CHROMSYMP. 1366

PURIFICATION OF CYTOCHROME *c* OXIDASE FROM CORN ROOT MITOCHONDRIA BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

NICHOLAS PARRIS*, HELEN A. GRUBER, SAMUEL M. MOZERSKY and ROBERT A. BARFORD

U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 1600 East Mermaid Lane, Philadelphia, PA 19118 (U.S.A.)

SUMMARY

Cytochrome *c* oxidase (COX) was isolated for the first time from corn root mitochondrial extracts by high-performance hydrophobic interaction chromatography with a 50 mM phosphate buffer (pH 7.2), containing 0.01% Tween 20. A 91% recovery of the total enzyme activity was achieved, along with a twelve-fold increase in specific activity. COX activity recovered from the column decreased rapidly with the age of samples. However, most activity was restored on the addition of phospholipid or non-ionic detergent. The crude oxidase was also fractionated with saturated ammonium sulfate in the presence of various detergents. Ionic and non-ionic detergents had variable effects on the release of the enzyme from corn root membrane as well as on its fractionation with ammonium sulfate.

INTRODUCTION

Cytochrome *c* oxidase (COX), a component of the electron transport complex of the inner mitochondrial membrane, has previously been isolated from mammalian and bacterial sources, but not from plant sources[1,2]. COX catalyzes electron transfer from ferrocytochrome *c* to molecular oxygen and functions as an energy transducer, which converts the energy of electron transfer to a driving force for ATP synthesis. Studies on the activity of cytochrome *c* oxidase have been complicated by functional dependence on its native hydrophobic environment. It is essential to establish optimum accessibility of cytochrome *c* binding sites without denaturing the protein complex. Removal of the enzyme complex from its membrane environment may lead to dissociation of protein subunits of COX or deplete the complex of phospholipids, which are essential for catalytic activity[3]. Most methods for its purification include fractionation with ammonium sulfate solutions. This paper describes, for the first time, the isolation of cytochrome *c* oxidase from corn root mitochondria with the aid of high-performance hydrophobic interaction chromatography (HPHIC). The effect of various detergents on release of the enzyme from the mitochondria and the loss of enzyme activity on fractionation with ammonium sulfate are also discussed.

MATERIALS AND METHODS

Isolation of cytochrome c oxidase

Corn root homogenates (50 g, fresh weight) were prepared as described by Nagahashi and Kane[4]. The corn roots were homogenized, and *ca.* 0.7 g wet weight of the mitochondria was isolated as a pellet after centrifugation by the method of Caldwell *et al.*[5]. The pellet containing the mitochondria was suspended in 4 ml of 50 mM Tris–HCl, 0.6 M sucrose, 1 mM histidine–HCl buffer (pH 8.0) containing 3% (w/v) detergent and release of COX activity was measured at 4°C with occasional agitation (Fig. 1).

The enzyme was purified by fractionation of the mitochondrial susension immediately, with ammonium sulfate in the absence of potassium chloride according to a previously described procedure[6]. Alternatively, the suspension was incubated in sodium cholate (24 h, 4°C) to release the enzyme and then clarified by centrifugation (7000 g, 20 min, 4°C). The resulting supernatant was injected into the HIC column.

Protein and enzyme activity assays

The protein contents of the corn root mitochondria fraction and purified COX were measured by the dye binding standard protein assay[7], using bovine serum albumin as the standard.

COX was determined essentially as described by Hodges and Leonard[8], except that dodecylmaltoside was substituted for digitonin as detergent. The absorbance was monitored for 3 min with a Shimadzu UV-240 spectrophotometer. Rates were linear and values were extrapolated to zero time to obtain initial velocity.

HIC

The chromatographic system used in this study consisted of an SP 8700 solvent delivery system equipped with an SP 4270 computing integrator (Spectra-Physics, Piscataway, NJ, U.S.A.) a 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a V[4] variable-wavelength absorbance detector (ISCO, Lincoln, NE, U.S.A.). Clarified enzyme, 25 μl, was injected into an LC-HINT (5-μm) column 100 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.); mobile phase, 50 mM sodium phosphate (pH 7.2), containing 0.01% Tween 20; flow-rate, 0.5 ml/min; detector, 0.2 a.u.f.s.; chart speed, 0.5 cm/min.

