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Simple and rapid purification of F_1-ATPase from mitochondria

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A simple, rapid method for isolation of F_1-ATPase from bovine heart mitochondria using the cation exchange resin Dynospheres PD-201-SCX was developed. The method is based on the fact that hydrophobic membrane proteins are adsorbed efficiently on this resin, whereas hydrophilic proteins, such as F_1-ATPase, are not. By this method F_1-ATPase of high purity and enzyme activity can be obtained from mitochondria in about 2 h.

ATPase, F_1; Purification, rapid; Dynosphere PD-201-SCX; Mitochondria; Oxidative phosphorylation

1. INTRODUCTION

H^+-ATPase catalyzes ATP synthesis in energy-transducing membranes, such as mitochondria, chloroplasts and bacteria (for reviews, see [1–7]). This enzyme consists of F_1 and F_0 portions. The F_1-portion (F_1-ATPase), consisting of 5 subunits, α, β, γ, δ and ϵ, catalyzes the synthesis and hydrolysis of ATP, while the F_0-portion, embedded in the membrane, acts as a gated channel for the H^+ necessary for ATP synthesis [8]. F_1-ATPase is hydrophilic and projects from the membrane into the aqueous phase. To understand the mechanism of ATP synthesis, studies on F_1-ATPase at the molecular level are very important. However, the methods available for isolation and purification of F_1-ATPase are complicated and tedious, taking more than 24 h.

The method most widely used for isolation of F_1-ATPase from mitochondria is that of Caterall et al. [9], and consists of disruption of the mitochondrial membrane and detachment of F_1-ATPase by sonication, separation of F_1-ATPase by protamine fractionation, and its purification by DEAE-Sephadex column chromatography. This method is referred to as the reference method in this paper. A more rapid method was developed by Beechey et al. [10] and its modification was reported by Williams and Pedersen [11]. These methods, called the solvent-release methods in this paper, are essentially based on the release of hydrophobic proteins by extraction with an organic solvent, such as chloroform, leaving hydrophilic F_1-ATPase in the aqueous phase. However, the inverted membrane vesicles must be used as starting material [10,11] and an additional purification step by HPLC is required [11].

As F_1-ATPase is more hydrophilic than most membrane-bound proteins in mitochondria, its isolation based on the difference in hydrophobicity should be possible, as in the solvent-release method. This could also be achieved by adsorption of hydrophobic proteins on a resin that can bind hydrophobic proteins, but not hydrophilic F_1-ATPase. By applying this idea, we developed a new method for isolation of F_1-ATPase from mitochondria using the cation exchange resin Dynospheres PD-201-SCX (a porous sulfonated styrene-divinyl-benzene polymer). This method is very simple and rapid, requiring only about 2 h, and the F_1-ATPase obtained shows high purity and high activity.

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography

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2. MATERIALS AND METHODS

The resin used was Dynospheres PD-201-SCX (porous divinylbenzene polymer, 20 μm diameter, strong cation exchanger), produced by Dyno Particles AS, Lilestrom (Norway), and was a generous gift from Dr S. Aotani, Japan Synthetic Rubber Co., Tokyo (Japan). Lactate dehydrogenase and pyruvate kinase were purchased from Oriental Yeast Co. Ltd, Tokyo (Japan). Other reagents were of the highest grade commercially available.

Bovine heart mitochondria were isolated by the method of Smith [12] and frozen until use. F_1-ATPase isolated by the reference method [9] was used as a standard sample.

ATPase activity was measured spectrophotometrically at 340 nm as decrease in NADH by its oxidation catalyzed by pyruvate kinase and lactate dehydrogenase coupled with phosphorylation of the ADP produced by hydrolysis of ATP [13].

SDS-PAGE was carried out by the method of Laemmli [14] with a separating gel concentration of 12% acrylamide. After electrophoresis, proteins were stained with Coomassie brilliant blue R-250. The concentration of protein was determined by the method of Lowry et al. [15] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

The method for purification of F_1-ATPase described here involves the following steps: (i) elimination of water-soluble proteins from mitochondria by hypotonic treatment; (ii) solubilization of the membrane fraction by treatment with the nonionic detergent Triton X-100; (iii) resin treatment to adsorb hydrophobic membrane proteins and (iv) removal of the resin with adsorbed proteins by centrifugation.

