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Purification of the subunits of pea mitochondrial F_1-ATPase by high-performance liquid chromatography

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ABSTRACT

The subunits of the F_1-ATPase from pea cotyledon mitochondria were purified by reversed-phase chromatography. The resolution of the subunits was affected by several chromatographic parameters: a reversed-phase C_8 column was superior to the less hydrophobic Bio-Gel TSK Phenyl-5-PW column for the resolution of the subunits, acetonitrile was more suitable for good separation of the subunits than 2-propanol and the flow-rate had a significant effect on peak height but little effect on the column resolving power. Tandem chromatography on two reversed-phase chromatography columns with different hydrophobicities was used in an attempt to isolate F_1-ATPase subunits directly from soluble proteins extracted from submitochondrial particles.

INTRODUCTION

The proton-translocating ATPase reversibly couples ATP synthesis and hydrolysis to the translocation of protons across energy-transducing membranes. The enzyme contains two sectors: a hydrophilic portion, F_1, and a hydrophobic membrane portion, F_0. The F_1 sector contains the binding sites for nucleotides and phosphate and is responsible for catalytic activity. Depending on the biological source of the enzyme, the isolated F_1 sector can contain five or six different subunits. Current interest is focused on the structure and function of these subunits. Several procedures have been reported for the isolation of the subunits. The δ and ε subunits of *Escherichia coli* F_1-ATPase have been purified to homogeneity by treating the enzyme with pyridine and using molecular sieve chromatography [1,2]. The use of ion-exchange celluloses (DEAE- and CM-cellulose) has been applied to resolve all five subunits of the F_1-ATPase from the thermophilic bacterium PS3 [3,4]. A similar procedure, reported by Dunn and Futai [5], involves the use of the hydroxyapatite–DEAE-Sepharose method to isolate the α, β and γ subunits of the *E. coli* coupling factor ATPase [5]. Unfortunately, the procedures used are time consuming and problems of poor resolution and low protein yields are often associated with these traditional techniques. Saishu *et al.* [6] used reversed-phase high-performance liquid chromato-

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graphy (RP-HPLC) to isolate the ε subunit from the thermophilic F_1-ATPase, but the separation of the other subunits was poor.

In this paper, we describe the use of RP-HPLC columns for the purification of F_1-ATPase subunits. The F_1-ATPase was isolated from pea cotyledon mitochondrial membranes. The purified enzyme contained six subunits, designated α, β, γ, δ, δ′ and ε in order of decreasing molecular weight [7]. The subunits from the purified pea cotyledon F_1-ATPase were separated by RP-HPLC on a SynChropak C_8 column and an attempt was also made to isolate the subunits directly from the proteins extracted from submitochondrial particles by a low-ionic strength sucrose solution.

EXPERIMENTAL

Reagents

Unless stated otherwise, chemicals and solvents were of analytical-reagent grade. HPLC-grade trifluoroacetic acid (TFA) and acetonitrile were purchased from Pierce (Rockford, IL, U.S.A.) and Fisher Scientific (Fairlawn, NJ, U.S.A.), respectively, and HPLC-grade 2-propanol from Caledon Labs. (Georgetown, Canada). Doubly distilled water was purified by passing it through Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus

The instrumentation consisted of a Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) combined with a Varian CS401 data system and coupled to a Varian UV-50 variable-wavelength detector. Three different HPLC columns were used: (1) SynChropak RP-8, 250 × 4.6 mm I.D., particle size 6.5 μm, pore size 300 Å, carbon loading *ca.* 7.5% (SynChrom, Linden, IN, U.S.A.); (2) Bio-Gel TSK Phenyl RP + reversed-phase, 75 × 4.6 mm I.D. (Bio-Rad Labs., Richmond, CA, U.S.A.); and (3) Bio-Gel TSK Phenyl-5-PW, 75 × 7.5 mm I.D. (Bio-Rad Labs.).

