Chapter 3

Preparation of Ca^{2+}-ATPase1a Enzyme from Rabbit Sarcoplasmic Reticulum

Jesper V. Møller and Claus Olesen

Abstract

The SERCA calcium ATPase, probably the most well-investigated membrane protein both from a biophysical and structural view, can be purified from native source in substantial quantities, making it a favorable target when conducting biochemical experiments and structure determination, e.g., by X-ray crystallography.

Key words SR calcium ATPase, Native source purification, Membrane protein

1 Introduction

The Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is an active transporter of calcium ions from the cytoplasm to the intracellular sarco/endoplasmic reticulum (SR) store, thereby maintaining the Ca^{2+} gradient across the membrane of intracellular vesicles of all cells. This process is of particular importance in muscle cells in which Ca^{2+} triggers contraction by binding troponin C, allowing the myosin-actin cross-bridge interaction that facilitates the muscle contraction. The protein belongs to the P-type ATPase family with which the exploration started in 1957 with Jens Christian Skou's discovery of the Na/K-ATPase purified from crab legs, and subsequently a Ca^{2+}-ATPase was identified in rabbit fast twitch muscles by Hasselbach and Makinose (1959). SERCA functions by an ATP hydrolysis-driven transition between two major states: E1 with a high calcium affinity (Ca_2E1) where Ca^{2+}-ions from the cytosol are bound, and an E2 state with a low calcium affinity (H_nE2), where the Ca^{2+}-ions are released to the SR lumen. The two major states are further subdivided into a number of intermediate steps to account for the phosphorylation by ATP with occlusion of the calcium ions followed by their translocation.

Maike Bublitz (ed.), *P-Type ATPases: Methods and Protocols*, Methods in Molecular Biology, vol. 1377,
DOI 10.1007/978-1-4939-3179-8_3,

Since its discovery the Ca^{2+}-ATPase has been subject to extensive investigations, which has been made possible for a number of reasons such as availability and stability both in membrane and detergent solubilized form. This has allowed detailed studies of the intermediate states by activity measurements and other assays. Furthermore, the ability to produce both tight and leaky SR vesicles and to reconstitute the ATPase in proteoliposomes have been important tools in the biophysical characterization of the Ca^{2+}-ATPase, in calcium transport/uptake and phosphorylation experiments [1].

In the following, we present a purification protocol for isolation of SR vesicles from skeletal muscles, where SERCA1a is the predominant isoform. The protocol is based on a series of differential centrifugation steps that originally was developed by Hasselbach and Makinose [2] and later refined by de Meis and Hasselbach [3]. These are detailed in the present protocol which also includes an up-to-date account with our own modifications.

The latter part of this chapter includes a description of a protocol for a mild deoxycholate (DOC) extraction based on a procedure by Meissner et al. [4] for purification of Ca^{2+}-ATPase from the SR preparation described above, in which loosely attached proteins like calsequestrin and M55 glycoprotein are removed from the microsomes. It is important to note that this procedure has the further advantage that it makes the microsomes leaky to Ca^{2+}, a circumstance that is often helpful to avoid complications arising from the formation of Ca^{2+} transmembrane gradients in the interpretation of activity experiments.

2 Materials

Prepare all solutions by using ultrapure water (18 MΩ cm sensitivity). Filtrate all solutions by 0.22 μm filters and keep all buffers and solutions ice-cold for every step of the purification procedure (very important).

2.1 Preparation of SR Vesicles

1. Two rabbits (approx. 4–5 kg each).
2. Balance.
3. Blender (a household type is adequate).
4. Hand-operated meat grinder.
5. Scalpels, scissors, tinfoil.
6. Measuring cylinder 250 mL.
7. Ice.
8. Sorvall centrifuge, Sorvall SLA-1500 rotor, and tubes.
9. Sorvall centrifuge, SS-34 rotor, and clear tubes.
10. Beckmann Coulter centrifuge, Ti-45 rotor, and tubes.
11. Beckmann Coulter centrifuge, Ti-70 rotor, and tubes.

12. Timer.
13. Automatic homogenizer apparatus with ice cooling of the homogenizing glass.
14. 5 mL Teflon homogenizer.
15. 30 mL Teflon homogenizer.
16. Buffer A: 3 L of 100 mM KCl, 2.5 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2.0 mM EDTA.
17. Solution B: 150 mL of 1 M sucrose, 50 mM KCl.
18. Buffer C: 236 mL of 120 mL 2 M KCl, 8 mL 100 mM MgATP, pH 7.0, 108 mL water. The final solution is not pH adjusted. Leave the mixture on ice for 30 min to 1 h before use.
19. Solution D: 200 mL of 50 mM KCl.
20. Buffer E: 50 mL of 0.3 M sucrose, 5 mM Hepes, pH 7.4.