Frozen mitochondria were thawed and homogenized in a hypotonic medium consisting of 1 mM ATP and 5 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 30000 × g for 10 min at 2°C. The precipitated membrane fraction was homogenized in the hypotonic medium and recentrifuged (hypotonic treatment). SDS-PAGE of proteins in the supernatant and pellet (lanes 2 and 3, respectively, of fig.1) indicated that most hydrophilic proteins were recovered in the supernatant. The pellet was then solubilized in the hypotonic medium with 3.5% Triton X-100 at a final protein concentration of 13 mg/ml (solubilization). The turbid solution was allowed to stand for 5 min at room temperature, and then centrifuged at 30000 × g for 20 min at room temperature. The results of SDS-PAGE of the solubilized proteins are shown in lane 4 of fig.1. The F_1-ATPase thus solubilized seemed still bound to F_0. These proteins were subjected to vortex mixing with a known amount of the resin Dynospheres PD-201-SCX in an Eppendorf centrifuge tube at room temperature for 10 min, and the mixture was centrifuged in a Kubota KM-15000 microcentrifuge at 12000 rpm for 2 min at room temperature (resin treatment). After this treatment, F_1-ATPase was found in the supernatant, while most other proteins were adsorbed to the resin.

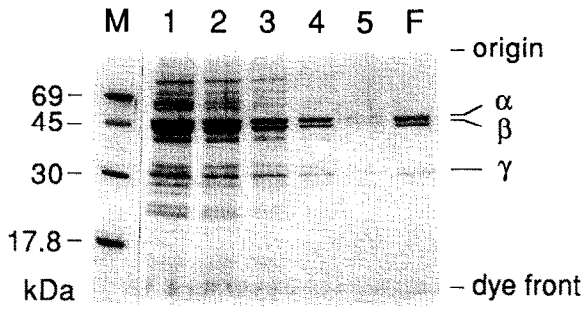

Fig.1. SDS-polyacrylamide gel electrophoresis of proteins at each step of purification. M, molecular weight markers (bovine serum albumin, ovalbumin, carbonic anhydrase and myoglobin); F, F_1-ATPase purified by the reference method [9]. Lanes: 1, whole mitochondrial proteins; 2, hydrophilic proteins from mitochondria obtained by hypotonic treatment; 3, sedimented proteins of the membrane fraction after hypotonic treatment; 4, proteins in the supernatant after solubilization with Triton X-100.

SDS-PAGE analysis showed that the purity of F_1-ATPase in the final supernatant was dependent on the weight ratio of proteins to resin as shown in fig.2. When the amount of proteins was more than 0.78 mg per 100 mg resin, the capacity of the resin was not sufficient and bands of contaminating proteins were seen on SDS-PAGE (lanes 1–3). On the other hand, when the amount of proteins was less than 0.39 mg per 100 mg resin, the intensities of the bands of subunits of F_1-ATPase were decreased, although the purity of F_1-ATPase seemed to be very high (lane 5). The best ratio for obtaining highly purified F_1-ATPase in high yield was 0.78 mg of protein per 100 mg resin, as shown in lane 4 of fig.2. In this electrophoreogram, strong bands of the α, β and γ subunits were clearly observed. However, under the conditions used, bands of δ and ϵ subunits were not clearly detected,

