Preparation of Gizzard Myosin

Day 1

Materials (all solutions at 4^{o}C)

1. 50 mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 3% (v/v) Triton-X 100, 0.2 mM DTT, 10 mM Tris-HCl, pH 7.5. Need 9 ml for each gram of tissue.

2. 100 mM KCl, 2 mM EGTA, 0.2 mM DTT, 10 mM Tris-HCl, pH 7.5. Need 9 ml for each gram of tissue.

3. Extraction buffer: 5 mM ATP, 4 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 40 mM imidazole, pH 6.8. Need 1.5 ml for each gram of tissue.

4. PBS, solution A, 2 liters (for thawing gizzards).

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

6. Scalpels.

7. Turkey gizzards, fresh or frozen at -80^{o}C. It is most convenient and efficient to handle ~200g from 4-5 gizzards.

8. GSA bottles, a lot of. Chill down on ice.

9. 1 M $MgCl_2$, 100 ml.

10. 100 mM ATP, pH 7.0, 10 ml.

11. Polytron, chill down the large generator.

12. Glass wool and funnel.

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

14. Large ultracentrafuge rotor and tubes (bottles), e.g. Type 35 or 50.2Ti or 60T.

Procedure

1. Wash/rinse turkey gizzards in cold PBS. Trim gizzards with scalpels to remove fat and connective tissue and cut gizzards into small pieces. To use frozen gizzards, thaw in a 4 liter

beaker containing cold PBS at room temperature, until the gizzards become pliable (about 1-1.5 h).

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

3. Mix tissue with 3 volumes of buffer 1 in a 2 liter beaker. Homogenize with a Polytron 4 x 15 sec. It gets very foamy but do not worry.

4. Centrifuge in a GSA rotor for 5 min at 4,000 rpm, 4^{o}C.

5. Resuspend pellets in 3 volumes of buffer 1. Homogenize with a Polytron 1 x 20 sec.

6. Repeat steps 4 and 5, then centrifuge as in step 4.

7. Resuspend pellets in 3 volumes of buffer 2. Homogenize with the Polytron 1 x 20 sec.

8. Centrifuge in a GSA rotor for 10 min at 9,000 rpm, 4^{o}C.

9. Perform steps 7 and 8 two more times.

10. Resuspend pellets in 1.5 volumes of extraction buffer. Blend with the Polytron 2 x 20 sec, then adjust the pH to 6.9. The solution is quite viscous and needs to be stirred with a glass rod. Soak the electrode in high salt after this step.

11. Stir by hand continuously on ice for 15 min.

12. Centrifuge in a GSA rotor at 12,000 rpm, 4^{o}C for 20 min.

13. Filter the supernatant through glass wool placed in a funnel. The filtrate may appear slightly turbid. Adjust the pH to 7.6 with 0.1 N KOH and measure the volume.

14. Add 1 M $MgCl_2$ dropwise while stirring the supernatant to obtain a final concentration of 150 mM $MgCl_2$ (0.177 volume of step 13). A peristaltic pump may be used. Then add 100 mM neutralized ATP to a final concentration of 2.5 mM (0.03 volume of step 13). The pH should be monitored and maintained at 7.6 with 0.1 N KOH immediately upon the addition of $MgCl_2$. Failure to do this may result in a reduced yield. Soak the electrode in high salt after this step.

15. Stir gently on ice for 10 min.

16. Centrifuged in a GSA rotor at 12,000 rpm, 4^{o}C for 10 min.

17. Collect supernatant and centrifuge in a Type 35 rotor at 31,000 rpm, 50.2Ti rotor at 29,000 rpm or 60Ti rotor at 32,000 rpm overnight.

Day 2

Materials

1. 1.5 mM EGTA, 5 mM ATP, 0.5 mM DTT, 10 mM (NaH_2PO_4+Na_2HPO_4), pH 7.6. Need ~0.5 ml for each gram of tissue.

2. 0.5 M KCl, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, 1 liter.

3. Cold distilled H_2O, 10 liters.

4. 1 M $MgCl_2$, 20 ml (from Day 1).

5. 40 ml Dounce homogenizer.

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

7. 50.2Ti or 60Ti rotor and tubes.

Procedure (performed at 4^{o}C)

1. Collect supernatant and measure the total volume. Dilute slowly with 10 volumes cold distilled water while stirring.

2. Centrifuge in a GSA rotor at 10,000 rpm for 10 min. Two loadings are required for a 200 g preparation, or set up a second Sorvall centrifuge.

3. Resuspend pellets in ~10 pellet volumes of buffer 1. Since the pellets stick tightly on the bottle, they may need to be gently teased with a glass rod then blown off with the buffer using a large-bore pipette.

4. Homogenize gently with a chilled Dounce homogenizer, using slow passage with the A-pestle.

5. Measure volume and adjust pH to 7.6 with 0.1 N KOH. While stirring and monitoring the pH, add dropwise 1 M $MgCl_2$ to obtain a final concentration of 150 mM $MgCl_2$ (volume x 0.177). Soak electrode in high salt after this step.

6. Centrifuge in a 50.2Ti rotor at 40,000 rpm (or a 60Ti at 45,000 rpm) for 2-3 h.

7. Collect supernatant and measure the volume. Dilute with 10 volumes of cold distilled water while stirring. Stir gently on ice for 10 min.

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

9. Resuspend pellets in ~10 pellet volumes of buffer 2 (see steps 3 and 4). Record the volume used.

10. Add cold distilled water while stirring to obtain a final KCl concentration of 0.1 M. Volume equals the volume of buffer 2 used in step 9 times 3.9. Stir for 15 min on ice.

11. Centrifuge in a GSA rotor at 12,000 rpm for 15 min.

12. Collect supernatant. Excessive pellet reflects a failure to maintain pH during earlier $MgCl_2$ precipitation steps. Measure volume and dilute 1:1 with cold distilled water while stirring.

13. Add 1 M $MgCl_2$ dropwise while stirring to obtain a final concentration of 10 mM (1/100 current volume) and place on ice for 1 hr. Don't panic, precipitation of myosin takes time.

14. Centrifuge in a SS34 rotor at 11,000 rpm for 15 min.

15. Resuspend pellets in buffer 2 (see steps 3 and 4). Use ~1 ml per 10 g of tissue. Dialyze overnight.

Day 3

Materials

1. 20 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 mM HEPES, pH 7.5. 1 liter.

2. 50Ti rotor and tubes.

Procedure (performed at 4^oC)

1. Clarify in a 50Ti rotor at 40,000 rpm for 1 h. Measure protein concentration by Lowry assay.

2. Dialyze against buffer 1 overnight. The solution turns cloudy due to myosin self-assembly. Store as aliquots in liquid nitrogen.