Purification of F_1-ATPase prior to HPLC

The pea mitochondrial F_1-ATPase was isolated as described by Horak and Packer [7]. Mitochondrial membranes were extracted with low-ionic strength sucrose solution (300 m*M* sucrose–2 m*M* tricine, pH 7.4). After centrifugation for 45 min at 100 000 *g* at 20°C, the supernatant was collected and the F_1-ATPase was purified by DEAE-cellulose column chromatography and by sucrose density gradient centrifugation [7]. The purified F_1-ATPase was subjected to HPLC.

High-performance liquid chromatography

The purified F_1-ATPase was incubated in 0.5% TFA in water for 2 h before injection. The samples were chromatographed at 22°C using a linear gradient of 1% B/min (solvent A was 0.1% TFA in water; solvent B was either 0.1% TFA in acetonitrile or 0.1% TFA in 2-propanol). The polypeptides collected from HPLC were dialysed against 50 m*M* Tris buffer containing 0.5% sodium dodecyl sulphate (SDS) (pH 7) to remove TFA and acetonitrile and then concentrated using Centricon membranes (Centricon microconcentrator; Amicon, Danvers, MA, U.S.A.).

Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [8] on a 1.5-mm slab gel consisting of a 13% separating gel and a 4% stacking gel. The gels were stained for protein with Coomassie Blue R250.

RESULTS AND DISCUSSION

Purification of F_1-ATPase subunits by reversed-phase HPLC

The native F_1-ATPase from pea cotyledon mitochondria was purified by DEAE-cellulose column chromatography and by sucrose density gradient centrifugation [7]. The enzyme, the molecular weight of which is *ca.* 400 000 daltons, is too large to be loaded into most commercially available reversed-phase columns. Therefore, a method for dissociating the enzyme complex into subunits was needed. The subunits of the F_1-ATPase were dissociated at room temperature in a 0.5% TFA–water solution (pH 2.0). The polypeptides were separated on a SynChropak C_8 column with a linear gradient of acetonitrile (1% B/min) at a flow-rate of 1 ml/min. The TFA–water to TFA–acetonitrile gradient has proved to be an excellent system for the RP-HPLC of peptides and proteins [9]. Under the chromatographic conditions used, the enzyme was well resolved into five peaks (Fig. 1). As shown in Fig. 1, all polypeptides were eluted from the reversed-phase column by a high concentration of acetonitrile, suggesting that the subunits are hydrophobic.

Because of their hydrophobic nature, the subunits recovered by lyophilization following HPLC purification were difficult to dissolve even in buffers containing detergents such as SDS or Tween 20. Therefore, an alternative method was used to improve the polypeptide recovery. The materials collected from the HPLC column were dialysed extensively against 50 m*M* Tris buffer containing 0.5% SDS (pH 7.0). The purpose of this step was to replace the TFA–acetonitrile solution directly with the Tris–SDS buffer.

The peaks on the chromatogram were labelled A, B, C, D and E in order of elution of the polypeptides (Fig. 1). The purity of the materials corresponding to peaks

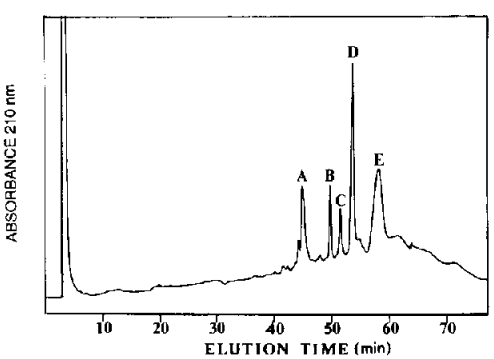

Fig. 1. Separation of subunits of the pea mitochondrial F_1-ATPase on a reversed-phase SynChropak C_8 column. Conditions: linear gradient (1% B/min) where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; sample load, 60 μg of the native F_1-ATPase which had been incubated in 0.5% TFA–water for 2 h prior to injection.

