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Analysis of covalent modifications of amyloidogenic proteins using two-dimensional electrophoresis: prion protein and its sialylation

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Abstract

A number of proteins associated with neurodegenerative disease undergo several types of post-translational modifications. They include N-linked glycosylation of the prion protein and amyloid precursor protein, phosphorylation of tau and α -synuclein. Post-translational modifications alter

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4. Notes

- I. It is important that an appropriate amount of sample is taken for 2D. Both overloading and underloading should be avoided. In Fig. 3, examples of inadequate sample loading are presented: the results of overloading are shown in the higher and middle rows, whereas an example of underloading is shown on the lower left panel. The following artifacts appear due to overloading: (1) smearing or “tailing”, (2) poor resolution of spots or (3) both effects. Underloading results in a high background and loss of a signal. All mentioned types of artifacts make the images unsuitable for profile building or calculations.
- II. It may be advisable to write down the numbers of the IPG strips and the samples they carry for convenience. Each IPG strip has its own unique number printed on its back. The strips can be thus easily identified afterwards.
- III. If a bubble is formed, try retrieving the strip from the channel and inserting it again, this time trying to push the bubble from the other end of the channel. If the bubbles are forming due to low sample volume, try adding some more rehydration buffer. It will not significantly dilute the sample.
- IV. The water is only providing a stable temperature so its purity is of minor importance
- V. It is important that each strip has contact with electrode wick for current to pass efficiently. Moreover, it is advisable that all strips are exposed on both ends and aligned similarly to simplify later analysis and comparison.
- VI. Note that the Bromophenol Blue contained in the rehydration buffer will start moving slowly towards the (+) electrode. If that is not observed in 10–15 minutes, check the current on the screen of the power supply. Check if there is water in the inner chamber. If so, stop the current, take off the lid and carefully discard the water from both the outer and inner chambers. Disassemble the module, blot the inner chamber surfaces with kimwipes, replace the soaked electrode wicks with the new ones, then start again from step 5.
- VII. According to the manufacturer, at this stage IPG strips can be frozen and kept at - 80°C in a sealed container. However, we have never done this and do not know whether this may interfere with PrP sialoform analysis.
- VIII. If the tray/cassette contact is leaking, it is possible to incubate strips in any clean container of an appropriate volume given that the strip numbers were taken to prevent subsequent confusion.
- IX. We have noticed that sometimes, due to high amount of material in the strip and/or the position of the strip relative to the electrodes, a spot may appear on either ((-) or (+)) side of the pI range on the Western Blot (Fig. 2, lower right panel). We advise, on subsequent runs, to decrease protein load on the strip and/or to verify its position in the cassette so that neither of its ends is past the respective electrode. In the latter case the (+) end of the strip will be colored with Bromophenol Blue after IEF.
- X. In the case of using the same sample in the marker lane, we advise to dilute initial 2D sample (step 1 from the section “Sample treatment for 2D”) 10–25 times in the SDS sample loading buffer prior to loading. It will thus not overpower the weaker signal from the IEF.

physical properties of proteins including their net and surface charges, affecting their processing, life-time and propensity to acquire misfolded, disease-associated states. As such, analysis of post-translational modifications is important for understanding comprehensive mechanism of pathogenesis. Recent studies documented that sialylation of the disease-associated form of the prion protein or PrP^{Sc} controls the fate of prions in an organism and outcomes of prion infection. For assessing sialylation status of PrP^{Sc}, we developed reliable protocol that involves two-dimensional electrophoresis followed by Western blot (2D). The current chapter describes the procedure for analysis of sialylation status of PrP^{Sc} from various sources including central nervous system, secondary lymphoid organs, cultured cells or PrP^{Sc} produced in Protein Misfolding Cyclic Amplification.

Keywords

prion proteins; prion diseases; amyloidogenic proteins; post-translational modifications; two-dimensional electrophoresis; sialylation; sialic acid; glycosylation

1. Introduction

A number of post-translational modifications of proteins associated with neurodegenerative disease including N-linked glycosylation of the prion protein or PrP^C and amyloid precursor protein, phosphorylation of tau and α -synuclein have been described over the years (1–4). Upon post-translational modifications, the physical properties of amyloidogenic proteins including their net and surface charges change affecting their processing, cellular localization, life-time and propensity to adopt misfolded, disease-associated states. The current chapter describes analysis of the glycosylation and sialylation status of the infectious, disease-associated state of the prion protein or PrP^{Sc}.

