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April 20th, 2018

Dear Doctor Mukherjee,

Please, find enclosed our manuscript "Isolation of F_1-ATPase from *Trypanosoma brucei*" by Ondřej Gahura and myself. We were invited to submit this manuscript and we hope you find it suitable for publication in Journal of Visualized Experiments.

Sincerely,

Alena Zíková, PhD.

TITLE:

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

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KEYWORDS:

F_1-ATPase, *Trypanosoma brucei*, mitochondrial ATP synthase, F-type ATPase, chloroform extraction, liquid chromatography

SUMMARY:

This protocol describes the purification of F_1-ATPase from the cultured insect stage of *Trypanosoma brucei*. The procedure yields a highly pure, homogeneous, and active complex suitable for structural and enzymatic studies.

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 a central and peripheral stalk.

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[2-6]. 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 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

1.1. Prepare the solutions listed below. Degas all buffers for liquid chromatography. Add ADP, benzamidine, and protease inhibitors just before use.

1.1.1. 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).

1.1.2. 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).

1.1.3. 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.

1.1.4. Prepare Q-column elution buffer: Q-column buffer with 1 M NaCl.

1.1.5. Prepare size-exclusion chromatography (S) buffer: 20 mM Tris-HCl pH 8.0, 10 mM $MgSO_4$, 100 mM NaCl, 1 mM ADP.

1.1.6. 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

2.1. 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.

2.2. Determine the protein concentration in the suspension by the bicinchoninic acid (BCA) protein assay[26] according to the manufacturer's instructions.

2.2.1. 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.

2.2.2. Calculate the total protein amount in the sample and bring the protein concentration to 16 mg/mL by diluting it with additional buffer A.

2.3. 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.

2.4. 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

3.1. 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.

3.2. Remove the sample from ice and, from now on, keep the sample and all solutions to be used at room temperature.

3.3. 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.

3.4. 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

4.1. 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).

4.2. 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.

4.3. 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.

4.4. Concentrate the pooled sample by membrane ultrafiltration using a spin column with a 100,000 MWCO PES filter to 200 - 500 µL. Proceed to S or store the sample overnight at room temperature.

5. Size-exclusion Chromatography

5.1. Equilibrate the S column attached to a liquid chromatography system with at least 48 mL (two column volumes) of the S buffer at a flow rate of 0.5 mL/min.

5.2. Apply the sample on the column and run chromatography at a flow rate of 0.25 mL/min. Collect 0.25-mL fractions.

5.3. 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.

5.4. 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 S, which eliminates residual impurities. The major contaminant is dihydrolipoyl dehydrogenase, which elutes from the S 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 AND TABLE LEGENDS:

Figure 1: Scheme of the purification procedure.

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 S (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.

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 S 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 S as the first and major purifying step, and the S 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 S 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.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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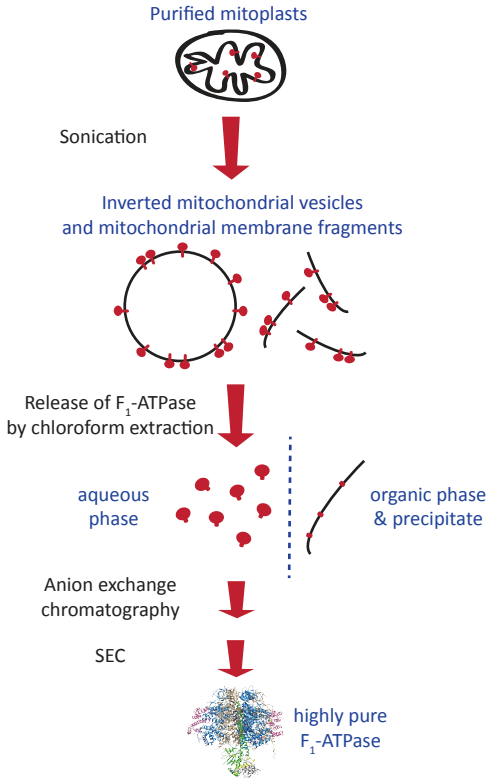

