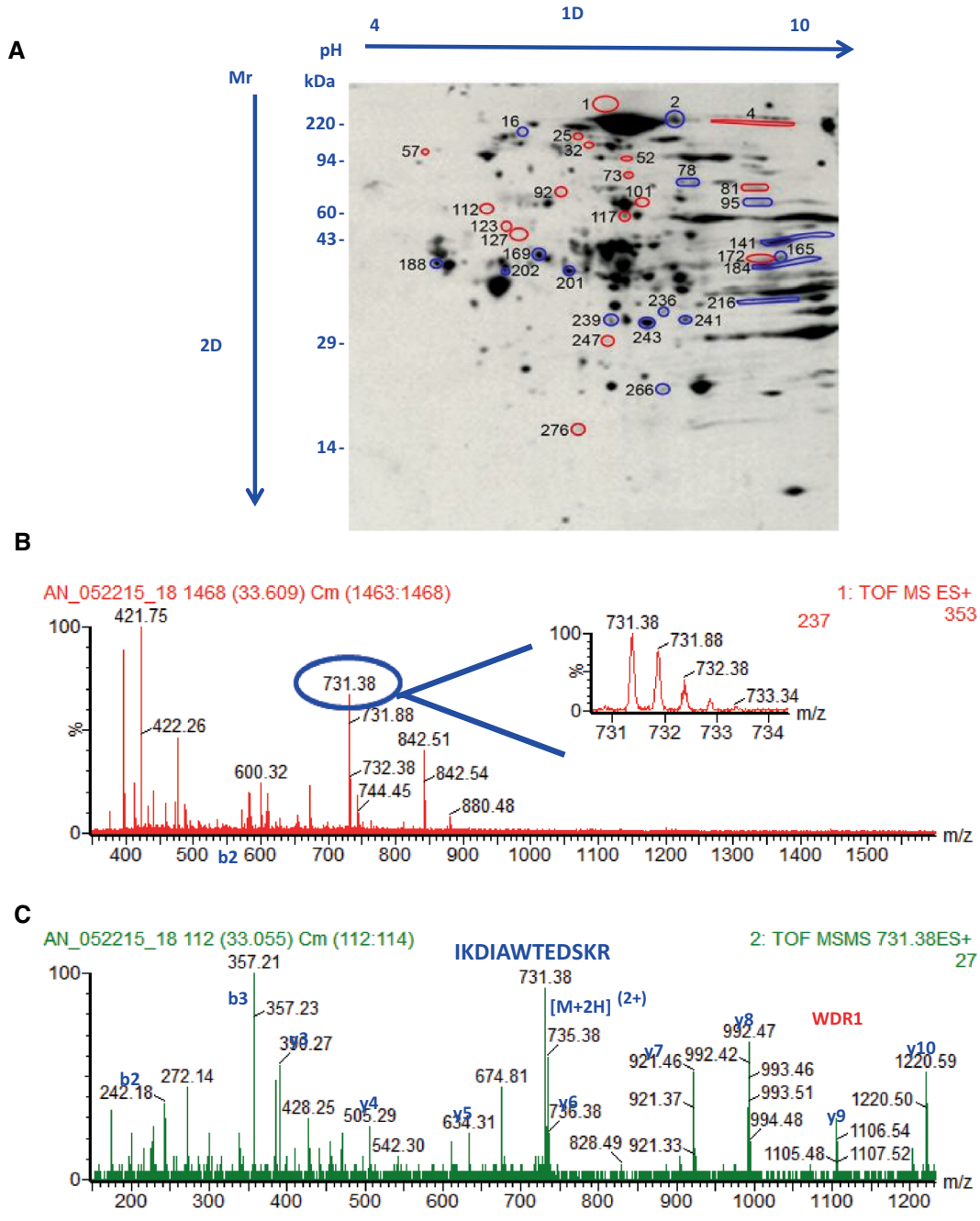



**Fig. 30.8** (**A**) 2D SDS-PAGE pSer/pThr WB of E20A (kinase-dead) cells showing spots with decreased reactivity (red outlines) relative to WT STK16 cells. Blue outlines show spots with increased pSer/pThr signal in E20A [16]. One of the former group, spot #101, was analyzed by LC–MS/MS. (**B**) The MS spectrum that contains the precursor ion with *m/z* 731.38(2+) corresponds to peptide IKDIAWTEDSKR, which is part of protein WDR1. (**C**) The MS/MS fragmentation of precursor ion with *m/z* 731.38(2+) produced a series of b and y ions, whose analysis confirmed the sequence of peptide IKDIAWTEDSKR and, therefore, the identity of protein WDR1. Note that protein WDR1 has two STK16 consensus sequences

