Preparation of Gizzard Alpha-actinin and Vinculin

Preparation of Alpha-actinin

Day 1-2

Materials

1. 0.5 mM PMSF, $4^{o}C$. Need 20 ml per gram tissue. Prepare by diluting 100 mM PMSF stock in 95% ethanol.

2. Buffer A: 1 mM EGTA, 0.5 mM PMSF, 2 mM Tris-HCl, pH 9.0 at room temperature, bring to $37^{o}C$ before use, 10 ml per gram tissue.

3. Buffer B: 20 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.02% NaN_3, 20 mM Tris-acetate, pH 7.6 at $4^{o}C$, 4000 ml, or 1000 ml of 5x without DTT.

4. PBS solution A, 2000 ml, $4^{o}C$.

5. 0.5 M acetic acid for titration.

6. 3 M $MgCl_2$ stock.

7. Ultrapure (enzyme grade) ammonium sulfate.

8. DE-52 column, 2.5x20 cm.

9. Scalpels and dissection scissors.

10. Miscellaneous glassware, 2-liter graduate cylinders, 4-liter beakers.

11. Two GSA rotors and Sorvalls.

12. Meat grinder, prechilled in a cold room and rinsed with 20 mM EDTA, pH 7.0 immediately before use.

13. High salt (e.g. 3 M KCl) for soaking electrode.

14. Polyron with prechilled large generator.

Procedure

1. Wash/rinse chicken or turkey (preferred) gizzards in cold PBS. Remove connective tissue with a scalpel. A typical preparation uses about 300 g (to fit into 2 GSA rotors). Extras can be stored frozen at -80°C. To use frozen gizzards, thaw for 1-2 hr in cold PBS at room temperature until the gizzards become pliable.

2. Pass dissected gizzards twice through a meat grinder, using first the coarse then the fine mesh. Weigh the tissue.

3. Homogenize with Polytron 3 times 10 sec bursts, in 10 volumes of 0.5 mM PMSF. Do this in an ice bucket. Adjust the speed to obtain the maximal vortex.

4. Centrifuge in a GSA rotor at 10,000 rpm, 4°C for 10 min. Discard supernatant and floating lipids.

5. Resuspend pellets in 10 volumes of 0.5 mM PMSF. Homogenize in a Waring blender for 15 sec at low speed.

6. Centrifuge in a GSA rotor at 10,000 rpm, 4°C for 15 min. Warm up rotor after the spin.

7. Discard supernatnat. Wipe lipid off the centrifuge bottle. Resuspend pellets in 10 volumes of buffer A at 37°C in a 4-liter beaker. Extract with gentle, constant stirring in water bath at 37°C for 1 hr.

8. Centrifuge in a GSA rotor at 7,000 rpm at room temperature for 10 min.

9. Collect supernatnat and measure volume. Titrate supernatant to pH 7.0-7.2 with 0.5 M acetic acid.

10. Bring the concentration of $MgCl_2$ to 10 mM. Use 3 M stock and add 1/300 of the volume measured in step 9. Stir at room temperature for 15 min.

11. Centrifuge in a GSA rotor at 7,000 rpm, room temperature for 10 min. Cool down the rotor to 4°C after this spin.

12. Carefully collect supernatant into a 4 liter beaker sitting on ice, avoid bubbles.

13. Bring ammonium sulfate to 40% saturation. Need 22.6 grams per 100 ml of volume in steps 9 and 10. The crystals should be added slowly with constant stirring, while monitoring and maintaining pH at 7.0-7.2. Soak the electrode in high salt after this step.

14. Let stand on ice for 45 min after all ammonium sulfate has dissolved.

15. Centrifuge in a GSA rotor at 7,000 rpm, 4°C for 15 min.

16. Resuspend pellets in 20-30 ml buffer B. Dialyze for 36 hr against 1 liter of buffer B, change buffer every 12 hr.

17. Equilibrate the DE-52 column with buffer B.

Day 3-4

Materials

1. Buffer B, 1500 ml, degased.

2. Buffer P: 0.2 mM DTT, 0.02% NaN_3, 50 mM (KH_2PO_4+K_2HPO_4), pH 7.0 at 4°C, 5 liters.

3. Hydroxylapatite column (BioRad DNA grade Bio-Gel HTP), 2.5x40 cm. The size is important since HTP has a limited binding capacity.