Figure 2

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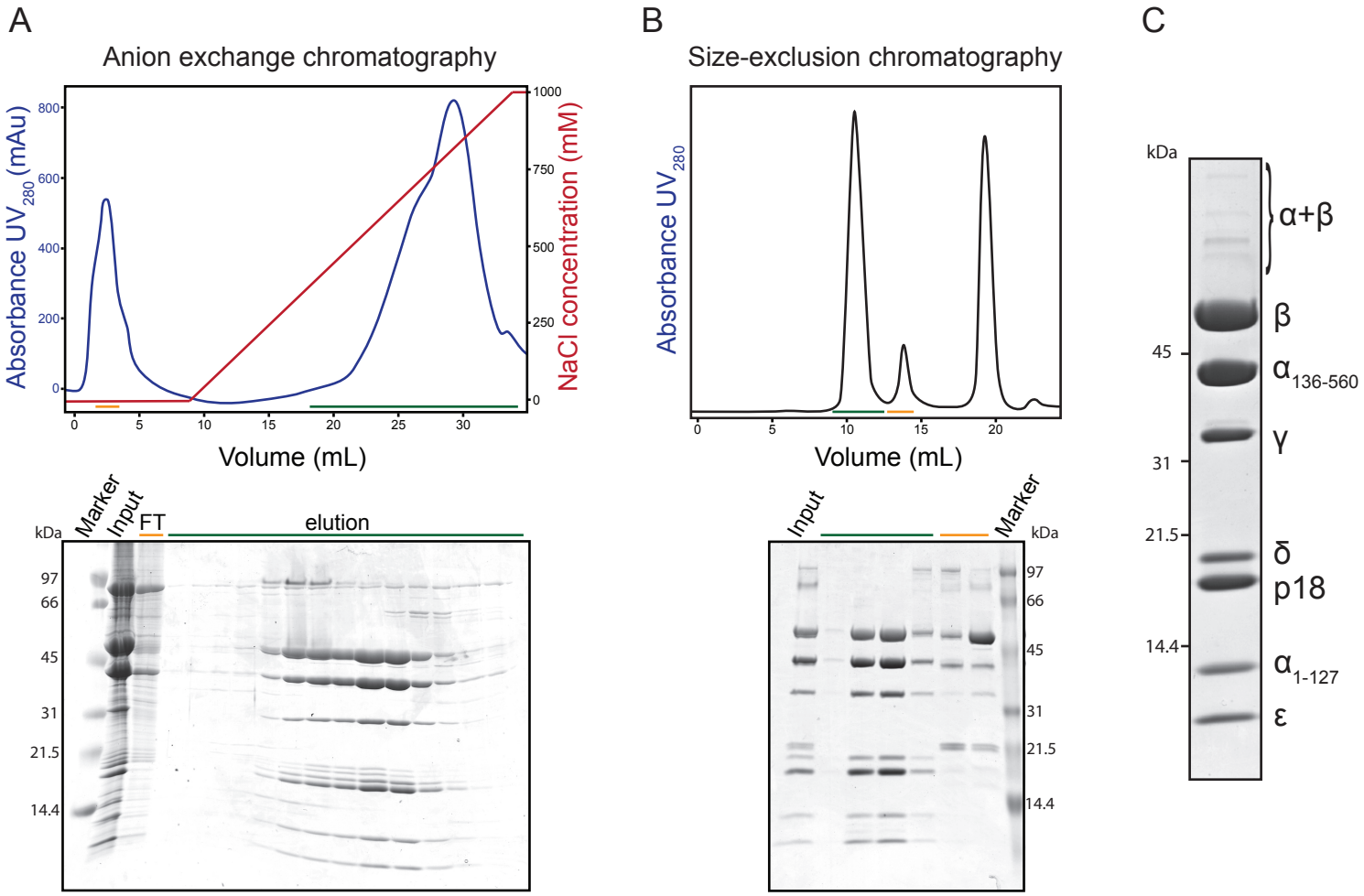

Table

	Protein concentration (mg/mL)	Total protein (mg)
Mitochondrial vesicles in buffer A	16.2	170
Mitochondrial membranes in buffer I	18.6	97
Chloroform extracted fractions	2.5	7.9
F_1-ATPase after Q-column	-	2.2
F_1-ATPase after gel filtration	-	1.6

Proportion of input material (%)	Activity ($\mu mol_{ATP} \times mg^{-1} \times min^{-1}$)	Azide sensitivity (%)
100	1.3	25-35
57	2.4	35-45
4.7	12	91-95
1.3	23	92-96
0.93	48	93-98