from the 16 spots included WDR1, spot 101, which contained two STK16 consensus sequences. The MS sequence of spot 101 is shown in Fig. 30.8. They concluded that WDR1 is a phosphoprotein that regulates secretion [21].

### 30.9.2 Changes in Protein's pI by Blocking Side Chain Amino and Carboxyl Groups

The charge of a protein, characterized by its isoelectric point (pI), depends on the primary and tertiary structures of the protein. It is well-known that manipulation of the carboxyl and amino groups within proteins can change their isoelectric point (pI). The number of conformers that a protein has is directly related to the number of charges induced by Arg, Lys, and His residues when analyzed by mass spectrometry (MS) in positive electrospray ionization mode [ESI(+)] and by Asp and Glu in negative electrospray ionization mode [ESI(−)]. Therefore, in order to change the behavior of a protein in MS, an obvious start is to control the number of proteins' charges that can be ionized by either neutralizing it (i.e., positive to neutral and negative to neutral) or by changing it to the opposite charge (i.e., positive to negative or negative to positive). These charges at the surface of the proteins are the first that are modified in an aging protein, and their neutralization due to aging is the main cause of decreasing structural stability and functional activity (i.e., enzymatic activity). One could test this by neutralizing these surface charges, isolation of individual conformers, and analyzing them for structural stability and/or enzymatic activity using MS. In particular, MS will identify, among others, the charged amino acid that is responsible for decreasing the enzymatic activity of the enzyme investigated.

Our labs can modify the proteins under investigation with particular molecules that can neutralize or change the charge of Lys or Asp/Glu residues, thus changing the proteins' pIs. To increase the protein's pI, we can add quaternary ammonium groups, thus making the protein positively charged. To decrease the protein's pI, we can add sulfonic groups. Both modifications can be done using commercially available reagents (i.e., changing the amino Lys charge to acidic charge can be done using linkers like *acid-dPEG®$_5$-NHS Ester*) [22].

In a recent study, using chymotrypsin as a model protein, we could modify the Lys residues using linkers like *acid-dPEG®$_5$-NHS Ester*. We also know that this enzyme has a maximum of 7 Lys residues on the surface of the enzyme that can be modified; hence, there are about seven conformers with similar mass but different pI, each with different enzymatic activity. Introduction of carboxylic groups by neutralizing Lys residues leads to a series of conformers that have a pI of 8.06, 6.86, 5.63, 5.05, 4.74, 4.53, and 4.37 for the derivatives with 1, 2, 3, 4, 5, 6, and 7 modified Lys residues. This example has been recently published, and our proteomics lab was the one responsible for the identification of the number of modifications per protein, as well as the location of these modifications within the amino acid sequence of this protein [22].

Another application of manipulation of the pI of a protein is in developing isoproteins, under denaturing conditions, that have different pI. This approach has many applications, including, but not limited to, pI standards. Kendrick Labs developed a method that allows them to modify a protein (bovine carbonic anhydrase) to have up to 20 different isoproteins, each of them with a different pI. Figure 30.9 shows such an example. This method involved boiling the protein (carbonic anhydrase) in urea, which produces mostly carbamoylation of the side chain amino groups of the lysine (and sometimes Arginine) residue. Other amino acids can also be modified, and our lab currently investigates these additional modifications.

