Short Communication

Cytochrome Oxidase of Cactus
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The present authors have isolated a particle-associated cytochrome oxidase from the phylloclades of cactus plant and studied its kinetics. To our knowledge, this is the first report on cytochrome oxidase from a cactus plant.

Tender phylloclades of *Nopalea depressa* Salm-Dyck were ground with 5 volumes of chilled medium in a Waring Blender operated first at low speed for 25 seconds and then at maximum speed for 5 seconds. The medium, essentially that of Wiskich and Bonner (10), consisted of: 0.32 M mannitol, 0.25 M sucrose, and 5 mM EDTA in 0.1 M tris buffer adjusted to pH 7.8. The suspension was squeezed through 2 layers of muslin into a chilled container and the filtrate (pH 7.0-7.2) subjected to differential centrifugation. The mitochondria sedimenting between 1600g and 15,000g during 30 minutes were collected and washed twice by suspending in a solution of 0.32 M mannitol and of 0.25 M sucrose and recentrifuging for 30 minutes at 15,000g. With the aid of a loosely fitting Potter-Elvehjem homogenizer, the final preparation was dispersed in sufficient 0.5 M mannitol to give a suspension equivalent to 2 g fresh tissue per ml. The protein content as determined by the method of Lowry et al. (4) was 1.4 to 2.0 mg per ml suspension. This method of isolation of mitochondria yielded the most active preparation from the point of view of oxidative phosphorylation. The preparation was free from ascorbate oxidase.

The spectrophotometric assay was carried out according to Cooperstein and Lazanow (1), at the optimum pH of 7.5. The initial rate of the reaction was calculated according to Yonetani (11).

The manometric assay of enzyme activity was according to Lieberman (3) with ascorbic acid as the hydrogen donor, but with the difference that ATP was not added and that phosphate concentration was higher (0.067 M). The rate of the reaction was linear from the tenth minute to the end of the hour. The activity was calculated from the total oxygen uptake at the end of 60 minutes. The oxygen uptake due to cytochrome oxidase action was obtained by subtracting the value for the autoxidation of ascorbate.

The kinetics of cactus cytochrome oxidase in the spectrophotometric assays was of the first order with respect to substrate at all concentrations tested (3.0-24.0 μM). However, the slope of log C / C₀ plot against time decreased with increasing concentration of ferrocytochrome c. When the initial rate of the reaction was plotted against the concentration of ferrocytochrome c, a rectangular hyperbola was obtained; the plot of the reciprocals gave a straight line (fig 1). The V_max was 0.285 μmole substrate per minute per mg protein at 20° and Km 30.8 X 10⁻⁶ M. When the assay with the above levels of ferrocytochrome c was carried out in the presence of 16.7 μM ferricytochrome c it was found that competitive inhibition occurred (fig 1). The K_i value for ferrocytochrome c was 27.4 X 10⁻⁶ M, which was nearly the same as the K_m for ferrocytochrome c.

The observed decrease in the first order rate constant with increase in concentration of ferrocytochrome c. the straight line relationship for the double reciprocal plot of initial rate and substrate concentration, the inhibition of reaction by ferrocytochrome c competing with ferrocytochrome c and the closeness of the values for K_m for substrate and K_i for inhibitor could be interpreted in terms of the expression arrived at by Yonetani and Ray (12) for heart muscle preparation:

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k_f = \ln \frac{s_0}{s} / t = \frac{V_{max}}{K_m + (s_0 + s_0)}
\]

![Fig. 1. Double reciprocal plot of initial rate in the presence and absence of 16.7 μM ferricytochrome c.](image-url)
where \( k_1 \) is the apparent first order rate constant, \( s_0 \) the initial substrate concentration, \( s \) the substrate concentration at any time \( t \) and \( p_0 \) the initial concentration of product. A plot of the concentration of total cytochrome \( c \ (p_0 + s_0) \) and the inverse of the first order rate constant gave a straight line (fig. 2). The values for \( K_m \), 33 \( \times \) 10\(^{-4}\) m and \( V_{max} \), 0.306 \( \mu \) mole substrate per minute per mg protein, arrived at by this plot were nearly the same as those derived from the Lineweaver and Burk plot.

![Graph](image)

**Fig. 2.** Plot of the reciprocal of apparent first order rate constant against the total concentration of cytochrome \( c \).

Cytochrome \( c \) was reported to inhibit soybean (6) and mung bean (2) cytochrome oxidase, but the nature of the inhibition was not defined. The present observation, therefore, constitutes the first published report on the competitive nature of the inhibition of plant cytochrome oxidase by ferricytochrome \( c \).

In manometric assays, the oxygen uptake followed a hyperbolic course at every level of enzyme \((0.1-0.3 \text{ ml})\) with 16.7 to 83.3 \( \mu \)m ferricytochrome \( c \). The double reciprocal plot yielded straight lines and the \( K_m \) value was a constant at 30.3 \( \times \) 10\(^{-6}\) m. The \( K_m \) value for cytochrome oxidase has been reported to be dependent on the concentration of the heart-muscle oxidase preparation (8) in assay systems based on oxygen uptake measurement. The relationship between \( K_m \) value and enzyme concentration was not studied in the case of plant cytochrome oxidase preparations by earlier workers. \( V_{max} \), calculated as \( \mu \) mole oxygen uptake per mg protein per hour at 37\(^\circ\)C, remained constant at 380.

The values for \( K_m \) and \( V_{max} \) for cactus cytochrome oxidase by the spectrophotometric and manometric methods were comparable. This is in agreement with Minneart (7).

With cytochrome \( c \) at a concentration of 16.7 \( \mu \)M, ascorbate was varied in the range 4 to 20 \( \text{mm} \) in the manometric assays. The maximum uptake of oxygen occurred with 10 \( \text{mm} \) ascorbate; at higher concentrations ascorbate tended to inhibit the activity. A double reciprocal plot with the data which obeyed Michaelian kinetics yielded a \( K_m \) value of 1 \( \times \) 10\(^{-2}\) m for ascorbate.

A concentration of 0.067 \( \text{m} \) phosphate was needed for optimum enzymic activity in both spectrophotometric and manometric assays. The stimulating effect of phosphate on cactus cytochrome oxidase activity was in keeping with its known effect on heart-muscle preparation (8) and preparations from plant sources (5, 9). Freezing at -17\(^\circ\) and thawing of the mitochondrial suspension did not result in either an increase or decrease of activity by spectrophotometric assay. Treatment of the mitochondrial suspension with digitonin, in amount of 1 mg per 1.5 mg mitochondrial protein, resulted in the stimulation of the cytochrome oxidase activity. Maximum stimulation (100\%) occurred in 15 to 20 minutes after which the activity gradually declined until at the end of 1 hour the activation effect of digitonin wore off. The activity of cytochrome oxidase was completely inhibited by 17 \( \mu \)M cyanide added at the time of incubation in spectrophotometric assays.

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**Literature Cited**