The importance of PrP^{Sc} sialylation for prion pathogenesis has been documented in a series of recent studies (5–9). Sialylation of PrP^{Sc} was found to control the fate of prions in an organism and outcomes of prion infection (5,7,8). Sialylation of prions is enhanced upon their colonization of secondary lymphoid organs, thus, prions may use strategies similar to other pathogens to camouflage themselves from the immune system, facilitating host invasion (6). In PrP^{Sc}, the glycans are directed outward, with the terminal sialic acid residues creating a negative charge on the surface of prion particles. In fact, electrostatic repulsion between sialic residues creates structural constraints that control prion replication rate and PrP^{Sc} glycoform ratio in a strain-specific fashion (10). Moreover, due to strain-specific structural constraints, prion strains recruit PrP^C sialoglycoforms selectively, i.e. according to their sialylation status rather than their relative expression levels (10,11). Analysis of differences in strain-specific sialylation pattern is also important for elucidating prion strain competition for a substrate and strain interference (11,12).

The fact that the N-linked glycans of PrP^C and PrP^{Sc} are sialylated was described more than 30 years ago (1). Sialic acids are linked to the terminal positions of the two N-linked glycans (13–15) and, upon conversion of PrP^C into PrP^{Sc}, are carried over giving rise to sialylated PrP^{Sc} (16,17). For assessing sialylation status of PrP^C and PrP^{Sc}, we developed reliable protocol that involves two-dimensional electrophoresis (2D) followed by Western blot

(5,6,10,18). In 2D electrophoresis, the first, horizontal dimension (isoelectrofocusing or IEF) separates molecules according to their pI, whereas the second, vertical dimension (SDS-PAGE) separates according to molecular weight. Native PrP^{Sc} particles are multimers of heterogeneous size. For analysis of sialylation status, cell or tissues homogenates containing PrP^{Sc} are first treated with proteinase K (PK) to remove PrP^C and other proteins. Then PrP^{Sc} multimers are denatured into prion protein (PrP) monomers and separated using 2D according to their charge and molecular weight (Fig. 1A). Individual PrP molecules could be un-, mono- or di-glycosylated that are separated in the vertical dimension of 2D according to their glycosylation status (Fig. 1A). Each of the two glycans carry up to five terminal sialic acid residues adding negative charges to individual PrP molecules (17). The distribution of charge isoforms in the horizontal dimension of 2D reflects sialylation status of individual PrP molecules. Heavily sialylated PrPs run toward acidic pH, while weakly sialylated toward basic pH (5) (Fig. 1A,B). Due to structural heterogeneity of the GPI anchors, unglycosylated PrPs also show several charge isoforms (19,20) (Fig. 1A,B). Nevertheless, sialylation of glycans shifts the distribution of charge isoform toward acidic pH for monoglycosylated PrPs and even more so for diglycosylated PrPs, when compared to unglycosylated PrPs (Fig. 1A,B).

Separation of PrP charge isoforms by 2D is typically followed by densitometry analysis, which is employed for quantification of isoforms according to their sialylation status. The individual intensity profiles of di- or mono-glycosylated isoforms are normalized and plotted as a function of pI (Fig. 2A) (18). This analysis is used for comparing the relative sialylation levels of PrP^C or PrP^{Sc} from different sources (Fig. 2A) or after desialylation using sialidases or acetic acid (Fig. 2C). However such analysis does not address the question whether the differences between different samples are statistically significant (18). According to alternative procedure, charged PrP isoforms on individual 2D profiles are separated arbitrarily into two groups. Isoforms located toward acidic pH from pI 7.5 are designated as hypersialylated and those toward basic pH are designated as hyposialylated (Fig. 2B) (10). The percentage of sum intensities of hypersialylated isoforms relative to the total intensities of all isoforms is used to analyze statistically significant differences between samples. Using this approach, mean and standard deviations can be calculated when sufficient number of samples within each group are analyzed (Fig. 2B). In addition, it is possible to plot the percentage of hypersialylation as a function of other parameters, for instance, the percentage of diglycosylated isoforms (Fig. 2B, bottom plot). The current chapter describes the procedure for analysis of sialylation status of PrP^{Sc} from various sources including brain, spleen, cultured cells or Protein Misfolding Cyclic Amplification (PMCAb) using 2D electrophoresis followed by Western blot.

