Phytochemistry, 1971, Vol. 10, pp. 977 to 980. Pergamon Press. Printed in England.

PURIFICATION OF CYTOCHROME OXIDASE FROM MITOCHONDRIA OF HIGHER PLANTS

HARI K. SRIVASTAVA* and IGOR V. SARKISSIAN
Institute of Life Science, Texas A & M University, College Station, Texas, U.S.A.

(*Received* 3 *August* 1970)

Abstract—A simple procedure for the purification of cytochrome oxidase from mitochondria of higher plants is described. The enzyme from wheat, beans, cauliflower and sweet potato was purified 20-, 15-, 20- and 11-fold respectively. Differences in specific activity and Km were observed for the different species. The qualitative differences in cytochrome oxidase activity may be due to the interspecific genetic differences.

INTRODUCTION

CYTOCHROME c oxidase is the principal terminal oxidase of high oxygen affinity in the aerobic metabolism of all animals, plants, yeast and some bacteria. It is clear that in plants cytochrome oxidase is the major terminal oxidase restricted to the mitochondria and that copper-containing oxidases, such as ascorbate oxidase or phenolases, play only a minor role.[1]

Cytochrome oxidase preparations have been obtained from many different organisms including higher plants.[2,3] However, since activity of the cytochrome oxidase system can be easily measured in intact plant mitochondria or submitochondrial particles, many investigators did not find it necessary to solubilize and purify the enzyme from the particulate material in their studies (for example see Refs. 4, 5). Ever since cytochrome oxidase was first solubilized from heart muscle preparations,[6] considerable progress has been made on purification of this enzyme from animal material.[7-9] Recently the cytochrome oxidase has been purified from yeast.[10,11] With the exception of one report where the cytochrome oxidase was partially purified from soybean roots,[12] no information is available in the literature concerning the purification of cytochrome oxidase from particulate fractions of higher plants. During our studies of mitochondrial complementation and heterosis,[13,14]

* Present address: Department of Biochemistry, The University of Edinburgh Medical School, Edinburgh, Scotland.

[1] M. R. LEMBERG, *Physiol. Rev.* **49**, 48 (1969).
[2] B. CHANCE, W. D. BONNER and B. T. STOREY, *Ann. Rev. Plant. Physiol.* **19**, 295 (1968).
[3] LUCILE SMITH and B. CHANCE, *Ann. Rev. Plant Physiol.* **9**, 449 (1958).
[4] B. CHANCE and W. D. BONNER, *Plant. Physiol.* **40**, 1198 (1965).
[5] A. A. KHAN, C. P. TEWARI, P. S. KRISHNAN and G. G. SANWAL, *Plant Physiol.* **43**, 1461 (1968).
[6] F. B. STRAUB, *Hoppe-Seylers Z. Physiol. Chem.* **268**, 227 (1941).
[7] D. E. GRIFFITHS and D. C. WHARTON, *J. Biol. Chem.* **236**, 1850 (1961).
[8] K. MINNEART, *Biochim. Biophys. Acta* **54**, 26 (1961).
[9] T. YONETANI, *J. Biol. Chem.* **236**, 1680 (1961).
[10] H. M. DUNCAN and B. MACKLER, *J. Biol. Chem.* **241**, 1695 (1966).
[11] I. SEKUZU, H. MIZUSHIMA, S. HIROTA, T. YOBISUI, X. MATSUMURA and K. OKUNUKI, *J. Biochem., Tokyo* **62**, 710 (1967).
[12] G. W. MILLER, H. J. EVANS and E. SISLER, *Plant. Physiol.* **33**, 124 (1958).
[13] I. V. SARKISSIAN and H. K. SRIVASTAVA, *Proc. Natl. Acad. Sci. U.S.* **63**, 302 (1969).
[14] I. V. SARKISSIAN and R. G. MCDANIEL, *Proc. Natl. Acad. Sci. U.S.* **57**, 1262 (1967).

we observed these two phenomena with respect to cytochrome oxidase activity in intact mitochondria. It was of interest then, to study the activity of purified cytochrome oxidase. In this paper we describe a simple procedure of purification of cytochrome oxidase from isolated mitochondria of several species of higher plants and show some kinetic properties of the enzyme prepared from these species.

RESULTS AND DISCUSSION

The results of a typical fractionation of cytochrome oxidase are summarized in Table 1. In general, a 10- to 20-fold increase in specific activity over the initial mitochondria was attained.

