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HPLC PURIFICATION OF THE NATURAL ATPase INHIBITOR FROM THE YEAST **Candida utilis**

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SUMMARY. A rapid method of preparation of the natural ATPase inhibitor (IF_1) from the mitochondria of the yeast **Candida utilis** has been developed. It involved high performance liquid chromatography (HPLC) as the final step of purification. An active form of **Candida utilis** IF_1 was obtained, free of contaminant. Its properties are compared with those of IF_1 from other sources.

The mitochondria from the yeast **Candida utilis** contain an ATPase inhibitor protein (IF_1). This protein of MW ≃ 7300 has been purified (1) and found to inhibit not only the ATPase activity of **C. utilis** F_1, but also that of beef heart F_1 (2). Inhibition of ATPase activities of both **C. utilis** F_1 and beef heart F_1 by the heart inhibitor has also been observed (2). This was surprising since the two inhibitor proteins show striking differences in molecular weight, isoelectric point and amino acid composition (1,3). To explore in more detail the cross-reactivity of **C. utilis** IF_1 and to obtain information on its interaction with F_1 at the molecular level, a rapid method of preparation of **C. utilis** IF_1 was set up ; it is based on the use of HPLC for the final purification step and allowed to prepare an active form of inhibitor, free of contaminant.

MATERIALS AND METHODS

To obtain a high yield of synthesis of IF_1, **C. utilis** (strain CBS 1516) was grown at 28°C under aerobic conditions in a liquid medium containing 2% bacto peptone (Difco), 1% yeast extract (Difco) and 3% glycerol as carbon source (1). Yeast cells were harvested at the plateau phase and mitochondria prepared from mechanically disrupted

Abbreviations: IF_1 : natural inhibitor of mitochondrial ATPase ; F_1 : catalytic sector of the ATPase complex ; HPLC : High performance liquid chromatography ; MW : molecular weight ; SDS-PAGE : polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate ; TFA : trifluroacetic acid ; TCA : trichloroacetic acid.

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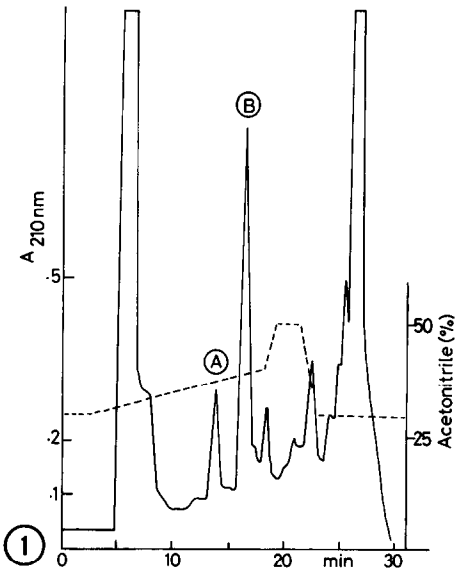
cells (4). The first steps of preparation of **C. utilis** IF_1 were essentially as described in (5) for beef heart IF_1, i.e alkaline treatment followed by ammonium sulfate precipitation at 41% and 68% saturation. The precipitate recovered at 68% ammonium sulfate was resuspended in 0.25 M sucrose, precipitated by 10% trichloroacetic acid (TCA), resolubilized at pH 5.0, centrifuged and brought to pH 7.0. At this stage, the protein solution was subjected to HPLC (Waters Associates S.A Instruments). 0.5 to 1 ml of the protein solution was injected into a preparative reverse-phase (RP) column C_{18} μBondapak (Waters) (7,8 x 250 mm) equilibrated at 32°C in 10 mM trifluoroacetic acid (TFA)(Fluka) and 25% acetonitrile (Carlo Erba). A gradient of acetonitrile was applied as described in the legend of figure 1, at a flow rate of 2 ml/min for 30 min. Elution was monitored at 210 nm. Each peak was collected separately and analyzed a) by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS-PAGE) ; b) by amino acid analysis after total hydrolysis ; c) by measurement of the inhibition of ATP hydrolysis by beef heart F_1.

The protein concentration was determined as described in (6), using bovine serum albumine as standard. SDS-PAGE was performed in a buffer system as described by (7), with 10% polyacrylamide as concentration gel and 20% polyacrylamide as separation gel. Proteins were stained by Coomassie brilliant Blue R 250. Protein samples were hydrolysed under vacuum in a TFA : HCl mixture (1 : 2, v/v) containing 0.01% phenol at 166°C for 25 or 50 min (8). Amino acid analysis was performed by HPLC with a CAT EX resin column (Waters) following the manufacturers instructions. Amino acid residues were post-derivatizated with O-phtalaldehyde (OPA) and detected by fluorescence at 420 nm, at 1 nanomole full scale. The proline content was measured after oxidation with hypochloride.