In order to identify the amino acids that are modified and their location within the protein, each protein spot was excised and digested by trypsin and then analyzed by MS in the Biochemistry & Proteomics Laboratories. Not only that we identified the location of the modifications within the protein, but we were also able to identify peptides unmodified or modified by one or two carbamoylations, and also in different locations within the protein (data not shown,

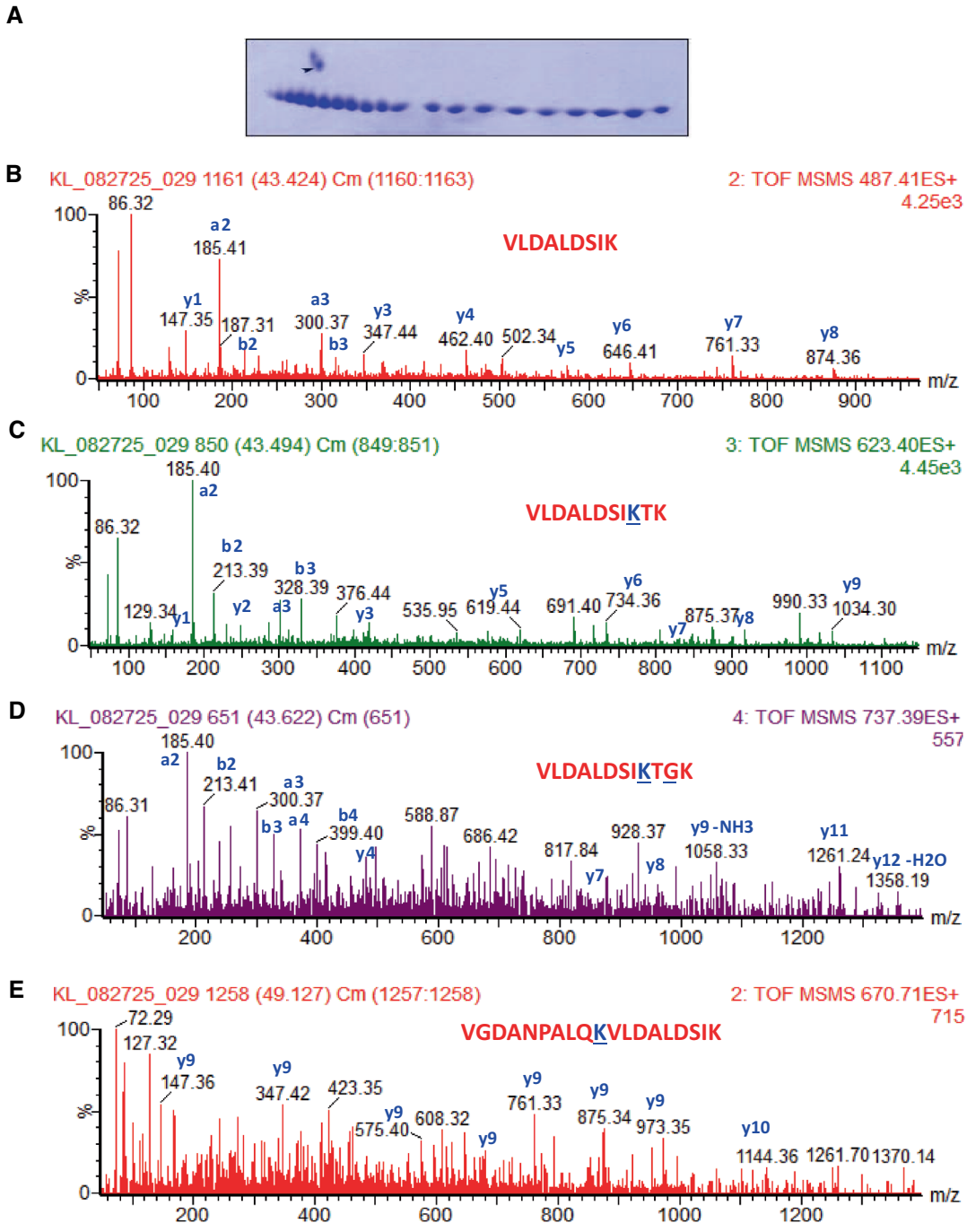



**Fig. 30.9** NanoLC–MS/MS analysis of different carbonic anhydrase isoproteins that have similar mass, but different pI. (**A**) 2D-PAGE gel showing the ~20 carbonic anhydrase isoproteins that have different pI. (**B–E**) MS/MS spectra of precursor ion peaks that correspond to peptides VLDALDSIK unmodified (**B**), or modified by urea at one lysine residue (VLDALDSIKTK) (**C**), two lysine residues (VLDALDSIKTKGK) (**D**), and upstream of the original peptide VLDALDSIK (VGDANPALQKVLDALDSIK) (E). MS/MS fragmentation of these precursor ions produced a series of b, a, and y ions, whose bioinformatics analysis allowed us to identify the amino acid sequence of the peptides, as well as to pinpoint the location within the peptide to a particular amino acid (lysine, marked in blue within the peptide's amino acid sequence)

currently, we are still investigating the carbamoylation mechanism). Several modifications are shown in the spectra from Fig. 30.9. These MS/MS spectra show peptide VLDALDSIK unmodified or modified by urea at one lysine residue (VLDALDSIKTK), two lysine residues (VLDALDSIKTKGK), and upstream of the original peptide VLDALDSIK (VGDANPALQKVLDALDSIK). All these peptides have VLDALDSIK within their amino acid sequence. Taken together, our labs' capability to modify proteins, so we can manipulate their pI, and then identify the modifications within these isoproteins at the amino acid level, shows strong complementary expertise in analysis and characterization of almost any protein.

### 30.9.3 Additional Examples

Aslebagh et al. used 2D SDS-PAGE in combination with MS to determine if milk proteins were affected by breast cancer [23]. Silver-stained 2D SDS-PAGE patterns of milk from a woman diagnosed with breast cancer was compared to that of normal milk from the other breast for differences. Statistically different spots were picked from matched Coomassie blue-stained gels for protein digestion followed by nanoLC–MS/MS. Five upregulated and five downregulated proteins in breast cancer versus control were identified. These potential biomarkers may provide a way to predict breast cancer aggressiveness [23].