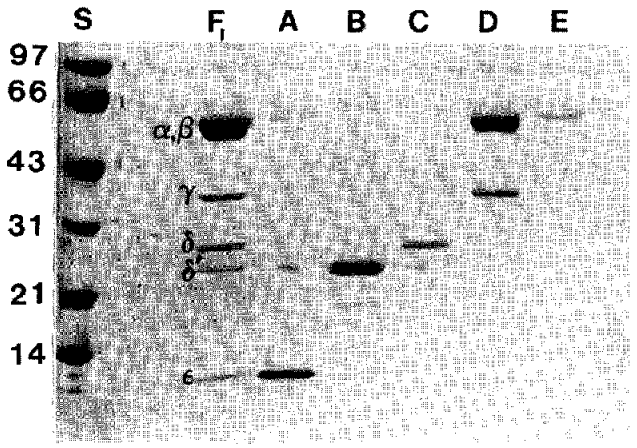

Fig. 2. SDS-PAGE of the subunits separated on the reversed-phase column in Fig. 1. Lanes: S = 10 μg of low-molecular-weight protein standards (Bio-Rad Labs.); F_1 = 10 μg of pea mitochondrial F_1-ATPase; A–C = 5 μg of the materials collected from peaks A–C in Fig. 1; D = 10 μg of the material from peak D; E = 2 μg of the material from peak E.

A–E was determined by SDS-PAGE (Fig. 2). The results indicate that the δ and α subunits are essentially pure after purification by RP-HPLC (lanes C and E, Fig. 2). The ε and δ' subunits contained minor impurities (lanes A and B, Fig. 2). Peak D contained two polypeptides corresponding to the β and γ subunits of the F_1-ATPase. To test the possibility of a disulphide link between these two subunits, the native enzyme was incubated in 5% β-mercaptoethanol and in 0.5% TFA before injection into the SynChropak column. The resulting chromatograms were identical, suggesting that a disulphide link was not responsible for the co-chromatography of these two subunits.

Comparison of reversed-phase columns which differ in their hydrophobicities

The TFA-treated F_1-ATPase was chromatographed on three different HPLC columns using identical eluting conditions. The three columns differ in their hydrophobicities in the following order: SynChropak RP-8 > TSK Phenyl RP+ > TSK Phenyl-5-PW. The SynChropak and TSK Phenyl RP+ columns showed much better overall resolution than the TSK Phenyl-5-PW column (Fig. 3). The SynChropak column was superior to the TSK Phenyl RP+ column for the resolution of the larger F_1-ATPase subunits (peaks D and E in Fig. 3).

Effect of the organic solvent on subunit separation

In our RP-HPLC system the protein samples were applied in water and eluted with a linear gradient of the organic solvent (acetonitrile or 2-propanol). Addition of a counter ion (TFA) in both water and organic solvent helped to minimize interactions between the silanol groups of the column and the positively charged groups of the protein [10,11]. Of the two organic solvents used, 2-propanol has a higher eluotropic strength than acetonitrile [12–14]. As seen in Fig. 4, the subunit elution times were