TABLE 1. FRACTIONATION OF CYTOCHROME OXIDASE OF FOUR PLANT SPECIES

Preparation	Specific activity μmole/min/mg protein	Purification, fold
Wheat shoot		
Mitochondria	0·318	
Deoxycholate-KCl Supernatant	0·622	
Ammonium Sulfate, 0–5%	1·590	
5–10%	6·36	20
Bean hypocotyl		
Mitochondria	0·188	
Deoxycholate-KCl Supernatant	0·432	
Ammonium Sulfate, 0–5%	1·320	
5–10%	2·820	15
10–15%	1·076	
Cauliflower		
Mitochondria	0·125	
Deoxycholate-KCl Supernatant	0·375	
Ammonium Sulfate, 0–5%	0·400	
5–10%	0·625	
10–15%	1·075	
15–20%	1·500	
20–30%	2·570	20
Sweet potato tuber		
Mitochondria	0·0325	
Deoxycholate-KCl Supernatant	0·0715	
Ammonium Sulfate, 0–5%	0·0487	
5–10%	0·3575	11

Preparation of cytochrome oxidase from wheat shoot mitochondria catalyzed the oxidation of 4–6 μmoles of ferrocytochrome c/min/mg of protein when assayed as described in the Experimental, representing a 20-fold increase in specific activity over the starting material. Preparations of cytochrome oxidase of the next highest purity were from mitochondria of cauliflower and bean hypocotyl and with specific activity of 7·8 and 2·6, respectively, representing a 20- and 15-fold increase in specific activity over the starting material. Preparations of the lowest purity (specific activity of 0·36) were from mitochondria of sweet potato tuber. The results emphasize differences between the species with regard to cytochrome oxidase. Cytochrome oxidase from wheat, bean and cauliflower exhibited about 18, eight, and seven times greater specific activity, respectively, as compared

to that of sweet potato enzyme. This may mean that wheat shoot, bean hypocotyl or cauliflower head have an efficient cytochrome oxidase system. This is understandable since these are, in essence, growing tissues, while the sweet potato tuber is a storage tissue.

Table 1 demonstrates differences in the recovery of cytochrome c by ammonium sulfate from the different species. The enzyme from wheat, bean and sweet potato was recovered in the 5–10% fraction while the cauliflower enzyme appeared in the 20–30% fraction. These results may be due to interspecific mitochondrial differences or enzymatic differences between these species. Similar suggestion has been made in case of cytochrome oxidase from cytoplasmic mutant strains of *Neurospora*,[15] where the enzyme of the poky mutant was recovered in a 12–16% ammonium sulfate fraction while the wild-type enzyme appeared in a 16–20% fraction.

Differences were observed in Michaelis constant for the enzyme isolated from various sources. Cytochrome oxidase from wheat and sweet potato showed relatively low Km (55 and 38·5 μM respectively) whereas bean and cauliflower enzyme exhibited higher Km's (100 and 142 μM). Difference in V_{max} of the enzyme prepared from wheat, bean and sweet potato was not pronounced (3·3, 4·0, 6·6 and 3·1 nmoles/min respectively). The V_{max} of the enzyme from cauliflower was higher as compared to the other species. While the small differences in V_{max} of cytochrome oxidase from different species were inconclusive, the data on Km support our view of existence of interspecific differences in cytochrome oxidase. Differences in Km for mitochondrial malic dehydrogenase in several mutant strains of *Neurospora* have led to the suggestion that the genetically specified structure of the protein determined the functional and kinetic properties of the enzyme.[16] An analogous situation may exist in the different species on which we report here. Although the nature of the components of cytochrome oxidase and their precise genetic origins in these species are not known, the results presented point out the existence of qualitative functional differences between cytochrome oxidases of different species. The methods described in this paper should make it possible to critically assess the cytochrome oxidase of higher plants.

EXPERIMENTAL

The following plant species were used: wheat (*Triticum aestivuum*, L), bean (*Phaseolus vulgaris*), cauliflower (*Brassica oleracea*) and sweet potato (*Ipomea batatas*). Mitochondria were isolated from the following tissues: shoots (sprouts) of 2–3-day-old wheat seedlings which had been grown at 27° in the dark on moistened paper toweling; from hypocotyl of 5–6-day-old bean seedlings grown in the dark in vermiculite at 23°; from heads of cauliflower obtained from a local supermarket and from tubers of sweet potato which had been stored at 15°.

Isolation of Mitochondria

All steps of the mitochondrial preparation were carried out while maintaining the preparation between 0° and 4°.

Wheat and bean. The procedure of isolation of mitochondria from wheat and bean was similar to that described earlier.[17] In each experiment, approximately 30 g of wheat shoots and 60 g of bean hypocotyls were used for mitochondrial preparation. The rapidly harvested and immediately chilled tissues were homogenized in 3–5 min in a mortar with 10 ml of grinding buffer per g of tissue. The buffer was composed of 0·5 M sucrose, 1 mM EDTA, 67 mM K phosphate buffer, pH 7·2. The homogenate was strained through nylon fabric (mesh *ca.* 50 μ) and centrifuged 2 min at 18,000 rev/min in the Sorvall SS-34 rotor. The supernatant fluid was removed by aspiration and fresh grinding buffer was added to the centrifuge tube. The tubes were centrifuged 2 min at 13,000 rev/min. Following this centrifugation the mitochondria were ready for the preparation of cytochrome oxidase.

