

Purification of Na,K-ATPase from Pig Kidney

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Abstract

The method of purification of Na,K-ATPase from pig kidney is based on a differential centrifugation and SDS-treatment of a microsomal preparation. The yield is 0.4 mg protein per 1 g tissue with the specific (ouabain-sensitive) activity of 25–28 $\mu\text{mol P}_i/\text{min}$ per mg protein and nucleotide binding capacity of 3 nmol/mg. The protein/lipid ratio is 1/1 (mg/mg) with a protein purity of ~80 %.

Key words Na,K-ATPase, Kidney, Microsomal fraction differential centrifugation, SDS-treatment

1 Introduction

Na,K-ATPase, a member of the P-type ATPase superfamily, maintains transmembrane gradients of Na^+ and K^+ , critical for cell homeostasis. The minimal functional unit of the Na,K-ATPase consists of a large catalytic α -subunit, defining its membership in the P-ATPase family (M_r 113 kDa), and a glycosylated β -subunit (M_r 55 kDa). A third regulatory subunit from the FXYD family is often present in a tissue-specific manner. The stoichiometry under normal conditions is $1\alpha/1\beta/\text{optionally } 1\text{FXYD}$.

The physiological significance of the Na,K-ATPase (Na-pump) has nourished the interest to investigation of its structure, function and regulation for over half a century. Protein chemistry research and in the recent years crystallization studies consume considerable amount of purified enzyme. Although present in most animal cells, the enzyme is particularly plentiful in secretory and excitable tissues. The best sources for purification are kidney, brain, salt glands, and electric organs. A collection of purification protocols from different sources is available in *Methods in Enzymology* [1]. Klodos et al. [2] described the large-scale preparation of partly purified Na,K-ATPase from pig kidney with precise estimation of person-hours of work and discussed the quality of the final preparation.

This chapter presents the above method [2] step by step, the protocol successfully used in our lab to supply material for functional as well as crystallization studies.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. All solutions must be stored at 4 °C. The volumes are sufficient for purification of the enzyme from 15 kg whole kidneys.

1. Buffer I: 30 mM Histidine, 250 mM sucrose; pH 7.3 (20 °C). Weigh 4.65 g L-histidine, 85.58 g sucrose, add 900 mL H₂O and adjust to pH 7.3 with 1 N HCl. Make up to 1 L with H₂O.
2. Buffer II: 25 mM Imidazole, 250 mM sucrose, 1 mM EDTA; pH 7.4 (20 °C). Weigh 10.21 g imidazole, 513.5 g sucrose, 1.752 g Na₂EDTA·2H₂O, add 5.5 L H₂O and adjust pH to 7.4 with 1 N HCl and make up to 6 L with H₂O.
3. Buffer III: 20 mM Histidine, 250 mM Sucrose, 0.9 mM EDTA; pH 7.0 (20 °C). Weigh 6.2 g Histidine, 171.15 g Sucrose, 0.526 g Na₂EDTA·2H₂O, add 1.5 L H₂O and adjust pH to 7.0 with 1 N HCl and make up to 2 L with H₂O.
4. 15 kg of fresh kidneys packed in plastic bags (maximum 10 kidneys per portion) at the slaughterhouse, immediately cooled, and transported on ice.
5. Stainless steel tissue press, with plates with 2 and 1.4 mm diameter holes.
6. Pestle homogenizer with Teflon pestle (Sartorius Potter S).

3 Methods

3.1 Kidney Dissection

1. Each kidney is cut longitudinally in half (Fig. 1).
2. Cut (dissect) the inner medulla away with a scalpel and discard it. This procedure gives access to the outer medulla (Fig. 1).
3. Dissect the outer medulla with the Rongeur tong (forceps) (Fig. 2); that is, take the pink and reddish tissue (Fig. 1) and place it in small beakers on ice with some amount of cold buffer I. The beakers with buffer are weighed beforehand in order to allow estimation of the amount of collected tissue. The volume of the buffer I and the amount of tissue per beaker should be approximately equal. Collect 330 g outer medulla tissue.