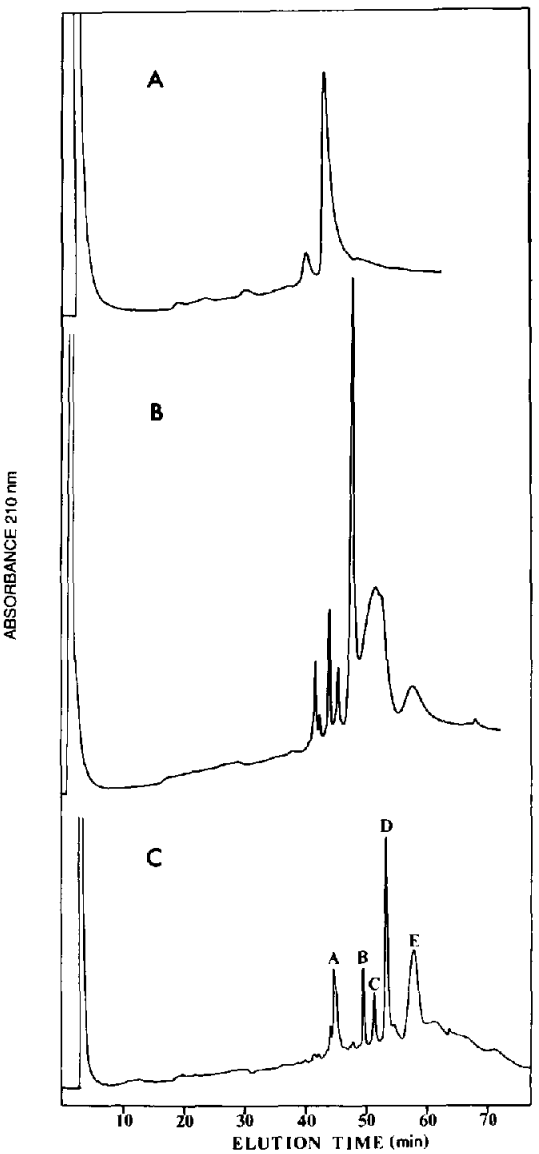

Fig. 3. Separation of subunits of the pea mitochondrial F_1-ATPase on three HPLC columns. Top, Bio-Gel TSK Phenyl-5-PW column; middle, Bio-Gel TSK Phenyl RP+ column; bottom, SynChropak C_8 column. Conditions: linear gradient (1% B/min) where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; sample load, 60 μg of the F_1-ATPase (top and bottom) and 160 μg of the F_1-ATPase (middle).

significantly reduced when acetonitrile was replaced with 2-propanol as the organic solvent. However, much better resolution of the subunits was obtained when acetonitrile was used.

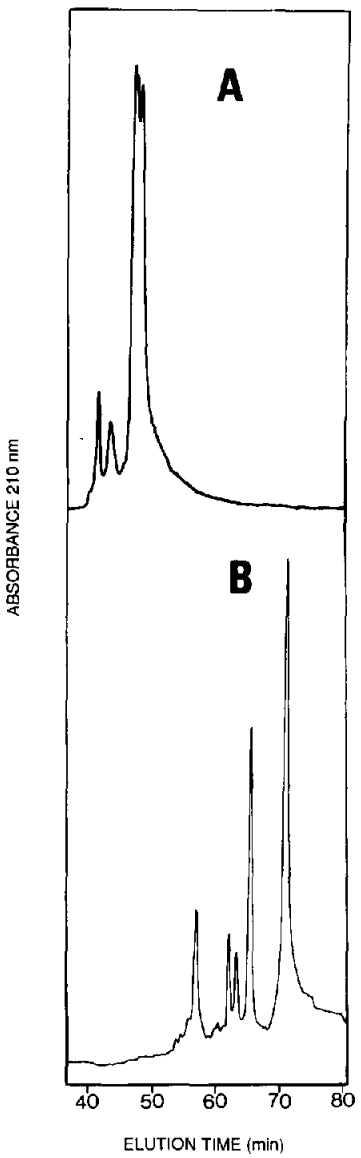

Fig. 4. Effect of the organic solvent on the separation of F_1-ATPase subunits on reversed-phase SynChropak C_8 column. Organic solvent: top, 2-propanol; bottom, acetonitrile. Conditions: linear gradient (1% B/min), where A was 0.1% TFA in water and B was 0.1% TFA in one of the above organic solvents; flow-rate, 0.5 ml/min; sample load, 60 μg of the F_1-ATPase for each injection.

Effect of flow-rate on subunit separation

F_1-ATPase subunits were separated on the SynChropak column using a linear gradient of 1% B/min at a flow-rate of 0.5 ml/min (Fig. 4B) or 1 ml/min (Fig. 1). The resolution of the polypeptides was similar when the flow-rate was changed from 1 to 0.5 ml/min, but the peak height was significantly increased.

