Video Article

Isolation of F_1-ATPase from the Parasitic Protist *Trypanosoma Brucei*

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Abstract

F_1-ATPase is a membrane-extrinsic catalytic subcomplex of F-type ATP synthase, an enzyme that uses the proton motive force across biological membranes to produce adenosine triphosphate (ATP). The isolation of the intact F_1-ATPase from its native source is an essential prerequisite to characterize the enzyme's protein composition, kinetic parameters, and sensitivity to inhibitors. A highly pure and homogeneous F_1-ATPase can be used for structural studies, which provide insight into molecular mechanisms of ATP synthesis and hydrolysis. This article describes a procedure for the purification of the F_1-ATPase from *Trypanosoma brucei*, the causative agent of African trypanosomiases. The F_1-ATPase is isolated from mitochondrial vesicles, which are obtained by hypotonic lysis from *in vitro* cultured trypanosomes. The vesicles are mechanically fragmented by sonication and the F_1-ATPase is released from the inner mitochondrial membrane by the chloroform extraction. The enzymatic complex is further purified by consecutive anion exchange and size-exclusion chromatography. Sensitive mass spectrometry techniques showed that the purified complex is devoid of virtually any protein contaminants and, therefore, represents suitable material for structure determination by X-ray crystallography or cryo-electron microscopy. The isolated F_1-ATPase exhibits ATP hydrolytic activity, which can be inhibited fully by sodium azide, a potent inhibitor of F-type ATP synthases. The purified complex remains stable and active for at least three days at room temperature. Precipitation by ammonium sulfate is used for long-term storage. Similar procedures have been used for the purification of F_1-ATPases from mammalian and plant tissues, yeasts, or bacteria. Thus, the presented protocol can serve as a guideline for the F_1-ATPase isolation from other organisms.

Introduction

The F-type ATP synthases are membrane-bound rotating multiprotein complexes that couple proton translocation across energy-transducing membranes of bacteria, mitochondria, and chloroplasts with the formation of ATP. Molecular details of the rotational mechanism of ATP synthesis are known mainly because of structural studies of purified bacterial and mitochondrial ATP synthases and their subcomplexes[1]. F-type ATP synthase is organized into membrane-intrinsic and membrane-extrinsic moieties. The membrane-extrinsic part, known as F_1-ATPase, contains three catalytic sites, where the phosphorylation of adenosine diphosphate (ADP) to ATP or the reverse reaction occurs. F_1-ATPase can be released experimentally from the membrane-intrinsic moiety while retaining its ability to hydrolyze, but not synthesize, ATP. The membrane-bound sector, called F_o, mediates protein translocation, which drives the rotation of the central part of the enzyme. The F_1 and F_o sectors are connected by the central and peripheral stalks.

The first attempts to purify the F_1-ATPase from budding yeast and bovine heart mitochondria date back to the 1960s. These protocols used extracted mitochondria, which were disrupted by sonication, fractionated by ammonium or protamine sulfate precipitation, followed by optional chromatography step(s) and heat treatment[2,3,4,5,6]. The purification was greatly improved and simplified by the use of chloroform, which readily releases the F_1-ATPase from the mitochondrial membrane fragments[7]. The chloroform extraction was then used to extract F_1-ATPases from various animal, plant, and bacterial sources (*e.g.*, rat liver[8], corn[9], *Arum maculatum*[10], and *Escherichia coli*[11]). Further purification of the chloroform-released F_1-ATPase by affinity or size-exclusion chromatography (SEC) yielded a highly pure protein complex, which was suitable for high-resolution structure determination by X-ray crystallography, as documented by the structures of F_1-ATPase from bovine heart[12,13] and *Saccharomyces cerevisiae*[14]. F_1-ATPase structures were also determined from organisms that are difficult to cultivate and, thus, the amount of the initial biological material was limited. In this case, the F_1-ATPase subunits were artificially expressed and assembled into the complex in *E. coli*, and the whole heterologous enzyme was purified by affinity chromatography *via* a tagged subunit. Such approach led to the determination of F_1-ATPase structures from two thermophilic bacterial species, *Geobacillus stearothermophilus*[15] and *Caldalkalibacillus thermarum*[16,17]. However, this methodology is rather unsuitable for eukaryotic F_1-ATPases since it relies on the prokaryotic protheosynthetic apparatus, posttranslational processing, and complex assembly.