RESULTS AND DISCUSSIONS

Previous reports regarding the purification of COX from mammalian and bacterial sources included solubilization of the enzyme in various detergents followed by fractionation with ammonium sulfate[1–4,6]. In this study, detergents were selected to simulate more accurately the natural environment of the membrane-bound protein in order to stabilize the protein structure and maintain enzymatic activity. Maximum release of COX from the mitochondrial preparation was obtained after 24 h for most detergents tested (Fig. 1). After 24 h, enzyme activity decreased rapidly in octyl glucoside. Optimal retention of activity was achieved by storage in Triton X-100 at – 10°C. Precipitation of the enzyme by ammonium sulfate was also influenced by the
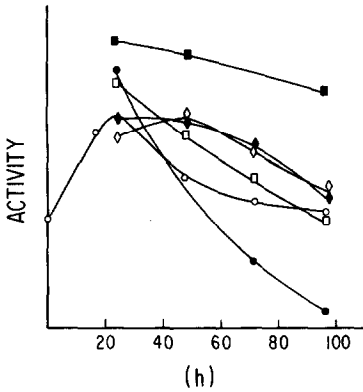
Fig. 1. Release of COX on incubation of mitochondria with various detergents. Detergents tested: sodium cholate (○); Brij 52 (◇); Tween 20 (◆); Triton X-100 (□); octyl glucoside (●); Triton X-100 (− 10°C) (■). All incubations were at 4°C except where otherwise indicated.

nature of the detergent (Table I). Most of the enzyme activity was precipitated by addition of 10% saturated ammonium sulfate in sodium cholate. Cytochromes precipitated from 35% saturated ammonium sulfate, and these were identified by spectral analysis. The bulk of the enzyme was soluble in 60% saturated ammonium sulfate in the presence of sodium deoxycholate and dodecylmaltoside. Incubation of the mitochondria in cholate buffer before ammonium sulfate fractionation (Table I) resulted in a change in the distribution of the enzyme; a significant amount of the activity was found in the soluble fraction. In addition, 75% of its activity was lost relative to the total activity recovered when fractionation was carried out immediately after the crude enzyme was isolated.

Since the ammonium sulfate fractionation greatly reduced the activity of the crude enzyme, we explored the potential of chromatographic techniques to purify the enzyme. HIC on phenyl- and octyl-Sepharose CL-4B has been used for the preparative-scale isolation of heart COX with retention of activity[1,5]. Since we estimated

TABLE I

FRACTIONATION OF COX ACTIVITY WITH AMMONIUM SULFATE IN THE PRESENCE OF VARIOUS DETERGENTS

Expressed as percent total recovered activity for single harvest.

Detergent (3%, w/v)	Ammonium sulfate fractions (% of saturation)*				
	10P	35P	60P	60S	
Cholate	95	4	1	0	
Cholate**	71	5	0	24	
Deoxycholate	20	0	0	80	
Dodecylmaltoside	8	0	0	92	

    * P = Precipitate; S = supernatant.
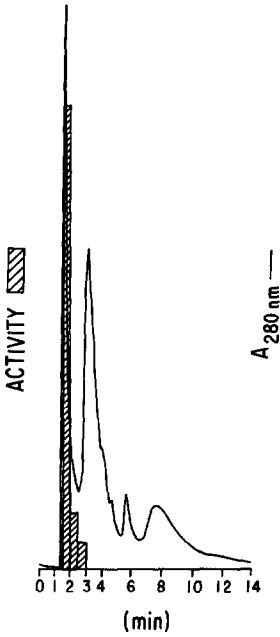   ** Mitochondria incubated in detergent for 1 h.