Purification of the F_1-ATPase subunits from the soluble proteins extracted from submitochondrial particles

Approximately 30% of the ATPase activity present in the low-ionic strength extract of mitochondrial membranes is recovered as the purified enzyme after sucrose density gradient centrifugation [7]. In view of the high resolving power of reversed-phase HPLC, we hoped that by using RP-HPLC the F_1-ATPase subunits could be purified with a higher recovery directly from the mitochondrial membrane extracts. However, we found that RP-HPLC on the SynChropak C_8 column alone was not sufficient to resolve the ATPase subunits from the complex mixture of polypeptides present in the extract (results not shown). To reduce the complexity of the polypeptide mixture applied to the SynChropak column, a preliminary fractionation of the mitochondrial extracts was performed on a Bio-Gel TSK Phenyl RP+ column (Fig. 5). The extracts were applied to the column directly without pretreatment with 0.5% TFA–water. Five protein fractions were eluted using conditions described in Fig. 5. The polypeptide composition of these fractions was analysed by SDS-PAGE. Fraction No. 4 contained all the subunits of the F_1-ATPase in addition to other polypeptides. The proteins from this fraction were incubated for 2 h in 0.5% aqueous TFA (pH 2) and then rechromatographed on the SynChropak column as described in Fig. 6A. Purified F_1-ATPase was also chromatographed on this column (Fig. 6B). The elution times of the subunits of purified F_1-ATPase were used to identify the corresponding peaks of the chromatogram of fraction No. 4. It is evident from Fig. 6A that the α, β and γ subunits of F_1-ATPase (peaks E and D, respectively; see also Figs. 1

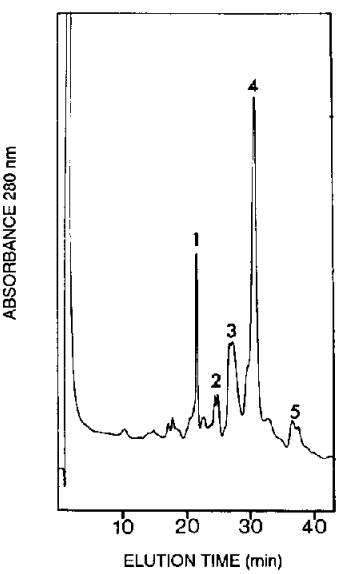

Fig. 5. Fractionation of soluble proteins extracted from pea mitochondrial membranes on a Bio-Gel TSK Phenyl RP+ column. Conditions: a linear gradient from 0 to 30% B in the first 20 min, then a gradient from 30 to 50% B from 20 to 60 min and a final gradient from 50 to 80% B from 60 to 80 min; solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; sample load, 440 μg of soluble proteins extracted from pea mitochondrial membranes.

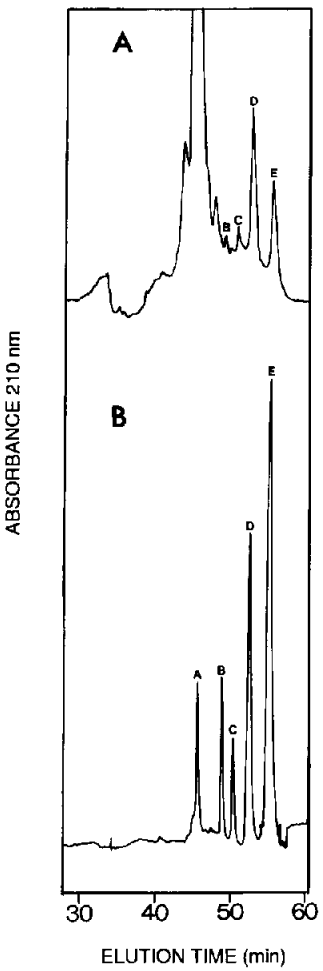

Fig. 6. Rechromatography of the proteins collected from peak 4 in Fig. 5 on a reversed-phase SynChropak C_8 column. Sample: top, proteins collected from peak 4 in Fig. 5; bottom, purified F_1-ATPase. Conditions: linear gradient (1% B/min), where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile; flow-rate, 1 ml/min.

and 2) are well resolved from the remaining polypeptides. The smaller F_1-ATPase subunits (δ, δ' and ε in peaks C, B and A, respectively) did not separate sufficiently from the other polypeptides that were present in fraction No. 4.

The results indicate that utilizing the HPLC methods described above, only the three larger subunits (α, β and γ) could be purified directly from mitochondrial extracts. However, we have shown that the smaller δ and δ' and ε subunits could be separated by RP-HPLC of the purified F_1-ATPase.

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