The chloroform-based extraction was previously used to isolate F_1-ATPases from unicellular digenetic parasites *Trypanosoma cruzi*[18] and *T. brucei*[19], important mammalian pathogens causing American and African trypanosomiases, respectively, and from monogenic insect parasite *Crithidia fasciculata*[20]. These purifications led only to a simple description of the F_1-ATPases, since no downstream applications were used to fully characterize the composition, structure, and enzymatic properties of the complex. This article describes an optimized method for F_1-ATPase purification from the cultured insect life cycle stage of *T. brucei*. The method is developed based on the established protocols for isolation of bovine and yeast F_1-ATPases[21,22]. The procedure yields highly pure and homogeneous enzyme suitable for *in vitro* enzymatic and inhibitory assays, detailed proteomic characterization by mass spectrometry[23], and structure determination[24]. The purification protocol and the

knowledge of the F_1-ATPase structure at the atomic level opens a possibility to design screens to identify small-molecule inhibitors, and aid in the development of new drugs against African trypanosomiases. Moreover, the protocol can be adapted to purify F_1-ATPase from other organisms.

Protocol

1. Buffers and Solutions

1. Prepare the solutions listed below. Degas all buffers for liquid chromatography. Add ADP, benzamidine, and protease inhibitors just before use.
    1. Prepare buffer A: 50 mM Tris buffer with hydrochloric acid (Tris-HCl) pH 8.0, 0.25 M sucrose, 5 mM benzamidine, 5 mM aminocaproic acid (ACA), and protease inhibitors (10 µM amastatin, 50 µM bestatin, 50 µM pepstatin, 50 µM leupeptin, and 50 µM diprotin A).
    2. Prepare buffer B: 50 mM Tris-HCl pH 8.0, 0.25 M sucrose, 4 mM ethylenediaminetetraacetic acid (EDTA), 5 mM benzamidine, 5 mM ACA, 1 mM ADP, and protease inhibitors (10 µM amastatin, 50 µM bestatin, 50 µM pepstatin, 50 µM leupeptin, and 50 µM diprotin A).
    3. Prepare Q-column buffer: 20 mM Tris-HCl pH 8.0, 4 mM EDTA, 10 mM $MgSO_4$, 5 mM benzamidine, 5 mM ACA, and 1 mM ADP.
    4. Prepare Q-column elution buffer: Q-column buffer with 1 M NaCl.
    5. Prepare SEC buffer: 20 mM Tris-HCl pH 8.0, 10 mM $MgSO_4$, 100 mM NaCl, 1 mM ADP.
    6. Prepare chloroform saturated with 2 M Tris-HCl pH 8.5. Mix chloroform with 2 M Tris-HCl pH 8.5 in approximately 1:1 ratio in a screw-cap bottle, shake, let the organic and aqueous phases separate, and measure pH in the upper aqueous layer with a strip of pH-indicator paper. Store at room temperature. Just before use, shake again and let the phases separate. Use the lower chloroform layer. CAUTION: Chloroform is volatile and irritating to eyes and skin. Work in a fume hood. Use safety spectacles when shaking.

2. Preparation of sub-mitochondrial Particles

1. Resuspend mitochondrial vesicles (mitoplasts) isolated by hypotonic lysis[25] from 1 x 10^{11} to 2 x 10^{11} cells of procyclic *T. brucei* in 5 mL of ice-cold buffer A. Keep the sample chilled until step 3.2.
2. Determine the protein concentration in the suspension by the bicinchoninic acid (BCA) protein assay[26] according to the manufacturer's instructions.
    1. Use a bovine serum albumin (BSA) dilution series in ultrapure water to construct the standard curve. Dilute a small amount of sample 20 - 100 times with ultrapure water to fit into the range of BSA standards.
    2. Calculate the total protein amount in the sample and bring the protein concentration to 16 mg/mL by diluting it with additional buffer A.
3. Fragment mitoplasts into inverted vesicles and membrane pieces by sonication of the suspension 7x for 15 s with a total energy of 70 to 100 J per impulse with a microtip with a diameter of 3.9 mm. If the ultrasonic homogenizer does not display the energy output, start the optimization at 50% of the maximal power. Incubate the sample on ice for 30 s between impulses. After the sonication, the suspension becomes slightly darker.
4. Sediment the membrane fragments by ultracentrifugation at 54,000 x g for 16 h or at 98,000 x g for 5 h at 4 °C. Decant the supernatant and proceed with the chloroform extraction, or flash-freeze the sediment in liquid nitrogen and store it at -80 °C.

