THIN LAYER ISOELECTRIC FOCUSING OF FISH PROTEIN

NG CHER SIANG

INTRODUCTION

Proteins are made up of many units of amino acids. All the amino acids have both positive and negative charges in their dissociated form. The net charge of a protein molecule in a given media is either positive, neutral or negative. These charged states are determined by the dissociation characteristics of the component amino acids in that protein. Under the influence of an electric field, the differently charged proteins will move towards the point of neutrality within the media. This point of neutrality is called the isoelectric point of that protein.

APPARATUS

The apparatus described in this procedure include the electrophoretic chamber (LKB 2117 Multiphor II), power supply (LKB 2197 Power Supply) and the water bath (LKB 2219 Multitemp II).

REAGENTS

1. **Fixing solution**

Methanol (MeOH) .... 150 ml
Distilled water .... 350 ml
Sulfosalicylic acid, dihydrate ($C_7H_6O_6S.2H_2O$) .... 17.25 g
Trichloroacetic acid (CCl_3COOH) .... 57.50 g

2. **Destaining solution**

Ethanol (C_2H_5OH) .... 500 ml
Acetic acid (CH_3COOH) .... 150 ml
and dilute to 2,000 ml with distilled water.

3. **Staining solution**

Coomasie Brilliant Blue R-250 .... 0.345 ml
Destaining solution .... 300 ml
Stand for 48 hours and filter before use.

4. **Preserving solution**

Destaining solution .... 500 ml
Glycerol ($C_3H_8O_3$) .... 50 ml

5. **Phosphate buffer (pH 7.0), 0.01 M**

1.79 g Sodium phosphate, dibasic $Na_2HPO_4.12H_2O$ in 300 ml distilled water. Add 1M H_3PO_4 dropwise to adjust to pH 7.0 and dilute to 500 ml with distilled water.

6. <u>Anode buffer</u>

1M H_3PO_4

7. Cathode buffer

1M NaOH

8. Kerosene

9. Thin layer polyacrylamide gel

Polyacrylamide gel concentration (T)	= 5%
Degree of cross linking (C)	= 3%
Ampholine concentration	= 2.4% (w/v)
Gel dimension 245 X 110 X 1 mm	

Commercially prepared gels are used as a matter of convenience. For laboratory preparation of gel, refer to LKB Handbook.

SAMPLE PREPARATION

1) Homogenise approximately 1 g fish muscle with 10 ml distilled water or phosphate buffer (pH 7.0).

2) Centrifuge homogenised sample at 10,000 rpm for 10 mins (0°-10°C).

3) Collect supernatant for use. Supernatant can be frozen until required.

PROCEDURE

Setting the electrophoresis apparatus

1) LKB 2219 Multitemp II

Set the cooling water bath between 5°C and 10°C.

2) LKB 2197 Power supply

Set Power at constant of 25W
Set Current at maximum (50mA)
Set Voltage at maximum (1500V)

Set the apparatus to run at constant power for maximum electrofocusing efficiency.

Isoelectric focusing

1) Switch on the cooling unit.

2) Place the paper template provided on the cooling plate.

3) Adjust the position of the electrodes (attached to the electrode holder) to the appropriate width.

4) Apply a layer of kerosene on the template for good contact.

5) Place the polyacrylamide gel on the template, with the plastic base in contact with the paper template.

   CAUTION

   If the gel is wrongly placed, and has been in contact with kerosene, **DO NOT USE**. The kerosene may catch fire under the high voltage during the run.

6) Wet a paper wick with the anode buffer, and separately wet another wick with the cathode buffer. Wet the wicks in excess buffer and blot away the excess buffer.

7) Place the wet wicks on the anode and cathode ends of the gel.

   CAUTION

   Wicks placed wrongly will cause short circuit and is very dangerous. Always check before placing the wicks on to the gel.

8) Wet the applicator strips with the protein solution and place on the gel at about two thirds distance from the anode end of the gel.

9) Connect the electrodes to the power pack (for anode, use the outer port).
   See Precaution 1.

10) Run for 30 mins., recording the voltage and current every 5 mins. **See Precaution 2.**

11) Stop the run by switching off the power pack. Remove the connection between the power pack and the gel chamber before opening the chamber cover.

12) Using forceps remove the applicator strips carefully.

13) Resume the run for 60 mins. **See Precaution 3.** Stop the run as before.

14) Remove the two strips of wicks and gels. Cut one corner of the anode end to mark the direction of the anode.

15) To proceed with the pH measurement, cut off a strip of the gel before fixing the main gel (see later section).

16) Transfer the gel to fixing solution. Keep for 45 mins.

17) Drain off the fixing solution. Rinse twice with destaining solution. Soak gel in destaining solution for 45 to 60 mins.

18) Drain off destaining solution. Introduce preheated stain (50°C). With gentle agitation, stain for 5 mins. **See Precaution 4**.

19) Discard stain. Rinse twice with destaining solution. Soak gel in destaining solution at room temperature, with occasional agitation. Destain overnight. **See Precaution 5.**

20) The end point is when the background of the gel is clear. Remove destaining solution, and replace with preserving solution. Keep for 1-2 hours.

21) When the gel is satisfactorily destained, tape the gel and plastic support onto a preheated glass surface. Dry at 50°C for 45 mins. The gel should not be too dry.
See Precaution 6.

22) Carefully place the protective cover over the gel, ensuring no trapped air bubbles. The gel is very sticky, and once the mylar sheet comes into contact with the gel, it cannot be removed without destroying the gel.

DETERMINATION OF THE pH GRADIENT

After the focusing and removal of the wicks, cut off a strip of the gel, approximately 1.5 cm broad breadthwise (i.e. from + to – ends). The small strip of the gel is overlaid onto a piece of graph paper. It is cut into 19 or 20 portions, each close to 0.5 cm. Each of these strips is placed in 10 ml of CO_2 free distilled water. It is covered and left to stand for an hour or so. Before measuring the pH, it is given a vigorous shaking, using a vibrator. The pH is then measured.

The distribution of the pH gradient by the ampholine is such that the anode end is acidic and the cathode end is basic. The pH gradient can be plotted on the graph provided.

PRECAUTIONS

1) Care should be taken when handling the unit. Always remove the connection between the power pack and the gel chamber before opening the chamber.

2) After setting the unit to run, keep an eye on the gel to ensure that the high voltage does not cause the gel to burn. If it happens, stop the run immediately.

3) Observe the cover placed directly over the gel for presence of condensation during the run. If much condensation occurs, it is better to stop the run temporarily, and wipe away the excess moisture before continuing. The usual procedure of disconnecting the power pack applies.

4) The stains should preferably be filtered before use. The temperature set at 50°C ensures rapid staining.

5) During destaining, it is necessary to agitate occasionally to ensure good de-staining. The destaining time is not fixed, but depends on each case.

6) During drying of the gel, the plastic support should be firmly taped to a good support to prevent it from buckling.

REFERENCE

Anders Winter (1977) : Analytical Electrofocusing in Thin Layers of Polyacrylamide Gels, LKB Application Note 250