The inhibitory activity of IF_1 on beef heart membrane bound ATPase was measured as described in (9).

RESULTS AND DISCUSSION

As reported in Methods, the last step of the purification procedure of **C. utilis** IF_1 was carried out by HPLC on a RP-column. Figure 1 shows the elution profile recorded at 210 nm and the gradient of acetonitrile. Only the results concerning peak A and B will be reported. Peak A eluted with a retention time of 13.8 min (36.5 % acetonitrile). It was analyzed by SDS-PAGE and found to contain a single protein which migrates with an apparent MW of 6000 (figure 2). Its amino acid composition (table I) indicates the absence of Met and Cys. A pHi value of 5.25 was determinated by isoelectrofocusing. The peak A protein was devoided of inhibitory capacity towards F_1-ATPase and had no effect on the interaction of purified IF_1 with membrane bound ATPase. The protein of peak B eluted with a retention time of 16.2 min (38.5% acetonitrile). It was identified as **C. utilis** IF_1 by the following criteria : a) it possessed the property to inhibit F_1-ATPase (figure 3) ; b) on SDS-PAGE a single band was stained by Coomassie Blue ; its migration corresponded to an apparent MW of 5000



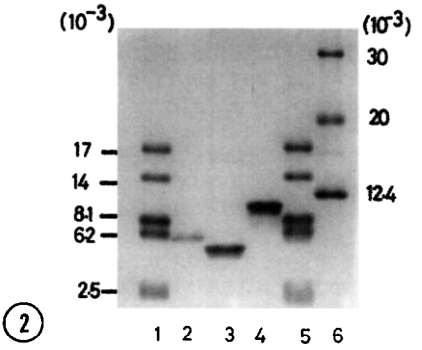


Figure 1 : Purification of **C. utilis** IF_1 by HPLC on RP-column : C_{18} µBondapak. Elution profile recorded at 210 nm with a gradient of acetonitrile obtained by mixing 50% acetonitrile in 10 mM TFA with 10 mM TFA (– – – – –). Flow rate : 2 ml per min.

Figure 2 : Separation by SDS-PAGE. Slots 1, 5 and 6 : MW markers ; slot 2 : peak A protein ; slot 3 : peak B protein (**C. utilis** IF_1) ; slot 4 : beef heart IF_1.

Table I : Amino acid composition of proteins from peaks A and B (**C. utilis** IF_1) eluted from HPLC RP-column, and from IF_1 of yeast **Saccharomyces cerevisae** (10) and beef heart (3)

	residues			
	Peak A	Peak B **C. utilis** IF_1	**S. cerevisae** IF_1	Beef heart IF_1
Asp	9	8	5	7
Thr	3-4	5-6	4	0
Ser	4-5	3	7	7
Glu	8	13	13	18
Pro	4	0	1	0
Gly	5	3-4	4	6
Ala	4-5	6-7	1	10
Cys	0	0	0	0
Val	3	0	2	2
Met	0	0	1	0
Ile	2-3	2	2	4
Leu	4	7	4	4
Tyr	2-3	1-2	0	1
Phe	3	1	3	2
His	1-2	1	1	5
Lys	5	10	8	10
Arg	2	3	7	8
Trp	nd	0	0	0
total	59-65	63-67	63	84
MW *	≃ 7 000	≃ 7 300	7 383	9 607
MW from gel	6 000	5 000	10 000	10 000
pHi	5.25	5.7	9.05	7.7

nd : not determinated ; MW * : calculated from amino acid composition.

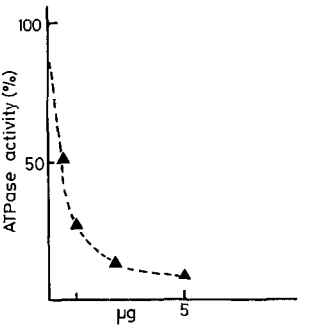


Figure 3 : Inhibition of beef heart membrane-bound F_1 by **C. utilis** IF_1 purified by HPLC.

(figure 2). The amino acid composition of **C. utilis** IF_1 indicated the presence of 63-67 residues, the absence of Pro, Met, Cys and Val. Based on the amino acid composition, a minimum MW of 7300 was calculated (table I).

In the present method of preparation of **C. utilis** IF_1, the number of purification steps was quite reduced, compared to the previously reported preparation (1), which involved DEAE chromatography, isoelectrofocusing and several TCA precipitations. These steps were advantageously replaced by HPLC, inasmuch as IF_1 is a minor protein. The yield of purification was considerably improved.