3. Release of F_1-ATPase from Membrane by Chloroform

1. Resuspend the pellet of mitochondrial membranes in buffer B with the aid of a small Dounce homogenizer. Calculate the volume of buffer B based on the total amount of buffer A used in steps 2.1 and 2.2 using the following formula: volume (buffer B) = volume (buffer A) x 12/21. Transfer the suspension to a 15- or 50-mL conical tube.
2. Remove the sample from ice and, from now on, keep the sample and all solutions to be used at room temperature.
3. Add chloroform saturated with 2 M Tris-HCl pH 8.5; the volume of chloroform to be added equals half the volume of the suspension. Close the cap tightly. Shake it vigorously for exactly 20 s. Centrifuge it immediately at 8,400 x g for 5 min at room temperature.
4. Transfer the upper cloudy aqueous phase to 1.6-mL microtubes. Add protease inhibitors (10 µM amastatin, 50 µM bestatin, 50 µM pepstatin, 50 µM leupeptin, and 50 µM diprotin A) to replace the inhibitors removed by the chloroform treatment. Centrifuge the samples at 13,000 x *g* for 30 min at room temperature. Transfer the supernatant to fresh microtubes and repeat the centrifugation to remove any insoluble material.

4. Anion-exchange Chromatography

1. Equilibrate the 5-mL anion exchange (Q) column attached to a fast-protein liquid chromatography system with the Q-column buffer at a flow rate of 5 mL/min until the absorbance at 280 nm and the conductivity stabilize (approximately 50 mL of buffer).
2. Load the supernatant from step 3.3 on the equilibrated column at a flow rate of 1 mL/min. Wait until the absorbance at 280 nm stabilizes at the background. Apply a 25-mL linear gradient of the Q-column elution buffer from 0% to 100% at a flow rate of 0.5 mL/min. Collect 1-mL fractions.
3. Assay the individual fractions corresponding to the major elution peak for ATP hydrolytic activity by the Pullman ATPase assay[2] at pH 8.0. Use 10 µL of each fraction per 1 mL of reaction mixture.Pool the fractions that exhibit ATPase activity. Optionally, separate 10 µL of each fraction on sodium dodecyl phosphate polyacrylamide gel electrophoresis (SDS-PAGE) and stain the gel by Coomassie Blue to visualize individual F_1-ATPase subunits and contaminating proteins.
4. Concentrate the pooled sample by membrane ultrafiltration using a spin column with a 100,000 MWCO PES filter to 200 - 500 µL. Proceed to SEC or store the sample overnight at room temperature.

5. Size-exclusion Chromatography

1. Equilibrate the SEC column attached to a liquid chromatography system with at least 48 mL (two column volumes) of the SEC buffer at a flow rate of 0.5 mL/min.
2. Apply the sample on the column and run chromatography at a flow rate of 0.25 mL/min. Collect 0.25-mL fractions.
3. Run 10 µL of the fractions that correspond to the peaks of the UV_{280nm} absorbance trace on SDS-PAGE and stain them by Coomassie Blue. The first major peak contains the F_1-ATPase. Assay the fractions corresponding to this peak for the ATP hydrolytic activity and azide sensitivity by the Pullman ATPase assay. Determine the protein concentration by the BCA assay.
4. Keep the purified F_1-ATPase at room temperature and use it within 3 d after purification for downstream applications. Alternatively, concentrate the sample using a spin column with a 100,000 MWCO PES filter to > 1.5 mg/mL, precipitate it by mixing it with saturated ammonium sulfate adjusted to pH 8.0 (1.2x the volume), and store it at 4 °C.