2.2 Preparation of Ca^{2+}-ATPase Membranes by DOC Extraction

1. Sodium deoxycholate (*see* **Note 1**).
2. Sorvall centrifuge, SS-34 rotor, and clear tubes.
3. Beckmann Coulter ultracentrifuge, Ti-45 rotor, and tubes.
4. Beckmann Coulter ultracentrifuge, Ti-70 rotor, and tubes.
5. Timer.
6. Plastic Pasteur pipet and glass rods.
7. Automatic homogenizer apparatus with ice cooling of the homogenizing glass.
8. 5 mL Teflon homogenizer.
9. 30 mL Teflon homogenizer.
10. Extraction buffer I: 0.3 M sucrose, 0.5 M KCl, 1 mM EDTA, 10 mM Tris, 0.01 mM $CaCl_2$, 1.25 mM $MgCl_2$, pH 7.9 (we prepare a large stock and keep it in the freezer in suitable aliquots for later use).
11. Extraction buffer II: 150 mL Extraction buffer I + DOC 0.5 mg/mL × 150 mL = 60 mg and DTT 0.5 mg/mL × 150 mL = 75 mg (freshly prepared).
12. Washing buffer: 60 mL of 5 mM TAPS (pH 7.5), 0.3 M Sucrose, 0.5 M KCl, 0.5 mM $MgCl_2$, 10 μM $CaCl_2$.
13. Buffer E: 50 mL of 0.3 M sucrose, 5 mM Hepes pH 7.4.

3 Methods

3.1 Preparation of SR Vesicles

1. Kill two fasting rabbits of approx. 4–5 kg by bleeding after a blow to the neck.
2. After removal of the skin, dissect the fast twitch muscles from the back and back limbs free with a scalpel and directly transfer them to the tinfoil placed on ice.

3. Carefully remove the fat tissue, fascia, etc. from the muscles with scissors, as they are being dissected from the rabbit. Be very careful not to contaminate the meat with blood and hair from the rabbit. The clean meat preparation is kept on ice until further use.

The following preparation is conducted in the cold room and all tubes and samples are continuously kept on ice!

4. Grind the meat twice using a hand driven meat grinder (to keep heating of the meat to an absolute minimum).
5. Mix portions of approx. 55 g of minced meat with 180 mL buffer A and then blend at full speed for 15 s followed by a 10-s break. Repeat this procedure three times (*see* **Note 2**).
6. Transfer and distribute the resulting rather viscous meat homogenate among plastic centrifuge flasks and centrifuge at 4 °C for 20 min and 6400 × *g*.
7. Filter the supernatant through 7–10 layers of gauze into a glass beaker placed on ice to further remove cell debris and fat.
8. Distribute the filtered sample in the plastic centrifuge flasks for medium speed centrifugation for 20 min at 4 °C at 9700 × *g*.
9. Once more, filter the supernatant through 7–10 layers of gauze into a glass beaker placed on ice to remove final remnants of cell debris.
10. Pour the supernatant into centrifuge tubes and centrifuge at 4 °C, and 47,800 × *g* for 60 min to sediment the microsomal pellet (sarcoplasmic reticulum) (*see* **Note 3**).
11. Discard the supernatant and resuspend the pellet by homogenization using a Teflon homogenizer 25 times at low speed (400–500 rpm) in 150 mL of solution B.
12. Transfer the suspension into centrifuge tubes and centrifuge for 30 min at 4300 × *g* and 4 °C.
13. Gently decant and save the supernatant and discard the pellet (*see* **Note 4**).
14. Mix the supernatant with KCl/ATP extraction buffer C and leave on ice or in the refrigerator for 30 min to 1 h (*see* **Note 5**).
15. The preparation is centrifuged in an ultracentrifuge for 90 min at 84,500 × *g* and 4 °C.
16. Discard the supernatant and resuspend the pellet in 6 × 70 mL washing solution D followed by gentle homogenization 25 times with a Teflon homogenizer at approx. 500 rpm.
17. The homogenized supernatant is distributed into ultracentrifuge tubes and centrifuged for 60 min at 84,500 × *g* and 4 °C.
18. Repeat **step 13**.
19. Discard the supernatant, resuspend the pellet and homogenize gently using a 5 mL Teflon homogenizer in 24 mL buffer E 25 times.

20. Transfer to cryo tubes in suitable aliquots (usually 1–5 mL), flash freeze in liquid nitrogen and store at −80 °C.
21. Determine the protein concentration, e.g., by the Lowry method [5] and subsequently measure the specific activity of the SR-Ca^{2+}-ATPase preparation (*see*, e.g., **Part II** in this book).

3.2 Preparation of Ca^{2+}-ATPase Membranes by DOC Extraction

This is a mild DOC extraction procedure for purification of Ca^{2+}-ATPase from the SR preparation protocol described above, in which the loosely attached proteins like calsequestrin and M55 glycoprotein are removed. It is important to note that if this procedure is done correctly, the DOC extraction will not solubilize the Ca^{2+}-ATPase but merely remove extrinsic proteins.