4. Gradient maker, fraction collector and UV monitor.

Procedure

1. Collect solution from the dialysis bag. Centrifuge in a 50.2Ti rotor at 45,000 rpm, 4°C for 45 min.

2. Apply to the DE52 column equilibrated in buffer B.

3. Wash column with 100-200 ml buffer B or until OD drops to baseline.

4. Elute with 300 ml 20-370 mM NaCl gradient. Fill the mixing side of the gradient maker with 155 ml buffer B, the reservoir side with 145 ml buffer B with 3.9 g NaCl. Collect 3 ml fractions at 20-25 ml/hr. Set monitor full scale at OD 2.0, chart speed 1.5 cm/hr. Wash the column with 1 liter of 2 M NaCl in buffer B when finish.

5. Locate peak fractions. Run SDS-PAGE of various peaks. Pool vinculin fractions near the beginning of the gradient and follow vinculin preparation protocols.

6. Pool alpha-actinin fractions towards the end of the gradient. Dialyze against 2 liters of buffer P for 24 hr. Change buffer at 12th hr.

7. Equilibrate the hydroxylapatite column with buffer P.

Day 5-6

Materials

1. Buffer P, 2000 ml, degased.

2. Buffer B, 5000 ml.

3. 1 M (KH_2PO_4+K_2HPO_4), pH 7.0, 100 ml.

4. Ultrapure (enzyme grade) ammonium sulfate.

5. Sepharose 6B-CL column, 2.5x90 cm.

6. Gradient maker, fraction collector and UV monitor.

Procedure

1. Collect solution from the dialysis bag. Centrifuge in a 50.2Ti rotor at 35,000 rpm, 4°C for 2 hr.

2. Apply to the hydroxylapatite column equilibrated in buffer P. Wash with 50-100 ml buffer P or until OD drops to baseline.

3. Elute with 500 ml 50-300 mM phosphate gradient. Fill the mixing side of the gradient maker with 255 ml buffer P, reservoir side with 180 ml buffer P and 65 ml buffer 3. Elute at 20 ml/hr. Collect 4 ml fractions. Set monitor full scale at OD 2.0, chart speed 1.5 cm/hr. Wash the column with 1 liter of 500 mM phosphate in buffer P when finish.

4. Locate peak fractions and run SDS-PAGE. Pool alpha-actinin containing fractions (central peak). Measure volume.

5. Precipitate with 45% saturation ammonium sulfate (26 g per 100 ml). The crystals should be added slowly with constant stirring, while monitoring and maintaining pH at 7.0-7.2. Soak the electrode in high salt after this step.

6. Let the solution stand on ice for 30-45 min after all ammonium sulfate has dissolved.

7. Collect precipitates by centrifugation in a SS34 rotor at 10,000 rpm, 4°C, for 15 min.

8. Resuspend pellets in 4 ml buffer B. Dialyze against 2 liters of buffer B for 24 hr. Change buffer at 12th hr.

9. Equilibrate Sepharose 6B-CL column with 1000 ml buffer B.

Day 7-8

Materials

1. Buffer B, 2000 ml.

2. 2 mM PIPES, 0.02% NaN_3, 3000 ml.

3. Ultrapure (enzyme grade) ammonium sulfate.

Procedure

1. Collect solution from the dialysis bag. Centrifuge in a 50Ti rotor at 40,000 rpm, 4°C for 45 min.

2. Apply to Sepharose 6B-CL column equilibrated in buffer B.

3. Elute with maximum pressure. Collect 3 ml fractions. Set monitor full scale at OD 2.0, chart speed 1.5 cm/hr.

4. Run SDS-PAGE of peaks to locate alpha-actinin (central peak). Pool fractions and measure volume.

5. Concentrate with CentriPrep or with ammonium sulfate precipitation: precipitate alpha-actinin with 45% saturation ammonium sulfate (26 g per 100 ml; the crystals should be added slowly with constant stirring, while monitoring and maintaining pH at 7.0-7.2. Soak the electrode in high salt after this step), let the solution stand on ice for 30-45 min after all ammonium sulfate has dissolved, and collect precipitates by centrafugation in a SS34 rotor at 10,000 rpm, 4°C, for 15 min.