Representative Results

A typical purification (**Figure 1**) starts with mitochondrial vesicles (mitoplasts) isolated on the Percoll gradient from hypotonically lysed 1 x 10^{11} to 2 x 10^{11} procyclic *T. brucei* cells[25] cultured in standard glucose-rich SDM-79 medium[27]. The mitoplasts are fragmented by sonication, spun, and the matrix-containing supernatant is discarded. Mitochondrial membranes are treated with chloroform to release the F_1-ATPase. After centrifugation, the organic phase and precipitated interphase are discarded. The aqueous phase is fractionated by ion-exchange chromatography on quaternary ammonium, a strong anion exchanger (**Figure 2A**). The fractions that correspond to the major elution peak and contain the F_1-ATPase are pooled and concentrated. This material serves as the input for SEC, which eliminates residual impurities. The major contaminant is dihydrolipoyl dehydrogenase, which elutes from the SEC column as a discrete peak, marked by the dark green bar in **Figure 2B**. The F_1-ATPase elutes in the first dominant, largely symmetric peak (**Figure 2B**).

The progress of purification is followed by the BCA protein assay (or another common protein assay), SDS-PAGE, and the monitoring of ATPase activity. The rate of ATP hydrolysis is measured by the Pullman ATP regenerating assay[2], based on the decrease of absorbance of NADH in the coupled reaction. Sodium azide, an established inhibitor of F_1-ATPase, is used at a 2-mM concentration to determine the proportion of the F_1-ATPase-specific ATP hydrolysis. Typically, the input material contains roughly 150 - 300 mg of mitochondrial protein, depending on the number of cells used as the source of mitochondrial vesicles. The azide-sensitive proportion of the total ATPase activity is around 30% to 40% at this stage. After the chloroform extraction, more than 90% of ATPase activity in the sample is contributed to the F_1-ATPase. The purified F_1-ATPase is virtually completely sensitive to the azide treatment (the minimal residual ATPase activity can be attributed to the background ATP autolysis) and represents around 1% of the input protein mass, with an approximate yield of 1 - 1.5 mg of F_1-ATPase per 1 x 10^{11} cells (**Table 1**). A typical band pattern after the separation of the purified F_1-ATPase on SDS-PAGE gel followed by Coomassie Blue staining is shown in **Figure 2C**. The proteins were identified by peptide mass fingerprinting and characterized in detail by various mass spectrometry approaches[23]. Sporadic weak bands visible above the β-subunit band represent subcomplexes of the $\alpha_3\beta_3$ headpiece (dimers and oligomers of α- and β-subunits) and are devoid of any contaminants detectable by sensitive mass spectrometry techniques. The purified F_1-ATPase can be stored for up to several days in the S buffer at room temperature. Alternatively, the F_1-ATPase concentrated to ≥2 mg/mL can be precipitated by an equal volume of saturated ammonium sulfate in the S buffer, with pH adjusted to 8.0, and stored at 4 °C. For at least six months after the precipitation, the active enzyme with no obvious degradation of any subunit can be obtained by redissolving the precipitated material in the S buffer or similar solution. However, storage longer than one month is not suitable for crystallization, as determined empirically.