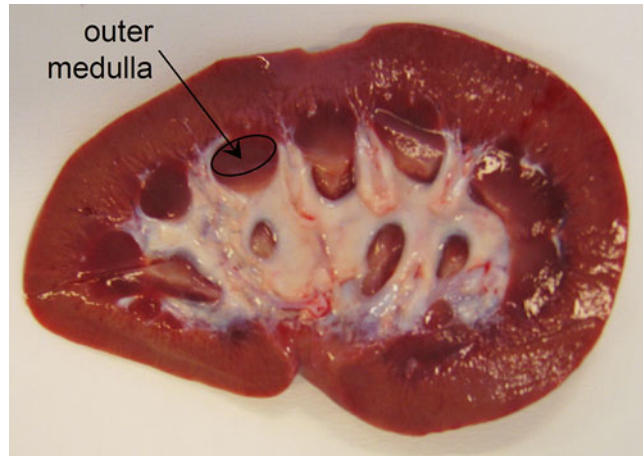


Fig. 1 Longitudinal cut over pig kidney

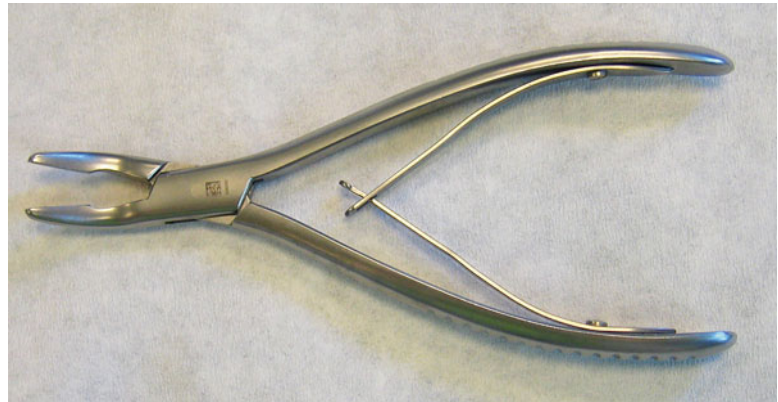


Fig. 2 Rongeur tong for outer medulla dissection

3.2 Disruption of Tissue

The following step is a disruption of the collected tissue by passage through a stainless steel tissue press.

1. Approximately 50 mL portions of tissue in buffer I are forced through first the plate perforated with 2.0 mm diameter holes and then through a plate with 1.4 mm diameter holes. The resulting suspension (approximately 500 mL) may be stored on ice in a refrigerator until the next day if necessary (*see Note 1*).

3.3 Preparation of Crude Microsomal Fraction by Differential Centrifugation

1. Add 500 mL of cold buffer II to the tissue suspension (500 mL) and homogenize in a pestle homogenizer with a Teflon pestle on ice at, e.g., 1500–2000 rpm, three times down and up (avoid foaming).
2. The homogenate (1 L) is diluted up to 3 L volume with buffer II; that is, the mixture contains 110 g tissue/L.

3. The homogenate is subjected to centrifugation at $3700\times g$ -average for 20 min at 4 °C (e.g., Sorvall GSA-rotor 6000 rpm).
4. Supernatant (S1) is kept on ice while the pellets are resuspended in 1 L of buffer II, homogenized as before.
5. The second homogenate is diluted to 3 L with buffer II and centrifugation is repeated as in **step 3**. Keep supernatant (S2) on ice and discard the pellet.
4. Combine supernatant S1 and S2 (ca. 5 L) and subject it to centrifugation $7400\times g$ -average for 20 min at 4 °C (e.g., Sorvall SS34 rotor 8500 rpm). Save the supernatant and discard the pellet.
5. In order to collect the microsomal fraction, centrifuge the supernatant at $38,000\times g$ -average for 40 min at 4 °C (e.g., Sorvall SS34 rotor 20,000 rpm). Save the pellet.
6. Resuspend the pellet in 200 mL buffer II and homogenize as described above with five strokes down and up. The microsomal fraction can be stored for months at -80 °C (*see* **Notes 2 and 3**).