2. Materials

2.1. Scrapie samples

Scrapie brain and spleen homogenates were prepared from terminally ill hamsters or mice inoculated i.c. or i.p. with brain scrapie material or PMCAb-derived products as previously described (6,21). PMCAb samples were produced as described elsewhere (5,22). N2a cells infected with 22L strain were collected in PBS.

2.2. Preparation of scrapie brain-or spleen-homogenates or cell lysates

1. Ice-cold phosphate-buffered saline (PBS) pH 7.4 supplemented with 5 mM EDTA
2. Ice-cold conversion buffer (Ca²⁺-free and Mg²⁺-free PBS, pH 7.4, supplemented with 0.15 M NaCl, 1.0% Triton, and 1 tablet of Complete protease inhibitors cocktail per 50 ml of conversion buffer)
3. Tissue grinder with pestle (30 ml size)
4. Cordless 12 V compact drill.
5. Tabletop centrifuge with cooling.
6. Thermomixer.
7. Misonix S-4000 microplate horn (Qsonica LLC, Newtown, CT)
8. Proteinase K (PK)
9. 1% SDS.
10. 4x LDS Sample Buffer
11. Water bath
12. Cell scraper (cat # SAR-83.1832, Sarstedt, Nümbrecht, Germany)
13. Cell media (MEM, cat # 10-010-CV, Corning, Corning, NY)
14. Pre-chilled acetone [Stored at -2 0°C.]
15. *Arthrobacter ureafaciens* sialidase (cat # P0722L, New England Biolabs, Ipswich, MA) [Stored at -20°C.]
16. Acetic acid, glacial.

2.3. Preparation of samples for 2D

1. Solubilization buffer: 20 mM Tris-HCl pH 8.0, 8 M Urea, 2% (w/v) CHAPS (3-[(3-**ch**olamidopropyl)dimethyl**ammonio**]-1-**p**ropanesulfonate), 5 mM TBP (tributylphosphine), (can be prepared and aliquoted in advance, stored at -20°C)
2. 0.5 M iodoacetamide (freshly prepared)
3. Pre-chilled methanol (stored at -20°C)
4. Tabletop centrifuge with cooling.
5. Rehydration buffer: 7 M urea, 2 M thiourea, 1% (wt/vol) DTT, 1% (wt/vol) CHAPS, 1% (wt/vol) Triton X-100, 1% (v/v) ampholyte, trace amount of Bromophenol Blue. (can be prepared and aliquoted in advance, stored at -20°C)
6. Fixed pre-cast immobilized pH gradient (IPG) strips with a linear pH gradient 3-10.
7. IPG Runner cassettes

2.4. Isoelectrofocusing

1. Power Supply.
2. ZOOM IPG Runner Mini-cell.
3. IPG strips with pi 3–10 linear gradient.

2.5. SDS-PAGE

1. Provision for equilibration buffers 1 and 2. In a 50 ml tube, prepare a solution consisting of 6 M Urea, 375 mM Tris 8.8, 20% v/v glycerol, 2% w/v SDS, dissolve on rotator. Add 0.3 g amberlite ion exchange resin (cat # A5710, Sigma-Aldrich, St. Louis, MO), incubate on rotator at room temperature for 1 h. Filter to get rid of amberlite particles. Divide into two 15 ml parts. This intermediate preparation can be stored at –20°C. Defreeze prior to use and add DTT and iodoacetamide as follows.
2. To make Equilibration buffer 1: to 15 ml, add 0.3 g DTT. Leave on rotator for 5–10 minutes. (Final composition 375 mM Tris-HCl pH 8.8, 6 M Urea, 20% (v/v) glycerol, 2% SDS, 130 mM DTT). To make Equilibration buffer 2: to 15 ml, add 0.375 g iodoacetamide. Leave on rotator for 5–10 minutes. (Final composition: 375 mM Tris-HCl pH 8.8, 6 M Urea, 20% (v/v) glycerol, 2% SDS, 135 mM iodoacetamide.)
3. ZOOM Equilibration Tray.
4. Laboratory shaker/rotator.
5. 4–12% Bis-Tris ZOOM SDS-PAGE pre-cast gels.
6. Power Supply 300 Plus 300V, 4–500 mA.
7. XCell SureLock Mini-Cell Electrophoresis System.
8. MES-SDS PAGE buffer, prepared from 20X Bolt MES SDS Running Buffer.
9. 0.5% w/w agarose prepared by melting powdered agarose in ultrapure water. Prior to SDS-PAGE, melt 0.5% agarose in a microwave oven and supply with MES-SDS buffer (1 part MES-SDS buffer for 19 parts 0.5% agarose, v/v). Keep at 55–65°C until used. Each IPG strip requires approximately 400 µl melted agarose.