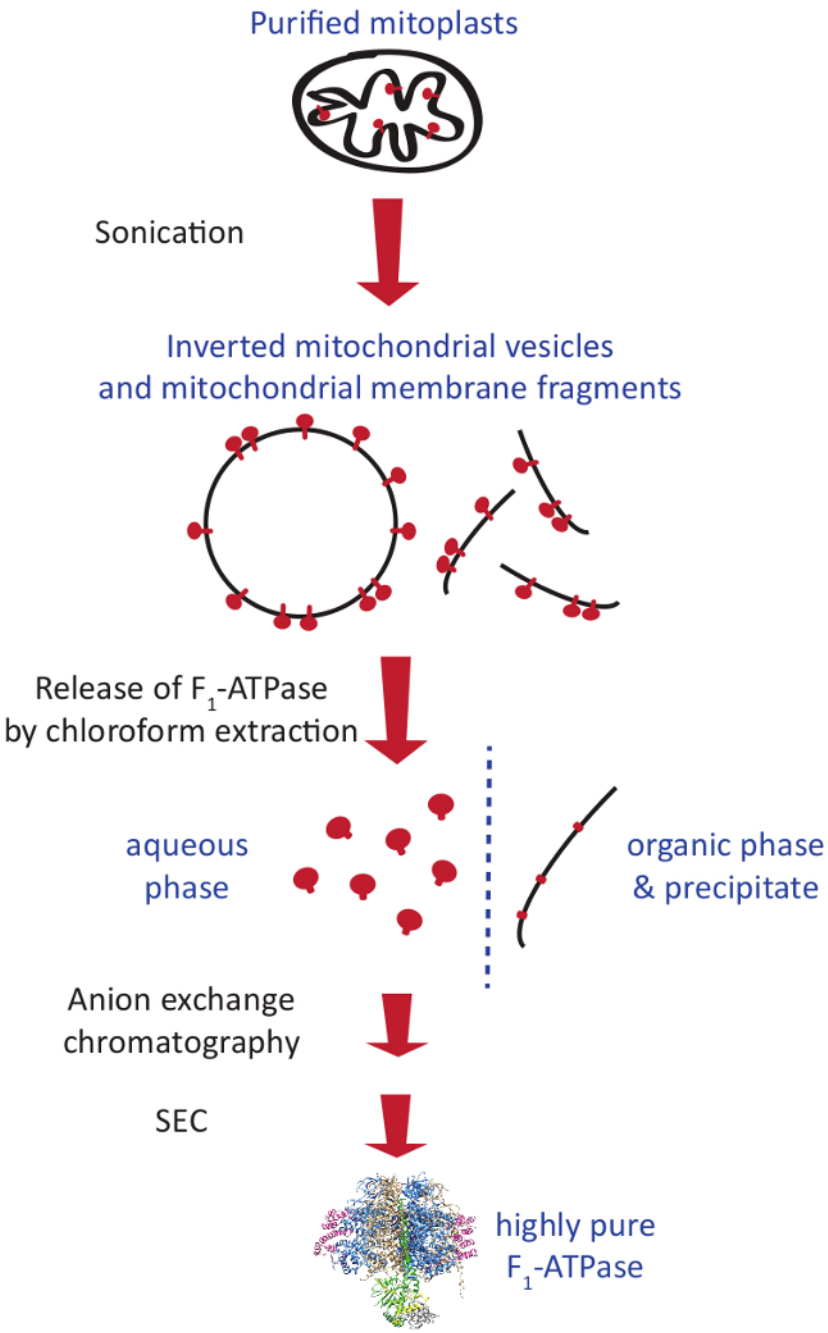

Figure 1: Scheme of the purification procedure. Please click here to view a larger version of this figure.

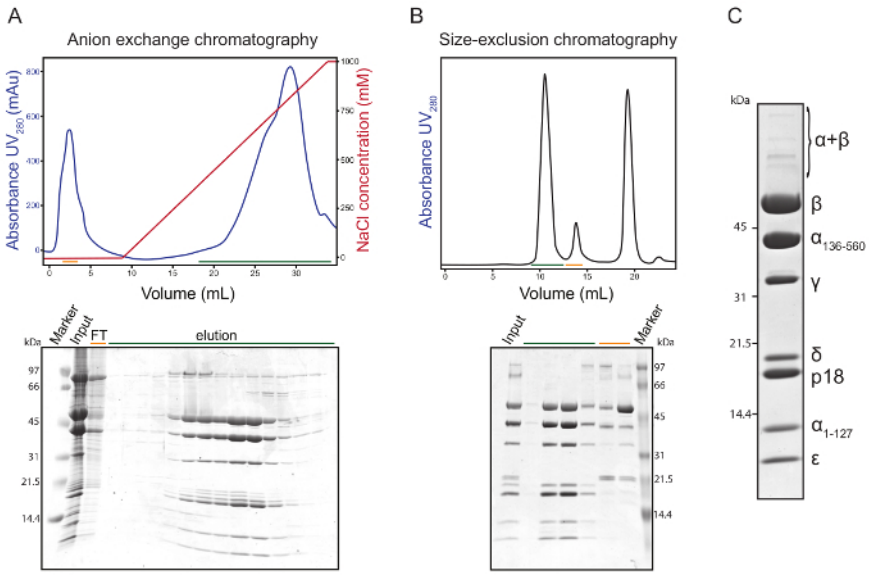

Figure 2: Two-step purification of the chloroform-released F_1-ATPase by liquid chromatography. (**A**) Elution profile of anion-exchange chromatography (upper panel) and selected fractions separated on the 10% - 20% Tris-glycine SDS-PAGE gel stained with Coomassie Blue dye (lower panel). Blue trace: UV absorbance at 280 nm; red trace: concentration of NaCl in the elution buffer; Input: the F_1-ATPase released by chloroform; FT: flow-through. (**B**) Elution profile of SEC (upper panel) and selected fractions separated on the SDS-PAGE gel stained with Coomassie Blue dye (lower panel). Input: pooled fractions from anion-exchange chromatography containing F_1-ATPase. The color-coded bars in panels **A** and **B** mark the fractions in the elution profiles that were analyzed by SDS-PAGE and the corresponding lanes in the respective gel. (**C**) Identities of individual proteins of the isolated F_1-ATPase as identified by mass spectrometry. Please click here to view a larger version of this figure.