1. Mix the extraction buffer I with SR protein, DTT and DOC, aim at the following concentrations: 2.5 mg protein/mL, 0.5 mg DOC/mL, 0.5 mg DTT/mL (*see* **Note 6**).
2. Incubate the solution for 10 min on ice and then distribute into Ti-70 tubes and centrifuge for 75 min at 4 °C at 181,000 × *g*.
3. Resuspend the pellet in 147.9 mL of extraction buffer II, and spin for 75 min at 4 °C at 181,000 × *g*.
4. Wash the pellet in 60 mL washing buffer (20 mL/125 mg protein) and centrifuge for 75 min at 4 °C at 181,000 × *g*.
5. Resuspend the pellet in approx. 7.5 % of the starting volume in buffer E (approx. 7 mL).
6. Aliquot the microsome sample in cryo tubes in portions of 100 and 300 μL, flash freeze in liquid nitrogen, and store at −80 °C.
7. Determine the protein concentration by the Lowry method and then measure and calculate the specific activity of the preparation. Check the purification by SDS-PAGE (*see* Fig. 1) and compare with the activity of the SR preparation after permeabilization with A23187 (Ca^{2+}-specific ionophore) or addition of $C_{12}E_8$.

4 Notes

1. We obtain reproducible good results with sodium deoxycholate from Sigma Aldrich (D6750).
2. This relatively brutal maceration and homogenization in Subheading 3.1, **steps 4** and **5**, is important to ensure a sufficient disintegration of the muscle tissue such that the connective tissue is broken up and, more importantly, the myofibrils are finely cut, preventing them from binding various cell organelles, in particular SR and thereby cause these to cosediment in the following low speed centrifugations steps (Subheading 3.1, **steps 6** and **8**) designed to remove the

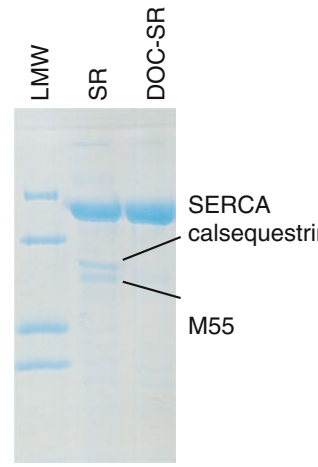

Fig. 1 SDS-PAGE of the SR and DOC-SR preparations

major part of the extracellular connective tissue, cell debris, nuclei and the majority of the myofibril and the mitochondria.

3. This will separate the major part of microsomal fraction from the soluble proteins.
4. This is an important and somewhat difficult step: The white/gray pellet is rather soft and loose and difficult to see when decanting, so be careful to avoid disturbing the sediment.
5. The KCl/ATP extraction procedure causes the actomyosine to dissociate and become soluble hence these components will not sediment and pellet in the following centrifugation.
6. As an aid to following the procedure, we have included an example based on a standard SR vesicle preparation result from the procedure described above. The starting point is an SR preparation which gave a yield of 28.44 mg protein/mL and had a total volume of 13 mL. 13 mL SR prep has a total protein content of (13 mL × 28.44 mg/mL) 369.72 mg. This is to be suspended in a total volume of (369.72 mg / 2.5 mg/mL) = 147.9 mL, i.e., by addition of (147.9 mL – 13 mL) = 134.9 mL extraction buffer I. If you prepare 150 mL extraction buffer I it should contain: (0.5 mg/mL × 147.9 mL × 150 mL / 134.9 mL = 82.2 mg DOC and (0.5 mg/mL × 147.89 mL × 150 mL) / 134.89 mL = 82.23 mg DTT in 150 mL.

   To use a correct concentration of DOC is of the utmost importance as: adding too little DOC leads to insufficient purification and adding too much may lead to extensive inactivation. Nevertheless you should expect that even a successful ATPase purification leads to some (10–20 %) inactivation.

Acknowledgement

We would like to thank Nikolaj Drachmann for preparing the gel for Fig. 1 and Ingrid Dach for critical reading and commenting on this book chapter. A special thanks goes to our technicians Lisbeth Nielsen and Birte Nielsen.

References

1. Møller JV (2010) The sarcoplasmic Ca^{2+}-ATPase: design of a perfect chemi-osmotic pump. Q Rev Biophys 43(4):501–566
2. Hasselbach W, Makinose M (1963) Uber Den Mechanismus Des Calciumtransportes Durch Die Membranen Des Sarkoplasmatischen Reticulums. Biochem Z 339:94
3. de Meis L, Hasselbach W (1971) Acetyl phosphate as substrate for Ca^{2+} uptake in skeletal muscle microsomes. J Biol Chem 246: 4759–4763
4. Meissner G et al (1973) Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+}-pump and Ca^{2+}-binding proteins. Biophysica Acta 298:246–269
5. Lowry OH et al (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275