IF_1 purified by HPLC retained its inhibitory capacity, even after elution with 38.5% acetonitrile. An amount of 0.5 µg of HPLC purified protein (peak B) was required to inhibit 50% of the ATPase activity of a preparation of mitochondrial particles depleted in inhibitor (25 µg protein, specific ATPase activity : 5-6 µmol Pi released/min per mg protein) (figure 3). When beef heart IF_1 was subjected to HPLC under the conditions described in figure 1 ; it eluted earlier than yeast IF_1 (retention time of 11 min ; 35% acetonitrile) ; it migrated on SDS-PAGE as a 10000 MW protein ; but it lost nearly 60% of its inhibitory capacity.

Two values of molecular weight have been determinated for **C. utilis** IF_1 : 7300 calculated from amino acid analysis and 5000 by SDS-PAGE estimated by comparison with comigrating standard peptides (BDH Chemicals). For molecular weights below 8000, the direct relation between the log of MW and the migration distance does not seem to hold rigourously. An abnormal behavior in SDS-SPAGE was also reported for

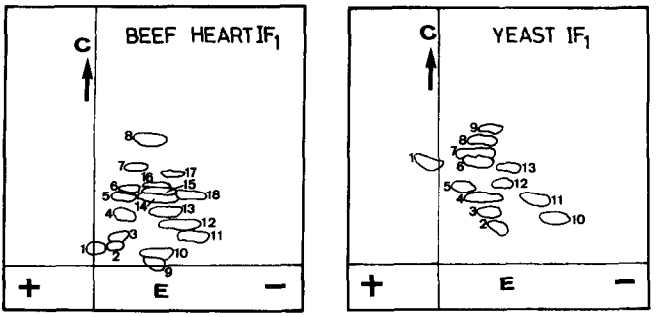


Figure 4 : Maps of tryptic digests of beef heart IF_1 and **C. utilis** IF_1 on cellulose thin layer (9). To obtain separation of peptides, electrophoresis (E) was run, for beef heart IF_1 at pH 4.4 (pyridine/acetic acid/acetone/H_2O, 20/40/160/800, v/v) and for **C. utilis** IF_1 at pH 6.5 (pyridine/acetic acid/H_2O, 25/1/475, v/v). The solvent for chromatography (C) was : butanol/pyridine/acetic acid/H_2O, (30/20/6/24, v/v). The peptides were revealed by ninhydrin staining.

the yeast **S. cerevisae** IF_1 : a MW of 7383 was calculated from the amino acid sequence, although the rate of migration corresponded to a MW of 10000 (10).

The amino acid composition of **C. utilis** IF_1 (table I) is slightly different from that given in (1), due to the removal of a contaminant, which was identified as the peak A protein by HPLC. The peak A protein contains Pro and Val, which are absent in the peak B corresponding to pure IF_1. From the amino acid composition and taking into account the content of Pro and Val given in (1), about 25% of contamination by peak A protein can be estimated in **C. utilis** IF_1, after isoelectrofocusing step as described in (1). The similarity of the pHi and MW values of peak A protein (5.25 and 6000 on gel), and pure IF_1 (peak B)(5.7 and 5000 on gel), easily explains the difficulty in separating pure IF_1 from the contaminant corresponding to peak A by conventional methods. By using HPLC, the peak B and peak A proteins were separated by more than 2 minutes during the elution of the column.

Although the F_1-ATPases from beef heart and **C. utilis** cross react with the corresponding ATPase inhibitors there appear to be significant differences in the amino acid compositions and tryptic maps of the two inhibitors (figure 4), suggesting that the amino acid sequences are different. The determination of the primary structure of **C. utilis** IF_1 purified by HPLC is currently in progress in this laboratory.

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REFERENCES

1. Satre M., De Jerphanion M-B., Huet J. and Vignais P.V. (1975) Biochim. Biophys Acta 387. 241-255.
2. Klein G., Satre M. and Vignais P.V. (1977) FEBS Lett. 84, 129-134.
3. Brooks J.C. and Senior A.E. (1971) Arch. Biochem. Biophys. 147, 467-470.
4. Lauquin G. and Vignais P.V. (1973) Biochim. Biophys. Acta 305, 534-556.
5. Horstman L.L. and Racker E. (1970) J. Biol. Chem. 245, 1336-1344.
6. Bradford M.M. (1976) Anal. Biochem. 72, 248-254.
7. Laemmli U.K. and Favre M. (1973) J. Mol. Biol. 80, 575-599.
8. Tsugita A. and Scheffler J-J. (1982) Eur. J. Biochem. 124, 585-588.
9. Klein G., Satre M., Dianoux A-C. and Vignais P.V. (1980) Biochemistry 19, 2919-2925.
10. Matsubara H., Hase T., Hashimoto T. and Tagawa K. (1981) J. Biochem. 90, 1159-1165.