2.6. Western blot

1. PVDF membranes.
2. Blotting paper.
3. Western Blot Transfer Buffer: 100 ml 10x Transfer Buffer [14.5 g Tris; 72.0 g Glycine, dissolve in ultrapure water and adjust volume to 1 L]; 200 ml MeOH; 700ml ultrapure water)
4. Power Supply 300 Plus 300V, 4–500mA.

5. XCell SureLock Mini-Cell and XCell II Blot Module.
6. PBST. Supply 1x PBS with 0.1% v/v TWEEN-20.
7. Blocking solution: 5% milk in PBST. Dissolve 2.5 g dry non-fat milk in 50 ml PBST by vortexing, and agitation at room temperature for at least 30 minutes
8. Laboratory shaker/rotator.
9. Primary antibodies: 1:10,000 3F4 (BioLegend, San Diego, CA) for hamster PrP^{Sc}, 1:5,000 Ab3531 (Abcam, Cambridge, MA) for mouse PrP^{Sc}.
10. Secondary antibodies, HRP-labeled: 1:10,000 Goat-anti-mouse (KPL, Gaithersburg, MD) was used with 3F4; 1:5,000 Goat-anti-rabbit (KPL, Gaithersburg, MD) was used with Ab3531.
11. Luminata Forte Western HRP Chemiluminescence Substrate (cat # WBLUF0500, EMD Millipore, Billerica, MA)
12. Western Blot Imaging System and software (we use FluorChem M equipment)

4. Methods

Unless stated otherwise, all the operations are done at room temperature (20–25°C). The whole experiment takes at least two days, depending on the desired incubation time in primary antibody. Prior to running a 2D, it is recommended to check the quantity of PrP^{Sc} in scrapie material using SDS-PAGE/Western Blot (I).

3.1. Sample preparation

3.1.1. Preparation of scrapie brain samples

1. Prepare 10% brain homogenate (w/v) from scrapie-inoculated animals using ice-cold in PBS pH 7.4, and glass/Teflon tissue grinders cooled on ice and attached to a drill.
2. Dilute 10% brain homogenate 10-fold in conversion buffer. Place 100 µl aliquot into thin-wall PCR tubes and sonicate for 30 seconds at 170W output in microplate horn.
3. Supplemented a 25 µl aliquot from last step with the same amount of PK solution in ultrapure water (final PK concentration of 20 µg/ml) and incubate at 37°C for 30 minutes.
4. Optional. In case of sialidase treatment, denature samples by incubating for 10 min at 95 °C in the presence of 0.5% SDS and 40 mM DTT. After letting the samples cool for 10 min at room temperature, supply with 10 % (v/v) of Sialidase buffer (supplied with sialidase by manufacturer) and 10 % vol/vol of sialidase. and incubate at 37 °C for 16 hours with 600 rpm shaking in thermomixer. Sialidase-treated samples show substantial shift on 2D toward basic pI (Fig. 2C).

5. Optional. In case of acetic acid treatment, denature samples by incubating for 10 min at 95 °C in the presence of 0.5% SDS and 40 mM DTT, then supplement with 1% (v/v) acetic acid and incubate at 100 °C for 1 h with 1,000 rpm shaking in thermomixer to achieve mild acid hydrolysis of sialic acids. Acetic acid-treated samples show substantial shift on 2D toward basic pI (Fig. 2C).
6. Take 19 µl from the last step, add 6 µl of 4x LDS and incubate for 10 minutes in a boiling water bath.