Table of Materials

Click here to download Table of Materials JoVE_Materials_fin_062218.xlsx

Name of Material	Company	Catalog Number
Chemicals		
Adenosin Diphosphate Disodium Salt (ADP)	Applichem	A0948
Amastatin Hydrochloride	Glantham Life Sciences	GA1330
Aminocaproic Acid	Applichem	A2266
BCA Protein Assay Kit	ThermoFischer Scientific/Pierce	23225
Benzamidine Hydrochloride	Calbiochem	199001
Bestatin Hydrochloride	Sigma Aldrich/Merck	B8385
Chloroform	Any supplier	
cOmplete Tablets, Mini EDTA-free	Roche	4693159001
Ethylenediaminetetraacetic Acid (EDTA)	Any supplier	
Hydrochloric Acid	Any supplier	
Ile-Pro-Ile	Sigma Aldrich/Merck	I9759
Leupeptin	Sigma Aldrich/Merck	L2884
Magnesium Sulfate Heptahydrate	Any supplier	
Pepstatin A	Sigma Aldrich/Merck	P5318
Protein Electrophoresis System	Any supplier	
Sodium Chloride	Any supplier	
Sucrose	Any supplier	
Tris	Any supplier	
Consumables		
Centrifuge Tubes for SW60Ti, Polyallomer	Beckman Coulture	328874
DounceTissues Homogenizer 2 mL	Any supplier	
Glass Vacuum Filtration Device	Sartorius	516-7017
HiTrap Q HP, 5 mL	GE Healthcare Life Sciences	17115401
Regenaretad Cellulose Membrane Filters, pore size 0.45 µm, diameter 47 mm	Sartorius	18406--47------N
Superdex 200 Increase 10/300 GL	GE Healthcare Life Sciences	29091596
Vivaspin 6 MWCO 100 kDa PES	Sartorius	VS0641
Name of Equipment	**Company**	**Catalog Number**
AKTA Pure 25	GE Healthcare Life Sciences	29018224
Spectrophotometer Shimadzu UV-1601	Shimadzu	
Ultracentrifuge Beckman Optima with SW60Ti Rotor	Beckman Coulture	

Ultrasonic Homogenizer with Thin Probe, Model 3000	BioLogics	0-127-0001

Comments/Description
Protease inhibitor cocktail tablets
For pH adjustment
Alias Diprotin A
Degasing solutions for liquid chromatography
Anion exchange chromatography column
Degasing solutions for liquid chromatography
Size-exclusion chromatography column

Comments/Description
Or similar FPLC system
Or similar spectrophotometer with kinetic assay mode
Or similar ultracentrifuge and rotor

Or similar ultrasonic homogenizer

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Author(s): ONDŘEJ GAHURA, ALENA ZÍKOVÁ

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Department: INSTITUTE OF PARASITOLOGY

Institution: BIOLOGY CENTRE ASCR, v

Article Title: The isolation of F_1-ATPase from Trypanosoma brucei

Signature: 

Date: 20/4/2018

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Point-by-Point Rresponse to editorial and reviewers comments
on manuscript entitled " The isolation of F1-ATPase from parasitic protist *Trypanosoma brucei*".
JoVE58334

We would like to take this opportunity to thank both reviewers for taking time out of their busy schedules to provide us with fair criticism that we believe has helped us to improve the manuscript.

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Table 1: Please provide the unit for protein concentration in the table.

The unit (mg/mL) was stated in the originally submitted table.

3. Figure 1: Please fix the typo “chlorform”, which should be “chloroform”.

Corrected.

4. Please provide an email address for each author.

The missing email address was added to the Authors and Affiliations section.

5. Please define all abbreviations before use.

Several abbreviations were defined.

6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

The abbreviations were changed. However, it is now in discrepancy with the requirements specified in the Instructions for Authors provided by JoVE (see the Technical Language section).

7. 1.1.1-1.1.6: Please write the text in the imperative tense in complete sentences.

Changed according to the requirements.

8. 2.2: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

Details and a relevant reference were added.

9. 4.1/5.1: What volume of buffer is added and how long does it take to equilibrate?

The volume of buffer and the recommended flow rate were added.

10. 4.2: How is the flow rate controlled?

The flow rate is one of the parameters that are set on the liquid chromatography instrument (e.g. AKTA Pure or similar).

11. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

The reference list was generated using EndNote and the style file provided by JoVE and manually corrected (e.g. the scientific names were italicized). The abbreviated journal titles are dictated by the reference style and are also used in recent JoVE publications as well as in the example references in the Instruction for Authors (the manuscript template file). Shall we use the full journal titles in spite of these facts? The volume and issue number are absent only from the reference #1 that is a book chapter, which does not have these specifications.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Gahura et al., describe a standard protocol for the purification of the F1 portion of the membrane-bound F1Fo ATPase which they have adapted for use with the human parasite Trypanosoma brucei. The authors clearly describe in the introduction the history behind the development of the purification protocol and what the final product can be used for. The protocol itself represents a standard protocol which is clear to follow but may not be in the correct style for a joVE article. The representative results are also clear apart from the sentence on line 202 and figure 2 (see below). The discussion is also adequate the pros and cons of the method. Thus in conclusion, the article represents a nice description of the protocol required to purify the F1 portion of F1Fo ATPases from T. brucei and illustrates how the protocol can be adapted for other organisms. When completed, this video will complement several other jove videos e.g. the purification of mitochondria and preparation of F1Fo ATPase vesicles for patchclamping.

