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PROPERTIES OF *o*-DIPHENOL:O_2 OXIDOREDUCTASE FROM *MUSA CAVENDISHII*

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Abstract—Banana fruits (*Musa cavendishii*) contain a non-particulate *o*-diphenol:O_2 oxidoreductase that has been partially purified. The enzyme is inactivated during its catalytic action with a half time of 135 sec. The activation energy for the catalytic process is 4445 cal/mol. Classical thermal denaturation takes place only above 70°, with an entropy change of about 190 cal/mol °K at pH 6·0.

INTRODUCTION

There is a relatively high level of *o*-diphenol:O_2 oxidoreductase activity in banana fruit (*Musa cavendishii*). A review of the literature showed only a few papers dealing with banana (*Musa acuminata*) polyphenol oxidase [1, 2] and banana (*Musa sapientum*) tyramine hydroxylase [3]. Tyramine hydroxylase seems to differ from the polyphenol oxidase. In some aspects Palmer's results [1] differ from ours. According to Palmer, releasing the enzyme into solution was only accomplished by extraction of the pulp with buffered detergent solution, while our enzyme was extracted without detergent and remained in the supernatant of 10000 *g* 10 min [4]. The presence of multiple forms of the enzyme was indicated by other properties. A more detailed study seemed to be justified. A report on the extraction and activity measurements has been published elsewhere [4].

RESULTS

Localization. The enzyme activity (Table 1) was comparatively high only in pulp, and varied with the degree of ripening. The sp. act. of fruit peel was only 10% of pulp and leaves. Plant bark had the lowest sp. act.

Purification. Extraction with K–Pi buffer 0·025 M, pH 7·0 resulted in extracts with twice the specific activity of that of aqueous extracts. Differential acetone precipitation with more than 40% acetone yielded sediments with specific activities twice that of the initial extract. The supernatant after 50% saturation with $(NH_4)_2SO_4$ had 2·5 times the initial sp. act. About 60% of the total activity was recovered. No purification was achieved by differential precipitation in the 50–70% saturation range. Generally, the dialyzed enzyme from the supernatant of the precipitation with 50% saturation of $(NH_4)_2SO_4$ was used in the measurements of enzyme activity.

Molecular gel filtration on Sephadex G-100 showed the presence of multiple forms of the enzyme. Very often, the number and situation

Table 1. Distribution of the enzyme in the banana

Zone	Total activity (units/g weight)	Sp. act. (units/mg protein)
Peel	22·4	1·5
Peel from green fruits	9·1	0·7
Pulp	108·2	12·0
Pulp from green fruits	40·6	7·1
Leaves	10·5	0·9
Plant bark	4·9	0·6

The units of activity and details of the extraction are given in the Experimental. Each value is the average of at least four determinations.

of the active peaks were variable, but two important fractions were always present: the main peak (V_e 105 ml) had K_{av} 0·23 and the other (V_e 150 ml) had K_{av} 0·615, corresponding to MW of approximately 60000 and 12000, respectively. Maximum sp. act. was in the range of 250–300 units/mg protein, representing a total purification of 50–60 times in relation to the aqueous extracts.

The polymeric nature of the enzyme can be deduced from the fact that 4M urea treatment of the enzyme prior to molecular gel filtration produced a pronounced reduction of the band corresponding to MW 60000, and simultaneously that of MW 12000 was notably intensified. Disc electrophoresis also gave at least two well-defined bands [4].

Inactivation. Ingraham *et al.* [5,6] found an inactivation of polyphenol oxidase accompanying oxidation of the substrate. Kinetics were of the first order for enzyme concentration. Our findings were similar to the *o*-diphenol : O_2 oxidoreductase from banana (Fig. 1). The rate of product formation was not proportional to but diminished with the reaction time with first-order kinetics. In 135 sec, one half of the activity of the enzyme disappeared. This inhibition was not dependent on the presence of ascorbic acid in the reaction medium. Using the iodometric method [7] to measure product formation in the presence of ascorbic acid, we found the results to be similar to those obtained using a polarographic oxygen electrode in the presence or absence of ascorbic acid. The ascorbic acid/dehydroascorbic acid relation varied during the course of the reaction but had no effect on activity measurements, as indicated below.