3.1.2. Preparation of scrapie spleen samples

1. Prepare 10% spleen homogenate (w/v) from scrapie-inoculated animals using ice-cold in PBS pH 7.4, and glass/Teflon tissue grinders cooled on ice and attached to a drill.
2. Dilute 250 µL of 10% (wt/vol) homogenate 2-fold with PBS. Aliquot into thin-wall PCR tubes and sonicate for 30 seconds at 170W output in microplate horn.
3. Combine all aliquots in one centrifuge tube and centrifuge in pre-chilled (4°C) tabletop centrifuge for 30 minutes at 16,000 g. Discard the supernatant.
4. Resuspend the pellet in 25 µl of 1% (wt/vol) Triton in PBS and treat with 20 µg/ml PK for 30 min at 37°C.
5. Take 19 µl from the last step, add 6 µl of 4x LDS and incubate for 10 minutes in a boiling water bath.

3.1.3. Preparation of PMCAb samples

1. Dilute a 10 µl aliquot of PMCAb material 10-fold in PBS, place into a thin-wall PCR tube and sonicate for 30 seconds at 170W output in microplate horn.
2. Supplement a 25 µl aliquot from last step with the same amount of PK solution in ultrapure water (final PK concentration of 20 µg/ml) and incubate at 37°C for 30 minutes.
3. Take 19 µl from the last step, add 6 µl of 4x LDS and incubate for 10 minutes in a boiling water bath.

3.1.4. Preparation of cultured cell-derived samples

1. Remove media from cultured cells, add 1 ml of fresh media and scrape cells with a scraper. Collect and spin-down 2,000 rpm for 3 minutes.
2. Re-suspend the pellet in 200 µl PBS and supply 1% v/v Triton X-100. Aliquot into thin-wall PCR tubes and sonicate for 30 seconds at 170W output in microplate horn.
6. Treat with 10 µg/ml PK for 30 min at 37°C.
3. Add 1,000 µl of pre-chilled acetone. Leave at -20°C overnight.
4. Discard acetone, let the pellet dry for 15 minutes. Re-suspend the pellet in 25 µl of 1x LDS and incubate for 10 minutes in a boiling water bath.

3.2. Sample treatment for 2D

1. Mix 25 μ l of LDS-containing sample with 200 μ l of solubilization buffer. Incubate for 1 hour (I).
2. Add 7 μ l of freshly prepared 0.5 M iodoacetamide, mix by inverting the tubes a couple of times. Incubate for 1 hour in the dark.
3. Add 1,160 μ l of pre-chilled methanol, mix by inverting the tubes a couple of times. Incubate for at least 2 hours at -20°C .
4. Centrifuge in pre-chilled (4°C) tabletop centrifuge for 30 minutes at 16,000 g. Discard the supernatant.
5. Dry the pellet by leaving the tube open for a maximum of 30 minutes; if needed, dry additionally with an air stream.
6. Re-suspend in 160 μ l of rehydration buffer by pipetting and, if needed, vortexing.
7. Put a new IPG cassette on a firm leveled surface. Load 155 μ l of each sample into a well on from the convex side of the cassette. The samples will immediately start entering into the sample channels. It is not necessary to use all the channels.
8. Peel the IPG strip from the blister with a forceps (II). Holding to its (+) marked end, with its printed side facing down, insert a strip into each channel containing a sample. Try not to introduce bubbles (III).
9. Seal the sample wells on both ends of the cassette by applying a sticker tape (provided with the cassettes).
10. Leave sample for rehydration overnight, or for at least 16 hours.

3.3. Isoelectrofocusing

1. Remove sealing tape with plastic sample loading wells from both sides of cassette thus exposing adhesive surface.
2. Make sure that portions of the blue-colored gel on IPG strips are exposed on both ends of the cassette; adjust with forceps if needed (IV).
3. Place electrode wick (provided with cassettes) over the adhesive. Use the black alignment marks on the cassette to properly place the wicks.
4. Evenly apply 600 μ l ultrapure water to each wick.
5. Assemble the IPGRunner sandwich with the help of the gel dummy (if using two cassettes at a time, the dummy is not needed). The electrode wicks must come in contact with the electrodes of the ZOOM IPG RunnerTM Core.
6. Place the assembled module into the Mini-Cell Chamber and secure with a gel wedge.