Major Concerns:
None

Minor Concerns:
1) Line 202 states "the organic phase and precipitated interphase are discarded". It would be important to show an image or video segment indicating what is meant by these two terms.

The Protocol describes this step in more detail (see 3.3), but we agree that it would be useful to visualize this step.

2) In figure 2 where the dark green line in fig 2A indicates the F1 containing fraction

but in figure 2B it is the light green colour. I think the color for the F1 containing fractions should be the same colour or all the shades of green should be different between the two images. Also a picture / diagram / or label should be included in the figure so that it is easy from the figure to determine which graph/gel image comes from which column.

The misleading color-coding in the figure was changed and the graphs were labeled for easier orientation.

Reviewer #2:
Manuscript Summary:
Authors provided comprehensive and well-written protocol. The F1-ATPase purification from unicellular parasite Trypanosoma brucei is explained clearly, results are presented and discussed well. All steps in the protocol are detailed, clear and simple to follow. Included references are relevant and provide essential reading list for researchers investigating FoF1-ATP synthase complexes. Overall, the methodology is easily transferable and can be used for purification of complexes from other organisms. Publication of the protocol in JoVE would be very helpful to researchers in the field.

Major Concerns:
Long abstract - line numbers 40 and 41: Can authors elaborate on long-term storage of ammonium sulphate precipitate? Is precipitated F1-ATPase stable for weeks or months?

We added details on the storage of ammonium sulfate precipitate in the last paragraph of Representative Results.

Step 2.2. - Assays for protein concentration determination have limitations in sensitivity, compatibility with chemicals, etc. Can authors suggest an alternative to BCA protein assay or explain why they have used it in the protocol (steps 2.2. and 5.3.)?

The solutions used in the protocol are devoid of any chemicals (e.g. reducing agents etc.).that interfere with any of the common protein assays. We routinely use the BCA assay, and therefore, we suggest it in the protocol, as each assay can give slightly different results. The data presented in the Table 1 were obtained with the BCA. However, principally, any assay can be used. We reflected this fact in the second paragraph of Representative Results.

Step 2.3. - Disruption of particles using sonication is an arbitrary method completely dependent on a homogenizer brand and thickness of used probe. Can authors provide more details regarding the type or diameter of the probe in step 2.3. or in the Equipment section? Thin probe is not sufficient information for replicating this step.

We agree that this information is essential. We added details on the probe in the step 2.3 and to the List of Material.

Step 5.3. and Representative results line numbers 215-217- Concentration of F1-

ATPase inhibitor azide used in the experiment is missing. Can authors provide it or insert a suitable reference?

The concentration of azide was added.

Minor Concerns:
Overall, I recommend thorough proofreading of the manuscript. In contains typos - for example the term "proton motif force" needs to be corrected.
I recommend changing the title to - "The isolation of F1-ATPase from unicellular parasite Trypanosoma brucei " - it would increase the visibility of the protocol for researches working with different protists.

We changed the title to "The isolation of F1-ATPase from parasitic protist Trypanosoma brucei"

Steps and buffers 1.1.1, 1.1.2, and 3.3. - Can authors provide a recipe for the cocktail of protease inhibitors or clarify the final concentrations used in the protocol? There is a discrepancy in concentrations of amastatin and bestatin between 1.1.2 buffer B and step 3.3 - is there any reason for increase concentration of these two inhibitors from 10μM to 50μM?

The discrepancy was corrected. In fact, in initial experiments we used 50 μM amastatin, but later we switched to sufficient 10 μM.

Discussion - line numbers 278-281 -Clarification is needed for: …"The F1-ATPase tends to adhere to the membrane of the spin column." … Is this claim based solely on the protein concentration measurements (Table 1) or can authors provide a reference for this sentence?

We slightly rephrased the statement. It is is based solely on our empirical experience with protein concentration measurement. The losses on the spin column were observed in each purification, but the extend was very variable. Reuse of the spin columns helps to decrease the losses, however, we do not want to recommend it in the protocol, as we are not sure how and how long the reused column can be safely stored.

Fig. 2 - Bands on Coomassie gels are labelled either by nomenclature of subunits or molecular weight of a protein marker. Can authors unify this or add or molecular weight labelling to panel C?

The MW labels were added to the panel C.