	Protein concentration (mg/mL)	Total protein (mg)	Proportion of input material (%)	Activity ($\mu mol_{ATP} \times mg^{-1} \times min^{-1}$)	Azide sensitivity (%)
Mitochondrial vesicles in buffer A	16.2	170	100	1.3	25-35
Mitochondrial membranes in buffer B	18.6	97	57	2.4	35-45
Chloroform extracted fractions	2.5	7.9	4.7	12	91-95
F_1-ATPase after Q-column	-	2.2	1.3	23	92-96
F_1-ATPase after gel filtration	-	1.6	0.93	48	93-98

Table 1: An example of the typical progress and yield of the F_1-ATPase purification from mitochondria isolated from 1 x 10^{11} procyclic *T. brucei* cells.

Discussion

The protocol for F_1-ATPase purification from *T. brucei* was developed based on previously published methods for the isolation of F_1-ATPase complexes from other species[13,14]. The method does not require any genetic modification (*e.g.*, tagging) and yields a fully active complex with all subunits present. The crucial step is the chloroform-facilitated release of the F_1-ATPase from the membrane-attached part of the enzyme. In purifications from all eukaryotic species described so far, the released subcomplex contained subunits α, β, γ, δ, and ε in a stoichiometry of 3:3:1:1:1. In *T. brucei*, the F_1-ATPase contains an additional three copies of the subunit p18, a novel component restricted to euglenozoan protists[23]. Furthermore, the euglenozoan α-subunit is proteolytically split into two fragments, both stably associated with the complex[24,28,29]. The subunit OSCP (oligomycin-sensitivity-conferring protein), which links the F_1-moiety to the peripheral stalk[30], is absent from the released complex, which is in agreement with F_1-ATPase purifications by chloroform extraction from other species[13,14].

The chloroform-released F_1-ATPase is further purified by liquid chromatography. In the case of the bovine F_1-ATPase, only one chromatography step, size-exclusion chromatography, suffices to obtain a highly pure and active complex[31]. However, the single SEC set-up was insufficient for the purification of the *T. brucei* F_1-ATPase, as the fractions enriched for F_1-ATPase contained additional protein contaminants, mainly delta-1-pyrroline-5-carboxylate dehydrogenase. Therefore, anion-exchange chromatography was introduced before the SEC as the first and

major purifying step, and the SEC serves as the subsequent polishing procedure. For crystallization experiments, the use of the Superdex 200 Increase column proved to be essential, since this column provided material that allowed growing crystals of good quality. It is likely that the resolution of the column enabled the separation of a small proportion of incomplete complexes that interfered with crystallization. However, for applications other than crystallization, the separation using the Superdex 200 column was equally satisfactory.

To protect the F_1-ATPase complex from partial proteolysis by unknown protease(s) present in the mitochondrial lysate, the initial buffers A and B contained a wide range of protease inhibitors. The impact of individual inhibitors on the proteolysis of F_1-ATPase subunits has not been tested and, most likely, the presence of some of the inhibitors is redundant. For the SEC step, the inhibitors are not added anymore, as the contaminating proteases are removed from the F_1-ATPase sample by the chloroform extraction or the first chromatography step.

The multistep protocol inevitably leads to partial losses of the F_1-ATPase. The most significant loss (25% - 45% of the total amount) occurs during the concentration step by membrane ultrafiltration on a spin column after the anion-exchange chromatography. The F_1-ATPase likely adheres to the membrane of the spin column. Thus, for some downstream applications that do not demand a highly pure and concentrated sample (*e.g.*, enzymatic assays and inhibitory screens), the F_1-ATPase can be used immediately after the anion-exchange chromatography (see **Figure 2B**, Input lane).

Although the purification of F_1-ATPase from different organisms varies in detail, the general workflow remains the same. Therefore, this protocol can serve as a guideline for the development of the F_1-ATPase isolation protocol of other abundant sources, such as tissues or cells cultivatable on a large scale.

Disclosures

The authors have nothing to disclose.

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