7. Fill outer chamber of the Mini-Cell with deionized or ultrapure water (V). Be careful not to spill water into the inner chamber.
8. Place the ZOOM IPGRunner Cell Lid on the ZOOM IPGRunner Core. The lid can only be positioned in one position, with the (–) electrode on the right.
9. Connect the electrode cords to the power supply.
10. Turn on the power supply and choose the IEF current conditions. We find that the standard scheme suggested by the manufacturer for broad range IPG strips works for our purposes. Specifically, 175 V for 15 minutes, then 175–2,000V linear gradient for 45 minutes, then 2,000V for 30 minutes (VI).
11. After the IEF program has finished, turn off the power supply, disconnect the cables and remove the lid.
12. Carefully discard the water from the outer chamber.
13. Disassemble the module by first taking out the gel wedge and then the cassette with the Runner Core.
14. Put the cassette on a paper towel with IPG wells facing up. Blot any extra liquid. Trying not to disturb the IPG strips, remove the film cover from the cassette (VII).

3.4. SDS-PAGE

1. Peel off adhesive liner from ZOOM Equilibration Tray. Carefully, trying not to touch the IPG strips, place the Equilibration Tray on the cassette: their outline shapes are similar and the cassette has protruding ribs which must come into the indentations in the edges of the tray. After placing the tray, secure it by applying pressure to the adhesive contacts of the tray.
2. Add 15mL of Equilibration Buffer 1 into the orifice of the tray. Incubate the strips with gentle agitation for 15 mins. Discard Equilibration Buffer 1 by gently turning the cassette/tray assembly upside down (VIII).
3. Add 15mL of Equilibration Buffer 2 into the orifice of the tray. Incubate the strips with gentle agitation for 15 mins. Discard Equilibration Buffer 2 by gently turning the cassette/tray assembly upside down.
4. Meanwhile, prepare the SDS-PAGE cells: unpack ZOOM gels, peel off the tapes, take out the combs. Rinse the wells with ultrapure water to remove any excess of polyacrylamide and blot excess water with blotting paper. If the marker well is bent it can be straightened with a help of a fine plastic tip or a forceps. Insert the gels into Mini Cell(s) with their Buffer Core and dummies, if needed. Secure with gel wedges.
5. Peel off the tray from the cassette. Blot any excess liquid.
6. Take each IPG strip and cut plastic ends. Do not cut off gel portions (IX).

7. Place the IPG into the gel with the gel part of the strip facing towards the outer chamber of the Mini-Cell. We use the position of the (+) part of the strip towards the marker lane; however, the reverse position is also possible. Align the strip horizontally, trying to avoid bubbles.
8. Seal the strip in the well by adding approximately 400 μ l of agarose (heated to 55–65°C) supplied with MES SDS-PAGE running buffer. Let the agarose set for a couple of minutes.
9. Add SDS-PAGE running buffer into the inner and outer chambers of the cell. If needed, add the marker into the marker lane (X).
10. Close the lid of the Mini-Cell and connect the electrode cords to the power supply.
11. Turn on the power supply and choose the SDS-PAGE current conditions. We use constant voltage of 170V for 60 minutes.
12. After the SDS-PAGE program has finished, turn off the power supply, disconnect the cables, remove the lid and take out the gels.

3.5. Western Blot

1. Pre-soak four pads in Transfer Buffer.
2. Prepare PVDF membrane: rinse marked PVDF membrane, in methanol for 20 seconds. Discard methanol, incubate in ultrapure water for 2 minutes, then incubate in Transfer Buffer.
3. Extract ZOOM gel, briefly rinse with ultrapure water
4. Assemble blot cell. Conduct transfer at 33 Volts for 1 hour on ice. After the program is finished, disassemble the cell and incubate membrane in 20 ml blocking solution for 30–60 minutes with gentle agitation.
5. Discard the blocking solution, incubate membrane in the primary antibody for either 1 hour at room temperature or overnight at 4°C.
6. Decant primary antibody (it can be re-used during 5–7 days if kept at 4°C).
7. Wash membrane in PBST twice for 15 minutes with gentle agitation.
8. Discard the PBST, incubate the membrane in the secondary antibody for 45–60 minutes with agitation.
9. Wash membrane in PBST three times for 10–15 minutes with gentle agitation.
10. Discard PBST, incubate the membrane briefly (10–15 seconds) in the developing solution and immediately proceed to developing. The developing solution can be used for several membranes during 1 hour.

