Molecular weight of unknown protein

ASSIGN BUSTER

Abstract

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used to separate protein molecules based on size. By using sodium dodecyl sulphate (SDS) and a gel made from acrylamide, protein shape, structure and charge no longer become factors as proteins migrate on to gels and protein bands are only affected by size. Together with Western Blotting (WB) both common laboratory techniques, they are used to further determine the presence of a given protein. Using this knowledge, this report will look at both techniques and also discuss how both techniques can determine the molecular weight of an unknown protein. Protein standards and an unknown protein sample will be prepared for SDS-PAGE using both a resolving and a stacker gel, and for WB analysis. The known protein standards are identified and act as markers for the unknown sample. The unknown sample migrates to a similar distance as the β- Galactosidase protein, as well as their Molecular Weights (MW) being very close together. It was concluded therefore, that the unknown protein was β- Galactosidase, but also both techniques, SDS-PAGE and WB are useful tools when determining the molecular weights of unknowns, but also identifying them too. Both variations of the same purpose were successful, however, it can be said that the SDS-PAGE can give a more quantitative and definite outcome, based upon the results in this report.

Introduction

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) is the most commonly used laboratory technique to separate proteins. It involves applying an electric current to a polyacrylamide gel matrix, allowing the proteins to migrate through the matrix. However, in order for proteins to migrate onto

the gel at a similar rate, or at all, the use of the detergent SDS is required in order to denature the proteins, which do so in the presence of SDS. Proteins become negatively charged, as they bind to SDS, forming a protein-detergent complex. Approximately 1. 4 g of SDS becomes bound to per gram of protein, giving the denatured proteins a similar charge to mass ratio. Therefore, since the denatured proteins no longer have a complex tertiary shape, but form similar rod-like structures and they all have a negative charge, differences in shape and charge are no longer factors for separation in the gel. Consequently, the rate at which the SDS coated proteins migrate in the gel is purely dependent on size.

Previous studies have involved using SDS-PAGE to separate urinary proteins by MW (1). The SDS-PAGE in this experiment was also carried out following the procedure described by Laemmli, who carried out an experiment using a stacking gel and SDS (2). Laemmli’s system has shown to be the most popular discontinuous buffer system. SDS-PAGE involves the use of discontinuous gels, consisting of a resolving or separating gel as well as a stacking gel. The resolving lower gel is where the proteins migrate to and separate into different sizes. The quantity of acrylamide in the resolving gel defines how well the separation takes place since higher concentrations of acrylamide restrict the mobility of proteins. The stacking upper gel is where the proteins are loaded and acts to concentrate large sample volumes, producing better band resolutions, compared to a gel with no stack. The difference in pH is one of the reasons why the proteins move from the stacking gel to the resolving gel, since the pH causes the molecules that were blocking the proteins moving through the stack, to separate, therefore

freeing the proteins to move down to the resolving gel. A molecular weight marker such as a known protein or standard that produces bands of known size, migrates to the resolving gel, and is used to help identify unknown proteins (1).

Western Blotting (WB) is a powerful and important procedure which follows successful electrophoresis for the detection of proteins, specifically those in low quantities (3). WB allows the transfer of proteins to a nitrocellulose membrane. Using the same principles as SDS-PAGE, an electric current is also used, but is applied at 90° to the gel, allowing the proteins to migrate out of the gel onto the membrane. WB can now begin to detect a target protein in a sample by using an antibody specific to that protein. Thus, revolutionising the immunological field, since prior to WB, the separated probes in the gel were difficult to access with molecular probes (3).

Therefore in this experiment, it was sought to determine the MW and identity of the Unknown protein using the two different techniques mentioned earlier, SDS-PAGE and Western Blotting.

Methods

SDS-PAGE Preparation

A 7. 5% resolving gel monomer solution was prepared combining 4. 85ml of Distilled Water; 2. 5ml of 1. 5M Tris-HCL (pH8. 8); 100µl of 10% (w/v) SDS stock and 2. 5ml of 30% Acrylamide/Bis. A comb is placed in the assembled gel sandwich and then carefully removed. 50µl of 10% ammonium persulfate and 5µl of TEMED were added to the monomer solution and mixed well, avoiding aeration. The solution was then poured to within 1cm of the comb.

The gel was overlain butanol and left to polymerise. These steps to create a discontinuous buffer system are that of Laemmli (2), and were already completed by staff. The preparation of the 4% stacking gel was performed by the students, and involved combining 6. 1ml of Distilled water; 2. 5ml of 0. 5M Tris-HC1 (pH 6. 8); 100µl of 10% SDS; 1. 3ml of 30% Acrylamide/Bis; 50µl of 10% ammonium persulfate and 10µ of TEMED. After the butanol had been removed from the top of the gel and the gel had been washed, it was filled with stacking gel. The comb was inserted and left to polymerise for 45-60 minutes. After this time, the comb was removed and the gel rinsed through with distilled water.