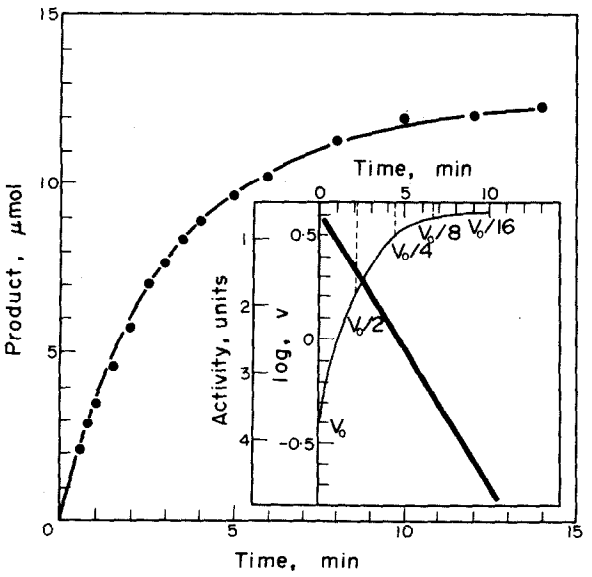

Fig. 1. Inactivation of the enzyme during its catalytic action: —— activity; ●—● product; ——log activity. An assay mixture was prepared with a volume 20 times greater than that given in Experimental. At different times, aliquots were removed from the assay medium, immediately poured into 10% H_3PO_4 solution and by titration with 1·25 mM I_2 soln the concentration of the reaction product was determined. A straight line representing log activity against time demonstrates the existence of first order kinetics.

Several authors have discussed the effect of ascorbic acid on the polyphenol oxidase enzyme. Baruah and Swain [8] found a clear inhibitory effect of ascorbic acid on polyphenol oxidase of potato; whereas Ingraham [9], and Scharf and Dawson [10] found neither an inhibitory nor an activating effect of this acid on other polyphenol oxidase systems. Likewise, with the banana enzyme, we found the presence of ascorbic or dehydroascorbic acid to be without effect. When concentrations of these acids in the reaction medium were varied from 0 to 12 mM, the results of the activity measurements were always coincident and independent of the different concentrations of the acids. The same results were obtained when different mixtures of these acids were added to the reaction medium in proportions ranging from 1:10 to 10:1 with a total concentration of 15 mM.

Activation energy. In the reaction there are two substrates, *o*-diphenol and oxygen. Under the conditions of the reaction, access to oxygen is not a limiting factor [4]. Under these conditions changes in the activity of the enzyme were produced when temperatures of the reaction were changed. An optimum temp. of 37° was obtained for enzyme activity. Between 9 and 29° the Arrhenius equation was followed and from the slope of the straight line graph produced an activation energy of 4780 cal/mol was deduced for the dissociation of an intermediate enzyme–substrate complex, whose nature is not known. A more exact calculation can be made using the variations of maximum velocity (V_M) values with temperature. The reaction kinetics were studied at different temperatures between 9 and 36° and for 5–50 mM substrate concentrations. The results were

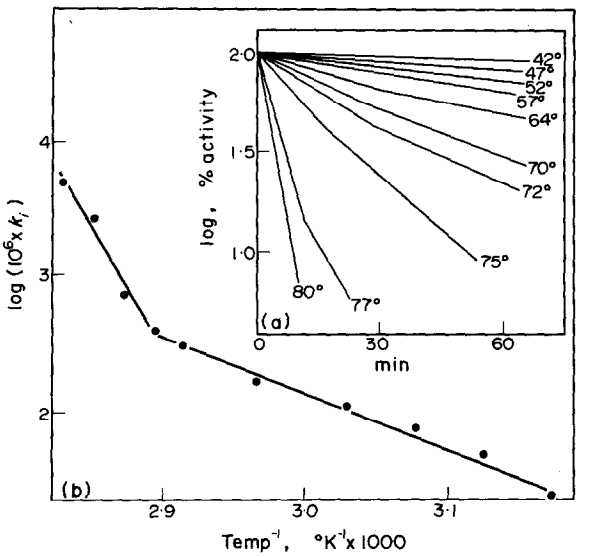

Fig. 2. Thermal denaturation of the enzyme. (a) Variation of log activity with time. (b) Relation between temperature^{-1} and logarithm of the velocity constants for thermal denaturation of the enzyme. Enzyme preparations were placed in thermostatic baths and aliquots were taken out at different times, their activity being measured by the standard method described in Experimental. Velocity constants for thermal denaturation of the enzyme (k_i) were determined at each temperature from the slopes of each line, considering the first straight portion only.

plotted in the Lineweaver–Burk representation and V_M values were deduced for extrapolation at each temperature. From the slope a "real" activation energy E_a = 4445 cal/mol was deduced.