3.4 Titration with SDS

1. Prepare a stock solution of 1 % SDS w/v (highest purity available) in buffer II and make the following dilutions according to the scheme:

Nr.	SDS %	1 % SDS in buffer II, μL	Buffer II, μL
1	0	0	1000
2	0.1	100	900
3	0.2	200	800
4	0.3	300	700
5	0.35	350	650
6	0.4	400	600
7	0.45	450	550
8	0.5	500	500
9	0.55	550	450
10	0.6	600	400
11	0.65	650	350
12	0.7	700	300
13	0.75	750	250
14	0.8	800	200

2. After determination of the protein concentration in the microsomal preparation (*see* **Note 3**), thaw the second aliquot and dilute the microsomes to 4.6 mg protein/mL with buffer II.

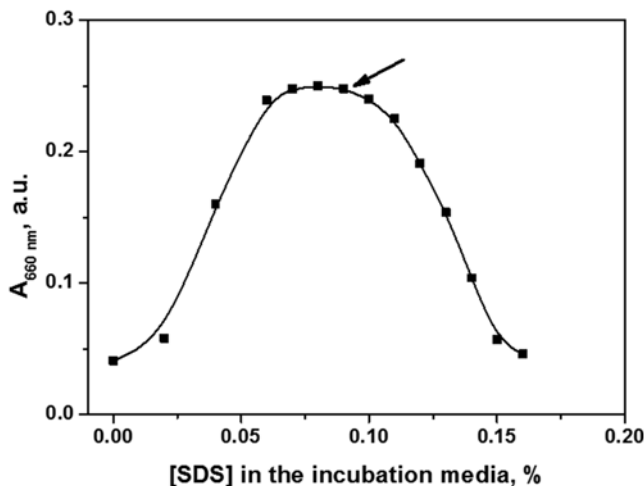


Fig. 3 Na,K-ATPase activity estimated colorimetrically from P_i release as function of SDS concentration in the incubation media. Optimal SDS concentration is marked by an *arrow*

3. Prepare 14 samples: To 80 μL microsomal fraction (4.6 mg/mL) add 20 μL of SDS solution in buffer II (according to the scheme above) and incubate the samples at 20 $^\circ\text{C}$ for 1 h on a water bath.
4. Measure Na,K-ATPase activity of each sample and plot the activity vs. final SDS concentration (Fig. 3). The Na,K-ATPase activity is defined as a difference in phosphate production in the absence or presence of 1 mM ouabain; *see* Chaps. 10–12 in this volume for protocols on colorimetric activity assays. Maximum of the bell-shaped curve, i.e., optimal SDS concentration for the activation of the microsomal fraction at the next step, is in the range of 0.08–0.1 % (*see* **Note 4**).

3.5 Treatment with SDS

1. Thaw the frozen microsomal preparation on a water bath at room temperature and dilute it with buffer II to a protein concentration of 4.6 mg/mL.
2. Add SDS dissolved in buffer II in the amount determined by the titration experiment and the relative volume as described above under continuous stirring (avoid foaming) (*see* **Note 5**).

Keep the suspension at 20 $^\circ\text{C}$ overnight.

3.6 Washing Procedure

1. Centrifuge the suspension at $127,000 \times g$ -average for 50 min at 10 $^\circ\text{C}$ (e.g., Beckmann Ti 45 rotor at 40,000 rpm). Discard supernatant.
2. For washing—resuspend the pellet in 400 mL of buffer III using tight pestle homogenizer and repeat centrifugation (four cycles).

3. Suspend the final pellet in 25 mL of buffer II (to an approximate protein concentration of 5 mg/mL). Take a small aliquot for determination of the protein concentration and specific Na,K-ATPase activity. Store both the aliquot and the main batch at -20°C .

4 Notes

1. Do not use a mixer or a blender. In our hands it decreased specific activity of the final enzyme preparation. However, other labs do use small blenders. In this case, the dissected tissue is drained on gaze, put it in the blender and blended with frozen buffer cubes. Ice cubes prevent foaming and therefore denaturation.
2. We freeze two aliquots (1 mL and 2 mL) of microsomal fraction for determination of protein concentration and optimal conditions for SDS-treatment conditions separately from the batch protein.
3. The protein concentration is measured according to Lowry et al. [3] against bovine serum albumin as a standard without correction for color factor.
4. In our hands, the shift of the bell-shaped curve towards higher SDS concentrations correlates with poor quality of the microsomal preparation and results in lower specific activity of the final preparation.
5. Add SDS very slowly under continuous stirring.

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