Rabbit Creatine Kinase Purification

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Sample Preparation

Steps Group Measurement Required Measurement

I-Crude Extract Preparation

1. 40-50g back and leg muscles of a **rabbit**
2. Blend with water
3. Homogenize sample
4. Stir 15 minutes at room temp(~20-26°C)
5. Prepare two 50mL centrifugal tubes (51.5mL each tube)
6. Centrifuge at 10,000rpm for 10 minutes, 0°C
7. Keep supernatant, discard precipitate
8. Transfer supernatant to microcentrifuge tubes, store at -20°C (27.501)

II-Ammonium Sulfate Precipitation

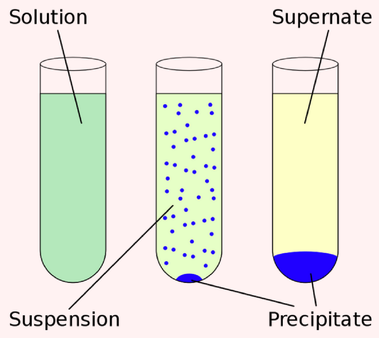
1. Add solid NH_4Cl to fraction 1 and stir (0.27g)
2. Adjust pH using NH_4OH until pH of 9.0 is achieved(16:09 o'clock)
3. Place sample in an ice bath
4. Gradually add in $(NH_4)_2SO_4$ (17.597g)
5. Stir until completely dissolved
6. Centrifuge at 10,000rpm for 10 minutes, 0°C to remove precipitated protein(56.8mL after)
7. Retain supernatant (S1)
8. Transfer S1 to a 250mL beaker, add more $(NH_4)_2SO_4$ to S1, stir until completely dissolves (5.827g)
9. Retain precipitate(P2) and discard supernatant(S2)
10. Add 1/2.5 volume of V1 20mM Tric-HCl to P2 until fully dissolved(Becomes V2)
11. Measure volume of V2(22.3mL)
12. Store in multiple microcentrifuge tubes(0.2mL per tube) at -20°C

III-Purification using Ethanol

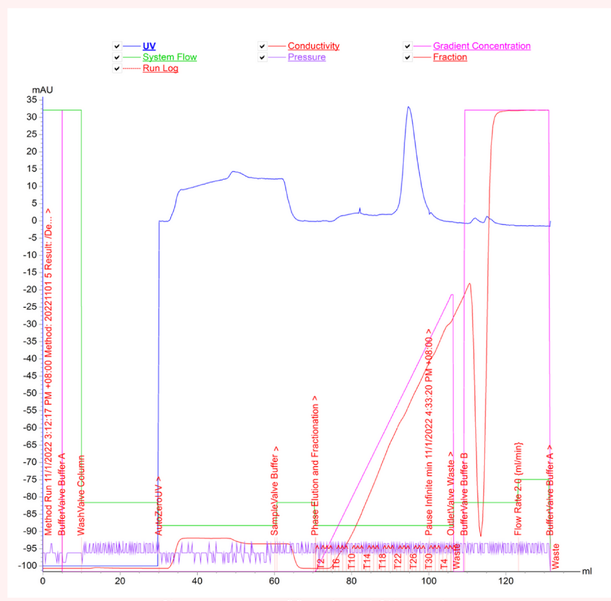
1. Slowly add 1.2x (volume of V2) of ice-cold ethanol to V2(containing 2mM β-mecapthoethanol)
2. Use stirring plate at low speed for 20 minutes, 20°C
3. Centrifuge at 10,000rpm for 10 minutes at -8°C
4. Retain supernatant (S3) and discard precipitate
5. Measure volume of supernatant 3(VS3):(41.6mL)

IV-Precipitation and Fractional Extraction

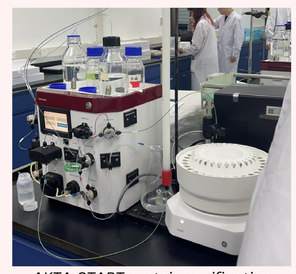
1. Add 0.5M of $MgSO_4$to S3 surrounded by an ice bath (3.617g)
2. Stir at low speed for 20-30 minutes
3. Centrifuge at 12,000rpm for 10 minutes, -8°C
4. Retain precipitate **(CK)**, discard supernatant
5. Store in -20°C



Ion Exchange Chromatography



Chromatogram of fractioned CK samples



AKTA-START protein purification

The protein requires anion exchange chromatography. We attached an anionic gel filter to the AKTA Start to assist in the purification process. Until about 116 ml, the net charge of the protein is below the isoelectric charge. Proteins bind to the gel column and begin elution at 70ml. Isoelectric charge increases at a steady rate, therefore giving each extracted sample the same protein concentration. A total of 40 protein solution samples were collected. We retained the first 20 and cut that down as well to the first 10. We used these as our samples in the later SDS-Page and gel electrophoresis experiments.