Thermal denaturation. At pH 6·0 the enzyme loses only 8% of its activity after 1 hr at 42°, as can be calculated from Fig. 2a. In the range of 42–80°, the kinetics of denaturation seem to be of the first order. Sometimes, two different straight lines appear at each temperature. This, possibly, reflects the existence—or interconversion—of two enzyme forms with different thermal properties. In each case, velocity constants k_i were calculated only from the first portion and log k_i was plotted against temp.$^{-1}$ (Fig. 2a). Two well-defined lines can be observed. From the slope of that which corresponds to the range 42–72° an activation energy of 18·4 kcal/mol was deduced; the temperature coefficient was Q_{10} = 3·0, similar to that of normal chemical reactions. In contrast to this, in the range 72–80° an abrupt increase in the slope was obtained, with E_a = 84·8 Kcal/mol, *i.e.* more than 4 times greater than the above value, and Q_{10} = 34, more than 10 times the other Q_{10} value. We can therefore assume that banana *o*-diphenol: O_2 oxydoreductase is thermally very stable at temperatures below 70°, the classical thermal denaturation taking place only above this temperature.

As can be seen from Table 2 the entropy changes between 40 and 70° are always less than 10 cal/mol °K, while between 72 and 80° they are above 190 cal/ml °K. This increment of 19 times means that only at high temperature does

Table 2. Thermodynamic characteristics of thermal denaturation of the enzyme at pH 6·0 at several temperatures

Temperature	Velocity constant for inactivation ($sec^{-1} \times 10^6$)	Activation energy (cal mol^{-1})	ΔH (cal mol^{-1})	ΔG (cal mol^{-1})	ΔS (cal mol^{-1} $°K^{-1}$)
40	12	18400	17774	14861	9·3
42	25	18400	17764	15098	8·4
47	55	18400	17754	15335	7·5
52	85	18400	17744	15572	5·9
57	122	18400	17734	15809	5·8
64	172	18400	17724	16046	5·0
70	322	18400	17714	16283	4·2
72	386	84840	84150	16378	196
75	782	84840	84144	16519	194
77	2640	84840	84140	16613	191
80	4780	84840	84134	16755	191

Enzyme samples in 0·025 M potassium phosphate buffer were placed in thermostatic baths. At different times, aliquots were taken and their activities were determined using the standard assay described in Experimental. Log activity was plotted against time, and velocity constants for inactivation (k_i) were deduced from the respective slopes. By plotting log k_i against temperature^{-1} ($°K^{-1}$) two different straight lines were obtained, one in the range 40–70°; another in the range 70–80°. From their slopes two different activation energies (E_a) were calculated, as indicated in Table 2. At each temperature, enthalpy (ΔH), free energy (ΔG) and entropy (ΔS) changes were obtained from the equations: $\Delta G = RT \text{ lin } K/h - \text{lin } k_i$; $\Delta H = E_a - RT$; and $T\Delta S = \Delta H - \Delta G$, in which: K = Boltzmann's constant; h = Planck's constant; k_i = velocity constant for inactivation; T = °K.

Table 3. Characteristics of thermal denaturation of the enzyme at two temperatures and several pH values

pH	Temperature	Velocity constant for inactivation ($sec^{-1} \times 10^6$)	Activation energy (cal mol^{-1})	Temperature coefficient $Q_{10} = (V_t + 10)/(V_t)$	ΔH (cal mol^{-1})	ΔG (cal mol^{-1})	ΔS (cal mol^{-1} °K^{-1})
5·0	50	200	20000	2·6	19350	15980	10
	80	11800	84250	11	83540	16750	189
6·0	50	58	18400	3·0	17750	15330	7
	80	5000	84840	30	84130	16750	190
7·0	50	191	15260	2·0	14630	15330	0
	80	5490	56450	11	55740	16750	110
8·0	50	32	29670	4·6	29040	15340	42
	80	7240	75900	23	75190	16750	165