Standard Sample and Unknown Protein Sample Preparation

2 tubes containing pre-stained protein standards mixed with sample buffer (0. 01M phosphate (pH 7. 2) containing 1% SDS, 10% glycerol, 0. 002% bromophenol blue and 0. 01M mercaptoethanol) in one, and pre-stained Unknown protein sample mixed with the same sample buffer in the other were given and 10µl of each sample were removed and placed in separate micro centrifuge tubes. The tubes were incubated in a boiling water bath for 3 minutes and then the contents of the tube containing the protein samples was added to 1 well, whilst the contents of the tube containing the unknown protein was added to another well. The gels were run at 200V, until the bromophenol blue band was 1cm from the bottom of the gel, which took approx. 55 minutes. After the electrophoresis was complete, the lining membrane was removed from the sandwich and the glass plates were lifted away from the gel. The gels were measured and then placed in transfer

buffer for 10 minutes. The proteins standards and their molecular weights were identified, calculated and given to the students.

Western Blotting

Nitrocellulose paper, scotch-brite fibre pads and filter paper, were cut to the size of the gel, and the latter two were saturated with transfer buffer. The nitrocellulose should be touched to the buffer and allowed to wet by capillary action. The Trans-Blot cell assembly was set up by placing a fibre pad onto the grey side of the gel holder, followed by a saturated piece of filter paper; the pre-equilibrated SDS gel and the nitrocellulose sheet. Air bubbles were removed before and after a further piece of saturated filter paper was placed on top of the nitrocellulose. Finally, a saturated fibre pad was placed on top of the filter paper. The gel holder was securely closed and placed in the half-filled Trans-Blot tank so that the grey panel of the holder was in the cathode side of the tank. The buffer tank was then filled to the bottom of the anode disk before electroblotting at 30V and 400mA, took place overnight at room temperature.

Probing the Membrane

At the next session, membranes were placed in the staining bath and were covered with ~20mls of Blocking solution for 10 minutes. Then, this was poured away and 5ml of Antibody solution was added to the membrane and it was incubated for 30 minutes at room temperature. The membrane was then washed three times in 10ml TTBS, before adding 5ml of anti-mouse IGG Alkaline Phosphatase Conjugate, and incubated at room temperature for a further 30 minutes. The membrane was washed as before, prior to 10ml of

colour development solution was added to the membrane and incubated at room temperature in the dark. After 5-20 minutes, colour development should be complete, and as a result the membrane should be rinsed with distilled water to stop the reaction. The membrane should be placed on filter paper to dry before analysing it.

Results

SDS-PAGE

Following SDS-PAGE, and the removal of the gel, protein standards were identified and the known Molecular Weights (Fig. 1), measured in kilo Daltons (kDa), were calculated and given to the class. The six standard proteins (Table 1) were identified as: Myosin (200 kDa), a motor protein found in eukaryotic tissues, and plays a key role in muscle contraction in the striated and smooth muscle cells along with actin; β-Galactosidase (116 kDa) a hydrolase enzyme, which catalyses the hydrolysis of β-galactosides into monosaccharides and an essential human body enzyme which is also produced in E. coli; Phosphorylase β (97 kDa) a contractile protein found in rabbit muscle; Albumin – Bovine (66 kDa) or Bovine Serum Albumin (BSA) is a serum albumin protein, which has many functions in laboratory techniques, such as ELISAs, Immunoblots and Immunohistochemistry, as well as being commonly used to determine the quantity of other proteins, by comparing an unknown quantity of protein to known amounts of BSA; Albumin – Chicken Egg (45 kDa) is a water soluble protein, but the best known form of albumin is egg white; and Carbonic Anhydarse (29 kDa) is an enzyme which catalyses the rapid conversion of CO2 into bicarbonate and protons and is responsible for the carriage of CO2 out of tissues. Table 1 also shows the calculation of

MW into log10, based upon kDa weights, and not Da weights. The distance the protein bands travelled on the gel were measured in centimetres (cm) (Table 1), as well as the distance the Unknown protein migrated on the gel, which was 1. 1cm. Protein standards measured the following distances: Myosin – 0. 4cm; β-Galactosidase – 1. 0cm; Phosphorylase β – 1. 3cm; Bovine Albumin – 1. 8cm; Chicken Albumin – 2. 3cm and carbonic Anhydrase – 3. 8cm.

Figure 1 shows the SDS-PAGE for both the standards and the unknown. The migration of the unknown was found to be similar to β-Galactosidase. Figure 1 allows us to estimate the molecular weight similar to that of β-Galactosidase, at 116 kDa, whereas Graph 1 allows a more quantitative analysis. It shows that the log10 MW of the unknown protein was approximately 2. 03. Removing log10, it can be calculated that the MW of the unknown protein was therefore, 107. 2 kDa. This MW is very close to that of β-Galactosidase, and therefore, β-Galactosidase must be the Unknown protein.

Western Blotting

The transfer of proteins from the polyacrylamide gel to the nitrocellulose membrane after SDS- PAGE analysis can be seen in Figure 2. The blue bands seen are the protein bands that have been transferred. The Western Blot involved membrane blocking, in order to reduce non-specific protein interactions between the membrane and the antibody. This was done by placing the membrane in BSA. The membrane was then incubated with the primary antibody, which is specific for the protein of interest, and we now

know that that protein was β-Galactosidase. The membrane was rinsed to remove any unbound primary antibody and then incubated with the secondary antibody, which binds to the primary antibody. The secondary antibody is modified in such a way, that it will bring about a reaction and give colour, allowing for visual identification of protein. Figure 2 shows the dark colour production and when analysed against the blue bands, it is clear that the unknown antibody had been identified and therefore the protein had been identified, due to the migration distance, which was similar to β-Galactosidase.

