Module:
Techniques in Neuroscience

Week 5
Molecular biology: Going inside the cell

Topic 1
An introduction to molecular biology methods – Part 2 of 3

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Now let's look at how we separate the proteins that we've produced using the recombinant DNA technologies described in the first section. SDS-PAGE stands for sodium dodecyl sulphate-polyacrylamide gel electrophoresis. SDS-PAGE is a method that uses electrophoresis to separate proteins in a sample by size - or to put it more accurately, by molecular weight.

Electrophoresis works by applying an electric field across a gel, in this case, made of a molecule called polyacrylamide. The first thing we do is break down the cell or tissue samples we're studying to release the proteins. We then denature the proteins in the sample and give them an electric charge. The sample containing the proteins is then placed in small wells at one end of the gel. When the electric field is switched on, the molecules are attracted to the end of the electric field that has the opposite charge with small lower molecular weight molecules migrating across the gel quickly, and big higher molecular weight molecules migrating more slowly. So, if you switch the field off at just the right time, you find that the proteins are spread out and have been separated. Okay, now I'll explain these steps in more detail.

For SDS-PAGE, we need all our proteins to be in solution, so we need to break open the cells or the tissue we're using. We can do this either mechanically or chemically. Mechanical breakdown works by homogenising the cells in a homogeniser or sonicating them by blasting them with ultrasonic vibrations. This tears the tissue apart and rips open the cell membranes, letting all the proteins out. Chemical breakdown is called lysis and works by mixing the cells with a buffer that will break the membranes apart.

The breakdown method we choose depends on the tissue we're working with and the protein we want to look at, though we often use a combination of methods. For example, if I were lysing cells that I had cultured in a monolayer, I'd scrape them into lysis buffer and pipette them up and down a bit to make sure they were in a single cell suspension. However, if I were lysing a chunk of tissue, I'd use a mechanical homogeniser because I'd need to break apart the structure of the tissue to allow lysis buffer to access all the cells. However, I might use a mechanical homogeniser rather than a strong lysis buffer if I wanted to preserve some cell structure. For example, if I were looking at membrane proteins, I might not want to use a strong lysis buffer as this would destroy

the membranes, whereas mechanical homogenisation would break the membranes up into small pieces but preserve their structure, meaning that most of the membrane proteins would stay within the membranes.

Breaking open the cells gives us a homogenate or a cell lysate. We now need to process the proteins further for two reasons: firstly, the proteins won't move through the polyacrylamide gel on their own, and secondly, different proteins are different shapes, which would confuse the results. For example, a long, thin protein would migrate differently on the gel to a small, dense, round one. It's important that any differences in migration are based on only one factor - molecular weight - and not a second factor, such as shape. To overcome these problems, we treat the cell lysate with a chemical called sodium dodecyl sulphate, or SDS, which acts as a denaturing agent. SDS has a long hydrophobic tail and a negatively-charged head.

The long hydrophobic tail binds the protein and pulls apart the protein structure, thereby unfolding it. Because the SDS molecule has a strong negative charge, once it's bound to the protein, it means the protein has a strong negative charge too. Because all the proteins in the sample now have a strong negative charge, we can use this to force them to move through the gel. At the same time as adding the SDS, we're also adding a blue dye- glycerol- and reducing agents. The dye, usually brilliant blue or bromophenol blue, enables us to see our samples when we're loading them and running them through the gel. The glycerol is to make the sample heavy so that it sinks into the well when we're loading the gel.

The reducing agent, either DTT or beta-mercaptoethanol, is used to break any disulphide bonds in the proteins. The samples are then boiled to denature the proteins further. Now the samples are prepared, we can move on to running the SDS-PAGE gel, which I'll explain next. The separation process begins with when the prepared samples are pipetted into wells on the gel. The gel is made from a molecule called polyacrylamide. Polyacrylamide is made of long chains, and by cross-linking lots of polyacrylamide molecules together, we create a gel mesh containing pores of many different sizes. The difference in pore size helps to separate the proteins as the larger molecules can't fit through the smaller pores so easily.

To make the gel itself, acrylamide is mixed with an SDS containing buffer. Then two catalysts, ammonium persulfate and TEMED, are added to initiate a polymerisation reaction that makes the acrylamide molecules cross link, thus forming the gel. This is diagram of how the electrophoresis set-up looks. The gel is made in between two glass plates and consists of a resolving gel and a stacking gel. The stacking gel has a lower pH and a lower percentage of acrylamide than the resolving gel. When the proteins in the sample hit the join between the stacking and the resolving gel, the change in pH and percentage of acrylamide slows them down. The proteins entering the resolving gel first are slowed down first, allowing the proteins behind to catch up.

The samples get stacked up, forcing them into a tight, thin band in the resolving gel. If we didn't use a stacking gel, the protein would all enter the resolving gel at slightly different times, resulting in very large and smeared bands. The resolving gel is where the action happens. In this gel, the proteins are separated by size as they're forced through the gel by the electric field. We can change the percentage of acrylamide in the resolving gel depending on the size of protein we want to look at. If we use a lower percentage acrylamide, the pore size will be bigger and this will give a good resolution of larger proteins but doesn't work so well for much smaller proteins.

If we use a higher percentage acrylamide, the pore size will be smaller which resolves small proteins well, but not large proteins. The electrophoresis is carried out in a tank containing a buffer that allows the current to flow through the gel. It contains SDS, the denaturing agent, and stabilises the pH of the system without reacting to any of the samples or the gel. We include some SDS in the gel and buffer to ensure the protein remains denatured throughout. The gel is mounted into a plastic cassette holder, the samples are loaded and then a lid is placed on top. The lid completes the electric circuit, connecting the electrodes to a power supply. Using the power supply,

we can alter the voltage passing through the gel to give us some control over how fast the proteins migrate. It usually takes between one and three hours for gel to run- that is, for the samples to reach the bottom of the gel. At the end of the SDS-PAGE, the proteins are spread through the gel according to their size.

We can't see the proteins, so to visualise them, we need to stain them. Here you can see a gel stained with Coomassie Blue, a dye that will bind to all the proteins in the sample. On the left side of the gel, there are standard-sized markers that will help approximate the molecular weight of samples in rows across the gel. The standard-sized markers contain a mix of proteins of which we know the sizes. We can compare the protein we're interested in with the size of the known proteins along the side of the gel to check what molecular weight it is. From this you can see that although we've managed to separate out the proteins by size, the proteins in the standard are all at different points along the gel. There are so many different proteins in the sample that we still cannot tell which one is the one we're interested in. This is where we could use another technique called western blotting. Western blotting is the subject of the third section of this topic.