[15] D. L. EDWARDS and D. O. WOODWARD, *FEBS Letters* **4**, 193 (1969).
[16] K. D. MUNKRES and D. O. WOODWARD, *Proc. Natl. Acad. Sci. U.S.* **55**, 1217 (1966).
[17] I. V. SARKISSIAN and H. K. SRIVASTAVA, *Plant Physiol.* **43**, 1406 (1968).

Cauliflower. The washed heads were chilled to 1° prior to cell disruption. A crude suspension was obtained by grating the outer 2 cm of the heads under the surface of 800 ml of 0·6 M sucrose, 1 mM $MgCl_2$, 5 mM EDTA and 0·05 M Tris-HCl, pH 7·4, until 700–800 g of tissue were disrupted. The resulting brei was pressed by hand through a tightly woven nylon bag and then centrifuged at 18,000 rev/min for 5 min. The supernatant fluid was removed by aspiration and the sedimented pellet was suspended by gentle swirling in 30–40 ml of 0·4 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, and 0·05 M Tris-HCl, pH 7·2. The suspension was centrifuged 2 min at 18,000 rev/min. Following this centrifugation the pelleted mitochondria were ready for the preparation of cytochrome oxidase.

Sweet potato. Sweet potatoes were peeled and refrigerated before use. Approximately 100 g of sweet potato tissue were grated into 300 ml of chilled medium containing 0·4 M sucrose, 1 mM EDTA, 67 mM K phosphate buffer, pH 7·2. The mixture was then homogenized for 2–3 min in a mortar. The homogenate was strained through two layers of nylon fabric and the filtrate was centrifuged at 3500 rev/min and the precipitate discarded. The supernatant suspension was recentrifuged at 18,000 rev/min for 5 min and the pellet was washed with a mixture of 0·25 sucrose and 0·35 M mannitol and resedimented by centrifugation at 18,000 rev/min for 2 min. Following this centrifugation the mitochondria were ready for the preparation of cytochrome oxidase.

Preparation of Deoxycholate Extract

Mitochondria from the different sources were separately suspended in 0·25 M sucrose, 25 mM Tris-HCl, 0·5 mM EDTA, pH 7·5, to a concentration of about 15 mg protein/ml. Approximately 33% vol. of 0·9% KCl was added to the mitochondrial suspension and frozen overnight. The mitochondria were then thawed, centrifuged at 17,000 rev/min for 10 min and the supernatant fluid discarded. The resulting pellet was suspended to a concentration of 8–10 mg protein/ml in 0·66 M sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 7·5.

The suspension has brought to 0·3 mg deoxycholate/mg protein by addition of Na deoxycholate in 1 M KCl. The suspension was stirred for 5 min and centrifuged at 17,000 rev/min for 10 min. The supernatant was decanted and the pellet was suspended in 0·5 M sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 7·5. The suspension was refractionated (in 1 M KCl) by adjustment to 1 mg deoxycholate/mg protein. The mixture was stirred for 5 min and then centrifuged at 17,000 rev/min for 15 min. The clear supernatant fluid containing most of the cytochrome oxidase was dialyzed for 3–4 hr against 10 mM Tris-HCl, pH 7·6.

Fractionation with Ammonium Sulfate

The dialyzed preparation was fractionated with saturated ammonium sulfate, solution neutralized with NH_4OH (Table 1). Preparations were then dissolved in a small volume of 10 mM Tris-HCl, 0·1% Na deoxycholate, pH 7·5 and stored frozen.

Cytochrome Oxidase Assay

Cytochrome oxidase activity was determined spectrophotometrically at 550 nm.[18] The reaction mixture in 1 vol. of 1 ml contained: 100 nmoles of K phosphate buffer, pH 7·2, 0·05 to 0·1 nmole of ferrocytochrome c,[19] and 10 μl of enzyme containing 8 to 10 μg of protein.[20] Reactions were run at 30° and the rate of oxidation of ferrocytochrome c was constant during at least 1 min. Specific activity is shown in nmoles of ferrocytochrome c oxidized/min/mg protein.

[18] S. J. COOPERSTEIN and A. LAZAROW, *J. Biol. Chem.* **189**, 665 (1950).
[19] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[20] O. WARBURG and W. CHRISTIAN, *Biochem.* **310**, 384 (1942).