Isoelectric Focusing (IEF) of Membrane Proteins

Introduction

Electrofocusing principle

Electrofocusing separates proteins on the basis of surface charge alone as a function of pH. The separation is done in a non-sieving medium (sucrose density gradient, agarose, or polyacrylamide gel) in the presence of carrier ampholytes, which establish a pH gradient increasing from the anode to the cathode. Since a protein contains both positive (amines) and negative (carboxyl) charge-bearing groups, the net charge of the protein will vary as a function of pH.

A pH gradient is established concomitantly with protein separation. As the protein migrates into an acidic region of the gel, it will gain positive charge via protonation of the carboxylic and amino groups. At some point, the overall positive charge will cause the protein to migrate away from the anode(+) to a more basic region of the gel. As the protein enters a more basic environment, it will lose positive charge and gain negative charge, via ammonium and carboxylic acid group deprotonation, and consequently, will migrate away from the cathode (-). Eventually, the protein reaches a position in th pH gradient where its net charge is zero (defined as its pI or isoelectric point). At that point, the electrophoretic mobility is zero. Migration will cease, and a concentration equilibrium of the focused protein is established.

Methodology

This method was designed for use with the Biorad Model 111 Mini IEF cell. For details on assembly and pouring of gels, consult the Instruction Manual, filed in the filing cabinet beside freezer #3, bottom drawer, under "Operation Manuals".

Gel Composition

- Polyacrylamide (T= 25% ; C= 40%) (5% T final ; 0.8%C)
- 2% detergent (TX 100)
- 2% ampholytes (stock =40%)

(you may also use up to 7.5% total monomer)

Stock Solutions

Polyacrylamide Stock

- (25% T/C = 40%)
- 24 g acrylamide (w/v)
- 1 g bis (w/v) to 100ml with dH2O

10% APS-100mg APS in 1ml H2O

TEMED

--use neat

TX 100 (10%) 1ml in 10 ml H2O (heat to dissolve) ampholytes (40%) from Biorad (these come in various pH ranges depending on the pI of the protein you are studying; if you don't know the pI then pH3/10 range is a good start.)

GEL POURING SOLUTION for 5ml (one IEF gel)

acrylamide stock	1mL
ampholytes (40%) 3/10	0.25mL
H2O	to 5mL

degas for 5 min., then add:

- 35(APS 10%
- 2.5(TEMED)

After the gel has polymerized, soak it in 10% Triton-X100 for 30 minutes. (Including the Triton in the gel mixture interferes with adherence of the gel to the hydrophilic backing.)

Running conditions:

- 15 min at 100 volts
- 15 min at 200 volts
- 60 min at 450 volts

(stepped increases in voltage prevent overheating and dehydration of the gel)

Gel processing:

1. fix for 15 min in

4% sulfosalicylic acid	10g
12.5% trichloroacetic acid	31.25mL of 100%
30% methanol	75mL
	250mL

2. stain for 30 min in:

27% isopropanol or ethanol	135mL
10% acetic acid	50mL
0.04% Coomassie R250	0.2g

0.5% CuSO4	2.5g optional
	500mL

(cupric sulfate helps to reduce the background staining of ampholytes; if you use it, dissolve it in water before adding the alcohol)

3. destain for 30 min in:

12% isopropanol or ethanol	60mL
7% acetic acid	35mL
0.5% CuSO4	2.5g optional
	500mL

Sample Preparation

Translate molecules in the presence of CRMs (canine rough microsomes) (see Translocation) separate RMs over CL2B column by collecting void volume. (see Separating membrane targeted molecules by gel filtration) label with sufhydryl modifying reagents. (see Labelling proteins with sulfhydryl modifying reagents) spin out RMs over equal volume physiological cushion, at 10psi for 5 minutes in the airfuge. resuspend in solubilization buffer (aim for 10eq of CRMs in 20(SB ;this gives ~ 2.5(g/(of total protein ; loading 2(will give 1eq of RMs which is equivalent to ~ 5(of T2 reaction.) load 0.5-2ml of sample (see Notes below). focus at 100V for 15 min ; 200V for 15 min; 450V for 60 min. use BIORAD IEF standards as PI markers

Solubilization Buffer (SB)

(1ml)

3% Triton X-100	300 (10% stock)
2% ampholytes	50(I (40% stock)
20mM DTT	20(I (1M stock)

Notes: tips for sample loading:

-if you use the sample templates you may load up to 2 ml of sample but I found that there is a lot of diffusion this way and the gels look rather messy -you can make small wells (hold up to 1 ml) by putting small rectangular pieces of tape on the casting tray; this works much better

Troubleshooting: see Mina