Gel Filtration Steps

1. Prepare 50mM phosphate buffer saline
2. Prepare AKTA-Start
3. Clean/prep the machine
   a. Wash buffer A (20mL)
   b. Wash buffer B (20mL)
   c. Wash fractionation tubing (10mL)
   d. Extrude air bubbles in tubing (flow rate: 5mL/min)
4. Connect HiTrap ion exchange chromatography column to the AKTA-Start (flow rate: 1mL/min)
5. Set equilibration to 0.5-1.0CV, flow rate: 1mL/min

Elution and Collection

1. Use buffer A to elute proteins (flow rate:0.8mL/min)
2. Eluted proteins do not need collection
3. Connect outlet valve to spinning fraction collector plate
4. Collect fractions of your desired protein
5. Equilibrate in ethanol and store at 4°C

SDS-PAGE

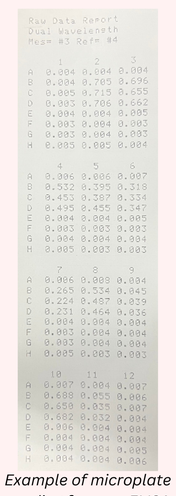
SDS PAGE (Sodium Dodecyl-Sulfate Polyacramide Gel Electrophoresis) is a method of analyzing protein in which the proteins in question are subject to SDS detergent. SDS causes the proteins to flatten out. The secondary, tertiary, and quaternary structures of the said protein and linear polypeptide chains covered in SDS molecules are produced. Proteins are then run through electrophoresis gel and are separated by size. Larger proteins above and smaller proteins below. It is only due to the application of sodium dodecyl sulfate that the proteins can be allowed to freely flow through the gel. Results can then be analyzed.

Steps

1. Separating Buffer
   a. 18.17 g Tris,
   b. 0.4 g SDS, pH 8.8 (HCl),
   c. Added H2O to 100 ml (1.5 mol/L Tris-HCl buffer)
2. Stacking buffer
   a. 6.06 g Tris,
   b. 0.4 g SDS, pH 6.8 (HCl),
   c. Added H2O to 100ml (0.5 mol/L Tris-HCl buffer)
3. 30% acrylamide
   a. 29 g Acrylamide,
   b. 1 g Bisacrylamide,
   c. Added H2O to 100ml
4. 10% w/v ammonium persulfate
   a. 0.1 g ammonium persulfate, Added H2O to 1 ml
5. Electrophoresis buffer
   a. 3 g Tris,
   b. 18.8 g glycine,
   c. 1g SDS,
   d. Added H2O to 1 liter.
6. Assemble the gel sandwichand pour separating buffer
7. Pour water
8. Pour stacking buffer
9. Place well comb in sandwich and wait for solidification
10. Dye protein samples with coomassie brilliant blue
11. Place sandwich in electrophoresis chamber, cover in prepared buffer
12. Pipette dyed proteins at desired dilutions and combinations into the wells
13. Attach positive and negative electrodes to each end and wait 30-50 minutes depending on width of gel
14. Remove gel and analyze
15. Perform western blot if necessary

Our SDS-PAGE gel electrophoresis

In the electrophoresis process, proteins being filtered



Example of microplate reading from an ELISA

CK Activity Measurement

Absorbance

CK activity is measured by the light absorbance of a protein at 595nm over time (ΔOD595nm/min).

In our experimentation, we did not have the chance to measure the activity of our creatine kinases. I will describe the process of measurement of this activity instead and provide accompanying figures.

Steps

1. Collect CK samples and centrifuge at 10,000g for 10 minutes, 4°C
2. Centrifuge will remove insoluble materialsSoluble materials can be assayed directly
3. Use serial dilution on a 96-well plate in the range form of a standard curve
4. Follow the concentration gradient of the example 96-well plate.
5. Each row has a marker as well as CK sample and buffers at different
6. concentrations
7. Put in a microplate reader and test at 595nm (595nm because of the Coomassie Brilliant Blue dye used)

	1	2	3	4	5
A	80 μL PBS	60 μL PBS + 60 μL 1/100 dilution F1	60 μL PBS + 60 μL 1/100 dilution F2	60 μL PBS + 60 μL 1/10 dilution F3	50 μL sample from A1
B	80 μL PBS	60 μL PBS + 60 μL A2 mixture (1/200 dilution F1)	60 μL PBS + 60 μL A3 mixture (1/200 dilution F2)	60 μL PBS + 60 μL A4 mixture (1/20 dilution F3)	50 μL sample from B1
C	60 μL PBS + 20 μL 0.1 mg/mL BSA	60 μL PBS + 60 μL B2 mixture (1/400 dilution F1)	60 μL PBS + 60 μL B3 mixture (1/400 dilution F2)	60 μL PBS + 60 μL B4 mixture (1/40 dilution F3)	50 μL sample from C1
D	60 μL PBS + 20 μL 0.1 mg/mL BSA	60 μL PBS + 60 μL C2 mixture (1/800 dilution F1)	60 μL PBS + 60 μL C3 mixture (1/800 dilution F2)	60 μL PBS + 60 μL C4 mixture (1/80 dilution F3)	50 μL sample from D1
E	40 μL PBS + 40 μL 0.1 mg/mL BSA	50 μL 1/200 dilution F1 from A2	50 μL 1/200 dilution F2 from A3	50 μL 1/20 dilution F3 from A4	50 μL sample from E1
F	40 μL PBS + 40 μL 0.1 mg/mL BSA	50 μL 1/400 dilution F1 from B2	50 μL 1/400 dilution F2 from B3	50 μL 1/40 dilution F1 from B4	50 μL sample from F1
G	80 μL 0.1 mg/mL BSA	50 μL 1/800 dilution F1 from C2	50 μL 1/800 dilution F2 from C3	50 μL 1/80 dilution F1 from C4	50 μL sample from G1
H	80 μL 0.1 mg/mL BSA	50 μL 1/1600 dilution F1 from D2	50 μL 1/1600 dilution F2 from D3	50 μL 1/160 dilution F1 from D4	50 μL sample from H1

Layout of 96-well plate for protein concentration determination

step 3; step 5; step 6; step 7; + step 9