6. Resuspend pellets in 3 ml of 2 mM PIPES with azide. Add DTT to 5 mM. Dialyze against 1000 ml of 2 mM PIPES. Change buffer twice over a period of 24 hr.

7. Clarify in a 50Ti rotor at 40,000 rpm, 4°C, for 45 min.

8. Store in liquid nitrogen as aliquots. Stable for months.

Preparation of Vinculin

Day 1-2

Materials

1. Buffer C: 20 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.02% NaN_3, 20 mM NaAc, pH 5.0 at 4°C, 5000 ml, or 1000 ml 5x without DTT.

2. 5 mg/ml PMSF, 5 mg/ml leupeptin in 95% ethanol, 0.3-0.5 ml.

3. CM-52 column, 1.5x40 cm.

Procedure

1. Carry through alpha-actinin preparation until day 3-4 step 5. Measure the volume of vinculin-containing fractions.

2. Add PMSF and leupeptin very slowly, while stirring, to a final concentration of 0.1 mg/ml.

3. Dialyze against 2 liters of buffer C for 24 hr. Change buffer at 12th hr. The solution turns cloudy transiently as it passes across the isoelectric point of vinculin.

4. Equilibrate CM-52 column with buffer C.

Day 3-4

Materials

1. Buffer C, 1000 ml.

2. Ultrapure (enzyme grade) ammonium sulfate.

3. 2 mM PIPES, 0.02% NaN_3 1500 ml.

4. High salt (e.g. 3 M KCl) for soaking electrode.

Procedure

1. Collect solution from the dialysis bag. Centrifuge in a 50Ti or 50.2Ti rotor at 35,000 rpm, $4^{o}C$ for 2 hr.

2. Apply to CM-52 column equilibrated with buffer C.

3. Wash with 50-100 ml buffer C or until OD drops to baseline.

4. Elute with 500 ml 200-500 mM NaCl gradient in buffer C. Fill the mixing side of the gradient maker with 255 ml of buffer C with 2.9 g NaCl, the reservoir side with 245 ml buffer C with 7.2 g NaCl. Elute at 10 ml/hr. Collect 4 ml fractions. Set monitor full scale at OD 0.5, chart speed 1.5 cm/hr. Wash the column with 2 M NaCl in buffer C when finish.

5. Locate peaks and run SDS-PAGE. Pool vinculin fractions (central peak).

6. Carefully bring pH close to 7.0 with 0.1 N KOH. Soak electrode in high salt after this step.

7. Concentrate with CentriPrep or concentrate with the ammonium precipitation method: measure volume and precipitate with 45% saturation ammonium sulfate (26 g per 100 ml;the crystals should be added slowly with constant stirring, while monitoring and maintaining pH at 7.0-7.2. Soak the electrode in high salt after this step). Let the solution stand on ice for 30-45 min after all ammonium sulfate has dissolved, collect precipitates by centrafugation in a SS34 rotor at 10,000 rpm, $4^{o}C$, for 15 min. Resuspend pellets in 300-700 µl of 2 mM PIPES with azide.

8. Add DTT to 5 mM. Dialyze against 500 ml of 2 mM PIPES. Change buffer twice over a period of 24 hr.

9. Clarify in a 50Ti rotor at 40,000 rpm, 4°C for 45 min, or in a 42.2Ti rotor at 25,000 rpm, 4°C for 20 min. Concentrate with Centricon if necessary.

10. Store in liquid nitrogen as aliquots. Stable for months. Also stable at 4°C for a couple of weeks.