Fig. 2. Isolation of COX by HPHIC.

that only 1–2 mg of the purified enzyme could be obtained from a single corn root harvest we decided to use a HPHIC system, which required much less sample. Separation of the clarified enzyme by HPHIC permitted COX to be eluted in 1.64 min, with a minimal overlap with other proteins, based on activity measurements (Fig. 2).
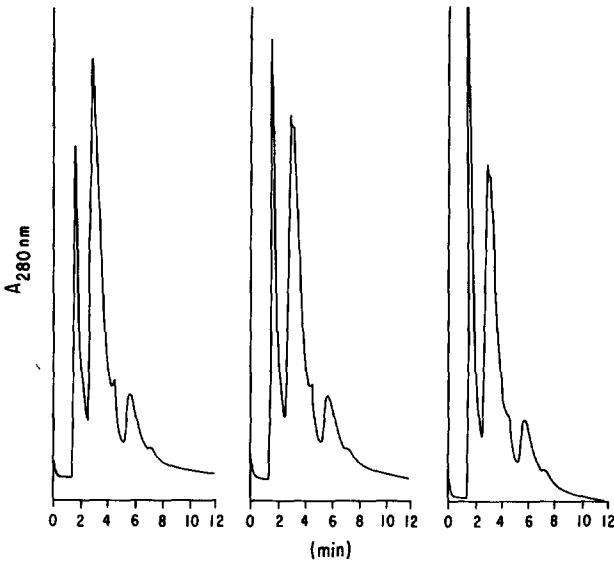

Fig. 3. HPHIC separation of COX after consecutive injections.

TABLE II

SUMMARY OF COX PURIFICATION

Source	Protein (mg/ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (mmol/min g)	Purification factor
Crude enzyme (mitochondria)	4.4	26.8	1.3	0.05	1 ×
Clarified enzyme	3.3	18.5	9.6	0.52	10 ×
HIC purified enzyme	0.02	2.2	13.7	6.1	122 ×

Clarified COX preparations were unstable. Their chromatographic profiles changed after 24 h with complete disappearance of the peak which was eluted in 8 min. Consecutive injections of the COX preparation resulted in an increase in the A_{280} values for the peak containing the enzyme (Fig. 3). This phenomenon was attributed to protein from the previous injection being eluted with the enzyme. This protein was removed by injections of 1% (w/v) aq. dodecylmaltoside between chromatograms. The specific activity of the crude enzyme increased ten-fold after clarification by centrifugation and increased by about another twelve-fold after purification by HIC (Table II). The specific activity of the HIC-purified corn root enzyme compares favorably with reported values between 15 and 20 mmol/min · g for beef heart cytochrome c oxidase[1]. HIC separations were carried out wihtin a few hours after the COX clarification step to maximize recovery of enzyme activity from the analytical column. Within three days, the total enzyme activity recovered from the column decreased from 91% to 10% of the applied sample. Loss of activity may be due to removal of enzyme bound lipid by the column. Boundary phospholipids reportedly were depleted from beef heart COX by HIC[1]. Addition of phosphatidylcholine or Tween 20 restored 70% and 50% of the corn root COX activity but phosphatidylglycerol had not effect.

In summary, purification of corn root COX by HPHIC avoids the use of ammonium sulfate, which greatly reduces the activity of the crude enzyme. The method is rapid and the enzyme can be recovered in good yields.

REFERENCES

1 M. Tanaka and T. Ozawa, Biochem. Int., 5 (1982) 67.
2 W. DeVrij,. A. Azzi and W. N. Konigs, Eur. J. Biochem., 131 (1983) 97.
3 N. S. Robinson, F. Strey and L. Talbert, Biochemistry, 19 (1980) 3656.
4 G. Nagahashi and A. P. Kane, Protoplasma, 112 (1982) 167.
5 K. D. Caldwell, S. M. Mozersky, G. Nagahashi and R. A. Barford, Sedimentation Field Flow Fractionation of Corn Root Membranes in Chemical Separation, Vol. 1, Litarvan Literature, Denver, CO, 1986, p. 59.
6 S. Rosen, Biochem. Biophys. Acta, 523 (1978) 314.
7 Bio-Rad Standard Protein Assay, Technical Bulletin, No. 1069, Bio-Rad, Richmond, CA, 1981.
8 T. K. Hodges and R. T. Leonard, Methods Enzymol., 32 (1974) 398.