3.6. Analysis of 2D images

1. Open the acquired images in the AlphaView software window.

2. To generate individual sialylation profiles for graphical representation, use the “Lane Profile” function. Select the lane of interest and follow the instructions in the dialog box. The resulting curve can be transferred to Excel or other graph building software for further analysis (see Fig. 2A, bottom plot).
3. For quantification of intensities of spot(s) of interest, use “Multiplex band analysis” option. First, draw a vertical line through carefully horizontally aligned 2D images at pI of ~7.5 (dash line Fig. 2B); this line is used to arbitrarily separate charge isoforms into hypersialylated (to the left from the line) and hyposialylated (to the right). In the “Multiplex band analysis” dialog box, choose a rectangle, circle or other shape and place it on the digitized blot to confine the spots of interest; subtract intensity of an equal background area from the same blot. The intensities of hyper- and hyposialylated isoforms combined are counted as 100% for each sample, and the percentage of hypersialylated can be easily derived from each 2D blot. After calculating mean and standard deviation from multiple repeats, a plot can be generated in Excel software. Fig. 2B shows the percentage of hypersialylated molecules versus the percentage of di-glycosylated, as previously reported for three mouse strains (10).

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Figure 1. Schematic diagram illustrating 2D analysis of PrP^{Sc}.

(A) Scrapie materials are treated with PK to clear PrP^C, denatured into monomers and then analyzed by 2D. In horizontal dimension of 2D, individual PrP molecules are separated according to their pI. Charge distribution of individual PrP molecules reports on contribution of sialoglycoforms in PrP^{Sc} particles. The charge distribution of monoglycosylated isoforms extends toward acidic pH beyond that of unglycosylated isoforms, and the charge distribution of diglycosylated isoforms extends toward acidic pH beyond that of monoglycosylated isoforms according to the sialylation status of individual PrP molecules.

For statistical analysis of sialylation status, diglycosylated charge isoforms are arbitrarily separated into two groups: hypersialylated (on the left of pI 7.5) and hyposialylated (on the right of pI 7.5). N-linked glycans are shown as blue lines, terminal sialic acid residues are shown as red diamonds. **(B)** Schematic representation of multiple charge isoforms observed in 2D. For non-glycosylated isoforms, multiple (up to 7) charge isoforms could be seen. These isoforms arise due to (i) structural heterogeneity of GPI anchor for which differences in GPI sialylation status contribute, and (ii) cleavage of PrP^{Sc} at alternative sites by proteinase K. N-linked glycans add to the charge heterogeneity of the mono- and diglycosylated isoforms as indicated by color arrows. Each charge isoform of unglycosylated protein give rise to multiple charge isoforms upon attachment of an N-linked glycans according to a distribution of the sialoforms of that glycan. Addition of each sialic acid results in a shift towards the acidic pI on the 2D strip.

Figure 2. Analysis of PrP^{Sc} sialylation by 2D analysis.

(A) Analysis of sialylation status of 22L brain-(BH), spleen-(SH) or N2a-derived material using 2D (top panels). Sialylation profiles of diglycosylated isoforms of 22L brain-(solid line), spleen-(gray line) or N2a-(dotted line) derived material (bottom panel). Profiles were built using densitometry analysis of 2D Western blots. The highest curve signal value was taken as 100%. (B) Analysis of sialylation status of RML, 22L and ME7 brain-derived material (BH) using 2D (top panels). Percentage of hypersialylated isoforms plotted as a function of percentage of diglycosylated glycoforms for brain-derived RML, 22L and ME7

scrapie material (bottom panel). For each strain, at least three values were acquired from independent brain materials; the variations were used to calculate mean and standard deviations. **(C)** Comparison of sialylation status of 22L BH mock-treated (top panel) or treated with either acetic acid (middle panel) or *Arthrobacter ureafaciens* sialidase (lower panel). Sialylation profiles on the right were built separately for di-, mono- and non-glycosylated forms of mock-treated (black thick line), acetic acid-treated (gray line) and sialidase-treated (thin line) 22L brain homogenate. The highest intensity value for each curve was taken as 100%. In panels **A**, **B** and **C**, black triangles, white triangles and arrows mark di-, mono- and non-glycosylated glycoforms, respectively.

Figure 3. Artifacts related to inadequate sample loading on 2D.

Inadequate sample loading on 2D results in "tailing" effect, poor spot separation, high background or spots at acidic pI. Black triangles, white triangles and arrows mark di-, mono- and non-glycosylated glycoforms, respectively.