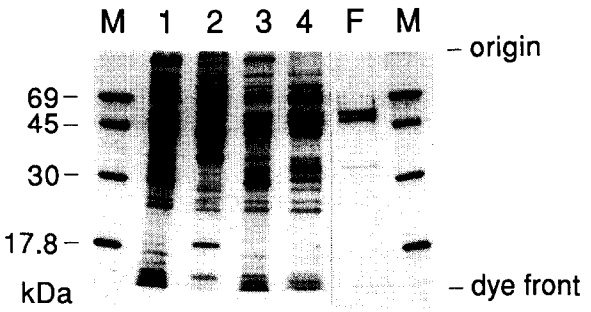

Fig.2. SDS-polyacrylamide gel electrophoresis of proteins in the supernatant after resin treatment. Lanes M and F are as for fig.1. Various amounts of proteins in the supernatant after addition of Triton X-100 (lane 4 in fig.1) were mixed with 100 mg of resin, and the resin was precipitated by centrifugation. Proteins remaining in the supernatant were then subjected to electrophoresis. Amount of proteins/100 mg resin (lanes): 1, 1.95 mg; 2, 1.56 mg; 3, 1.17 mg; 4, 0.78 mg; 5, 0.39 mg.

as was the case for F_1-ATPase isolated by the reference method (lane F in fig.2). In any case, under the optimal conditions, bands of contaminating proteins were very weak, indicating the high purity of the isolated F_1-ATPase.

Next, we measured the activity of F_1-ATPase obtained by the present method (table 1). In intact mitochondria, the specific activity was 0.32 units/mg protein, whereas at the final step of resin treatment using 0.78 mg protein/100 mg resin, the ATPase activity was 8.50 U/mg protein. Thus the specific activity was increased 27-fold, which is similar to the value obtained in the reference method (either 28- or 54-fold [9]) and better than that in the solvent-release method (21.9-fold [10]).

Table 1

Specific activity of F_1-ATPase at each step of purification

ATPase in	Protein[a] (mg)	Specific activity[b] (U/mg protein)	Total activity (U)
Mitochondria	7.75	0.32	2.48
Membrane fraction	5.12	0.41	2.10
Triton X-100 extract	1.56	0.24	0.37
Supernatant after resin treatment[c]	0.13	8.50	1.11

[a] Amount of protein calculated from that used for resin treatment

[b] One unit of ATPase activity is defined as that hydrolyzing 1 μmol of ATP in 1 min according to [9]

[c] F_1-ATPase obtained by treatment at 0.78 mg protein/100 mg resin (cf. lane 4 in fig.2)

The total ATPase activity decreased in the first two steps, but increased in the final step probably because of depletion of inhibitor proteins. A similar increase in total activity in the final step of isolation is observed in the solvent-release method [11]. The yield of F_1-ATPase from mitochondria by the present method was 1.6%, which is comparable to those in the reference method (0.94% [9]) and the solvent-release method (3.9% [11]). The higher yield obtained in the latter method is due to the fact that submitochondrial particles, used as starting material in this method, have already lost most hydrophilic proteins during sonication.

By using the cation exchange resin Dynospheres PD-201-SCX, we succeeded in purifying F_1-ATPase from bovine heart mitochondria in about 2 h. This time is much shorter than those required in other methods [9–11]. Under appropriate conditions, contamination of the purified F_1-ATPase with other proteins was very low and its enzyme activity was high. This rapid purification of F_1-ATPase with high ATPase activity was attributable to the fact that among the mitochondrial membrane proteins, F_1-ATPase is the only hydrophilic, water-soluble protein that is not trapped by the resin Dynospheres PD-201-SCX. We were unable to achieve similar purification of F_1-ATPase using other resins, such as Dowex 50W-X4. At present it is not clear why the resin Dynospheres PD-201-SCX is suitable for purification of F_1-ATPase. Probably the surface of the resin contains appropriate proportions of both the anionic sulfonate groups and non-polar styrene-divinylbenzene groups. A study on the adsorption mechanism of proteins solubilized by detergents is under way. In this study we purify F_1-ATPase on a small scale, but this method should also be applicable for its isolation on a large scale.

The enzyme solution obtained by this method contained Triton X-100, but the detergent did not affect the activity of F_1-ATPase purified by the reference method (data not shown). Moreover, in preliminary experiments, we isolated F_1-ATPase in the absence of the detergent, though with a lower yield. Further investigations are in progress to develop a better method for purification of F_1-ATPase without use of detergent.

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