Enzyme samples were placed in thermostatic baths in buffered media: 0·025 M citrate-phosphate (pH 5·0) and 0·025 M potassium phosphate (pH 6·0–8·0). Temperatures studied were 40, 45, 50, 55, 60, 65, 70, 72, 75, 77 and 80°. Deduction of velocity constants for inactivation at each temp, and calculation of activation energy, ΔH, ΔG and ΔS at 50 and 80° were made as indicated in Table 2.

the protein molecule of the enzyme suffer marked changes in its spatial configuration, passing from an ordered state to a disordered one without biological activity. The same situation was found at other pHs. Table 3 gives different thermodynamic characteristics calculated at 50 and 80° at four different pHs. In all cases E_a and ΔS at 80° were several times greater than at 50°.

DISCUSSION

In contrast to the banana (*Musa acuminata*) polyphenol oxidase reported by Palmer [1], the enzyme system described here can be easily extracted with buffered media without the addition of detergents. Pulp has 10 times the sp. act. of peel. The existence of multiple polyphenol oxydase forms has been demonstrated in other biological material, their number varying from at least two in chloroplasts of sugar beet [11] to eight or more in grapes [12]. In banana there seems to be at least two active forms.

The time-course of the enzymatic oxidation of catechol was similar to that described for dopamine oxidation with tyramine hydroxylase from banana [3]. Although Deacon and Marsh did not calculate it, a half-life of 160 sec can be assumed for the reaction–inactivation effect of the catecholase activity of their enzyme, as deduced from the corresponding graph. The kinetics also seem to be of the first order. Another noteworthy point is the thermodynamic characteristics of the thermal denaturation of the enzyme, particularly its stability below 70°. In tyrosinase from melanome [13] the enthalpy, free energy and entropy changes for one of the more important isoenzymes have been calculated at 55° their values being 61400 cal/mol, 16800 cal/mol and 136 cal/mol °K respectively. These figures are quite similar to ours in the temperature range which produces thermal denaturation.

EXPERIMENTAL

Extraction. Ripe bananas (*Musa cavendishii*) (7 g), purchased from the local market, were homogenized in a mortar with purified sea-sand and 0·025 M K–Pi buffer pH 7·0 (50 ml). Homogenate was centrifuged at 10000 *g* for 15 min. The supernatant was fractionated by precipitation with $(NH_4)_2SO_4$ until reaching 50% of saturation. The soluble material which remained after fractionation was dialyzed against 0·025 M potassium phosphate buffer pH 7·0. The preparative manipulation were performed at 0–4° C.

Molecular gel filtration. Enzyme (1 ml) was applied to Sephadex G-100 gel previously equilibrated with 0·025 M K–Pi buffer pH 6·0; then 5 ml fractions were collected. To determine the approximate MW of the enzyme pure polymers were used for calibrating the column: ribonuclease (MW 13000), trypsin (MW 24000), egg-albumin (MW 45000), bovine albumin (MW 67000 and 134000) and blue dextran (MW 2×10^6). $K_{av} = (V_e - V_0)/(V_t - V_0)$, in which V_e = elution volume; V_0 = void volume; V_t = total volume.

Enzyme assay. *o*-Diphenol:O_2 oxidoreductase assay was carried out as described previously [7]. The substrate, 12·5 mM catechol, was placed in 0·05 M K–Pi pH 6·0 buffer medium in the presence of 7·5 mM ascorbic acid. Good oxygen uptake was maintained with magnetic stirring, the reaction time was 180 sec and the total vol. 5 ml. Non-oxidized ascorbic acid was titrated with a 1·25 mM I_2 soln. The difference between the ascorbic acid concentration at the beginning and end of the reaction corresponds to the product of the reaction in that period. One unit of the enzyme is the amount which transforms 1 μmol of substrate in 1 min at 20°. The sp. act. is the number of units/mg of protein. When *o*-diphenol:O_2 oxidoreductase activity was determined by the use of a polarographic oxygen electrode, the medium reaction was aerated for 10 min and the reaction was initiated by addition of the enzyme solution.

Protein estimation. Protein determinations were carried out using the Lowry method [14]. For crude extracts it was

necessary to dialyze previously in order to eliminate some dialyzable substances which interfere with the measurements.

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