Shepard et. al. used 2D SDS-PAGE to study the magnitude of liver lysine acetylation in rats pair-fed ethanol and control liquid diets. These authors detected dramatic difference in the patterns between normal and ethanol-fed animals by using an acetyl-lysine antibody to western blot the 2D gels. Following up with MS, a total of 40 nonnuclear protein were identified, half from the cytosolic fraction and half from nonnuclear membranes. Interestingly, almost all the latter were from mitochondria. They hypothesized that chronic alcohol consumption leads to hyperacetylation of protective antioxidant enzymes, enhancing oxidative stress. If so, acetyltransferase inhibitors might be useful in treating alcoholic liver disease [24].

Wang et. al. investigated the role of alpha-synuclein (α-syn) in neurodegeneration. They found that α-syn is an efficient substrate for arginyltransferase and is arginylated in vivo. Lack of arginylation leads to α-syn aggregation with pathological consequences. This group used 2D SDS-PAGE of a radiolabeled substrate to convincingly show that α-syn is arginylated. MS was used to identify which α-syn amino acid residues were arginylated in native mouse brain [25].

## 30.10 Discussion and Outlook

The strength of standardized 2D SDS-PAGE deserves recognition as an orthogonal method for purification of proteins prior to MS, the only way to unequivocally identify PTMs that cause cancer. Phosphotyrosine (pTyr) especially is a marker for active receptor tyrosine kinases that are cancer drivers after dimerization and trans-phosphorylation, but completely inactive as the unphosphorylated monomer. In our opinion, no single method will suffice for identification of cancer driver biomarkers, rather, combined results from several proverbial blind men will lead to a true description of the elephant.

**Acknowledgments** We thank team members of Kendrick Labs and the Biochemistry & Proteomics Laboratories for their hard work and fruitful discussions. We also thank Drs. Ou and Tuma for allowing use of their published results in our Figs 30.6 and 30.8. Part of the work presented in this publication was supported by the National Institutes of Health under Awards Number 1R15CA260126-01A1 and 1S10OD040197-01. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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