TABLE 1:

Identified Protein Standards and their Molecular Weights

Protein Standards

M W

(kDa)

Log10 MW

Distance

Migrated (cm)

Myosin

200

2. 3

0. 4

β-Galactosidase

116

2. 06

1. 0

Phosphorylase β

97

1. 99

1. 3

Albumin – Bovine

66

1. 82

1. 8

Albumin – Chicken Egg

45

1. 65

2. 3

Carbonic Anhydrase

29

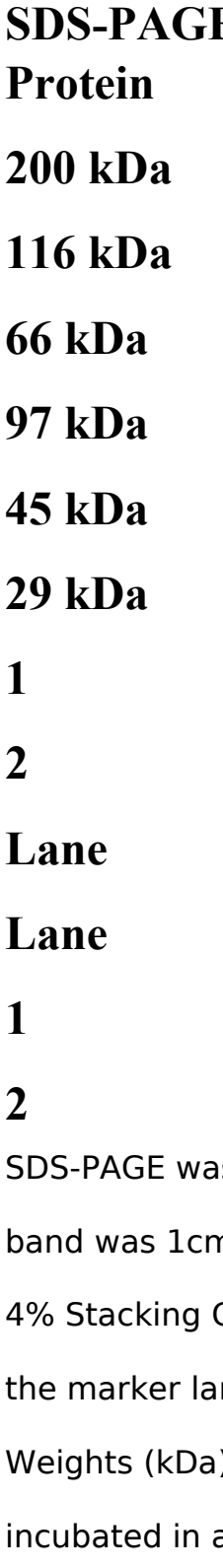
1. 46

3. 8

Table shows the known molecular weight (kDa), calculation of log10 molecular weights of the identified standard proteins (n= 6), and the distance protein bands migrated on the gel (cm) which were measured before the Western Blot procedure.

FIGURE 1:

SDS-PAGE image of Protein Standards and Unknown Protein



SDS-PAGE was run at 200V for approx. 55 minutes as the bromophenol blue band was 1cm away from the bottom of the gel (7. 5% Separating Gel and 4% Stacking Gel). Lane 1 shows bands for the Unknown Protein and Lane 2 is the marker lane, showing all bands of the Protein Standards. Molecular Weights (kDa) are highlighted on the side of the arrows. Both samples were incubated in a boiling water bath for 3 minutes, so as to denature the proteins and form the SDS complexes, so that they could migrate onto the gel. The Unknown protein band migrated to a similar point to the second

standard protein, at approx. 116 kDa, hence the unknown protein must be β-Galactosidase.

GRAPH 1:

Log10 MW and Migration Distance of Protein Standards

Myosin –

0. 4cm

β-Galactosidase –

1. 0cm

Phosphorylase β –

1. 3cm

Albumin – Bovine –

1. 8cm

Albumin – Chicken Egg – 2. 3cm

Carbonic Anhydrase – 3. 8cm

The graph shows the results of the SDS-PAGE. The chart at the side represents each of the Standards individually. The Log10 MW of Standards is shown against the Distance Migrated on the polyacrylamide gel allowing the MW of the unknown protein to be calculated. The distance travelled of the unknown protein was 1. 1cm, therefore, the log10 MW is approximately 2. 03. This is very similar to the log10 MW for β-Galactosidase, which is 2. 06. Again, indicating that the unknown protein is β-Galactosidase.

FIGURE 2:

Western Blot Analysis of Standard and Unknown Sample Separated by SDS-PAGE

Lane

1

2

The Western Blot was prepared with 5ml of a primary and secondary antibody – 5ml of anti-mouse IGG Alkaline Phosphatase Conjugate. Colour development occurred in approximately 20 minutes. Lane 2 shows the reaction of the second antibody to give a change in colour, and Lane 1 contains the proteins from the gel which transferred on the nitrocellulose membrane, and act as MW markers.

Discussion

In this experiment, it was concluded that the unknown sample was β-Galactosidase. This protein is a hydrolase enzyme, which catalyses the hydrolysis of β-galactosides into monosaccharides and is an essential human body enzyme. In E. coli, β-galactosidase is produced by activation of the lac operon, as the lacZ gene.

The SDS-PAGE technique highlighted the standard proteins very well, making any comparison very easy. Using the SDS-PAGE technique gave more of a definite result, as the MW of the unknown could be measured and read off the graph, whereas in the WB, it was a lot more difficult to define what the colour change represented. Only after several comparisons and careful

analysis of the western blot, could it be said that the antibody for the protein, β-galactosidase, was in fact the correct one.

Although the correct result was identified, using the two techniques; the experiment could be improved with more practice, and also perhaps doing more than one gel, or western blot. As well as this, the gel could have been run more slowly and carefully, as there were some handling errors.