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SHORT REPORTS

ASCORBATE OXIDASE FROM *CUCURBITA MAXIMA*

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Abstract—Ascorbate oxidase is present in homogenates of the flesh of *Cucurbita maxima* fruits. Its activity is independent of ascorbate concentration over the range 4–40 mM, is unaffected by 2 mM EDTA and it does not oxidize catechol. The enzyme has a MW of *ca* 150000, an optimum pH over the range 5.5–7 and an energy of activation of 8.8 kcal/mol. The enzyme was only weakly inhibited by azide and thiourea–copper complexing agents.

INTRODUCTION

The ability of plant tissue homogenates to oxidize ascorbic acid has been attributed to many factors present in the preparations. Hallaway *et al.* [1] have proposed a procedure based on kinetic characteristics to distinguish the specific enzyme [ascorbate oxidase (AO), EC 1.10.3.3.] from other catalysts of ascorbate oxidation. Nevertheless, this enzyme was first recognized as a protein with specific molecular properties by Lovett–Janison and Nelson [2], working with a highly purified preparation obtained from squash (*Cucurbita pepo condensa*). Several properties of AO from different sources have been studied since then, such as: MW [3]; influence of pH and temperature on the enzyme activity [4–7]; visible absorption spectrum [8]; energy of activation of the reaction [1]. Frieden and Maggiolo [9] reported two widely differing K_m values for the AO of squash (*C. pepo condensa*) when employing manometry and spectrophotometry to follow the enzymatic activity. Amon and Markakis [7] found closer K_m values but the data from the spectrophotometric method were still 5–6-fold higher than those from the Warburg method. AO has been identified in the soluble fraction [7, 9] and less often in the cell wall [1, 10] of the plant tissues. In the present paper, the AO from a tropical cucurbit (*C. maxima*) is studied and some physical and chemical properties are described.

RESULTS AND DISCUSSION

Homogenates obtained from the flesh of *C. maxima* fruits catalysed ascorbic acid oxidation which follows zero order kinetics with respect to ascorbate over the range 4–40 mM in the presence and absence of 2 mM EDTA. Furthermore, the preparations were not capable of oxidizing catechol and were completely inactivated after 1 min at 100°. According to these criteria, recommended by Hallaway *et al.* [1], the presence of AO at *C. maxima* fruits can be assumed. This enzyme has a MW of *ca* 150000 and an optimum pH in the range 5.5–7. These results agreed with those reported for the enzyme from other sources [4, 9]. However, unlike earlier findings [6], the enzymatic activity was not lost below pH 4, so that 25% of the activity observed in the optimal pH range was retained at pH 3. The steric properties of this enzyme may prevent loss of Cu from the protein molecule below pH 4.

The AO from *C. maxima* was relatively heat resistant. Its activity remained unaltered when it was incubated at temperatures ranging from 0° to 40° for 30 min. The energy of activation of the reaction catalysed by AO from cell wall and soluble fraction of cabbage leaves (*Brassica oleracea*) were 12 and 4.4 kcal/mol, respectively [1]. For vegetable marrow (*C. pepo*) these values were 6.7 and 3.5–4 kcal/mol, respectively. The enzyme from *C. maxima* was present in the soluble fraction only and showed an energy of activation of 8.8 kcal/mol, which is nearly twice that of the soluble enzymes from *B. oleracea* and *C. pepo*. Nevertheless, this value is *ca* 3 times less than the energy of activation required for the reaction catalysed by $CuSO_4$ (22 kcal/mol).

The values for K_m (expressed as L-ascorbic acid concentration) and V_{max} using the spectrophotometric procedure (ascorbate oxidation) were 200 μM and 3.9 μM/min per mg protein, respectively, whereas the polarographic method (O_2 consumption) yielded values of 166 μM and 3.0 μM/min per mg protein. The K_m values are of the same magnitude either following the enzymatic activity by ascorbic acid oxidation or by O_2 consumption. Such results support the explanation for the discrepancy of the K_m value reported by Frieden and Maggiolo [9] which is based on the large difference in enzyme–substrate concentrations in the two methods employed (spectrophotometry and manometry).

AO from *C. maxima* was only weakly inhibited by Cu-complexing reagents. Azide at 8 mM and thiourea at 0.3 M lost 28 and 55% of the control activity, respectively. EDTA which is a most effective complexing agent for Cu^{2+}, had no effect on *C. maxima* AO activity.

EXPERIMENTAL

Preparation of enzyme. The fresh fruits of *C. maxima*, known in Brazil as 'abóbora' or 'jerimum', obtained from a local food supplier—CEASA, was knife-peeled to a depth of *ca* 3 mm. The tissue was blended in 0.1 M citrate/Pi buffer, pH 6 for 5 min at 4°; keeping the ratio of 1 g tissue/1.5 ml buffer. The homogenate was centrifuged at 13000 *g* for 10 min at 0–4°. The ppt. was discarded and $(NH_4)_2SO_4$ was added to the supernatant to 40%. After

removal of the ppt. by centrifugation, $(NH_4)_2SO_4$ satn was increased to 80% and the ppt. was resuspended in Pi buffer, pH 7.6, to give a vol. equal to half of the initial vol. of the homogenate. The enzyme was further ppted in EtOH at −10° between 40 and 60%, yielding an activity of 16.0 μmol/min per mg protein. The two procedures gave a purification of *ca* 30 times and a yield of 30%. This purified enzyme was used throughout. The intracellular distribution of AO was established using the subcellular fractions obtained according to ref. [11].

Assay methods. AO activity was measured spectrophotometrically according to ref. [12] whereas the polarographic method was carried out as follows: 1.9 ml of pre-aerated L-ascorbic acid soln prepared in 0.1 M citrate/Pi buffer, pH 6, containing 2 mM EDTA was introduced into the electrode chamber at 25° of a Gilson KI-C apparatus with a Clark electrode, and after equilibration, 0.1 ml of enzyme prepn was added and O_2 uptake recorded.

K_m, V_{max} *and optimal pH.* Lineweaver–Burk plots were obtained using spectrophotometry and polarography. The enzymatic activity was polarographically determined at different pH values employing citrate/Pi buffer (pH 3–6); Pi buffer (pH 5.7–8) and Tris–HCl buffer (pH 7.6–9) at the same ionic strength.

Estimation of MW was determined according to ref. [13]. The Sephadex G-200 column was calibrated with trypsin (MW 23 000), amyloglucosidase (MW 100 000), glucose oxidase (MW 150 000) and xanthine oxidase (MW 320 000). V_e was plotted against log MW; a straight-line relationship was obtained and AO MW was calculated by interpolation.

Thermal stability. Portions (1 ml) of enzyme were incubated at temps ranging from 0 to 100° for 30 min. Immediately after treatment the samples were equilibrated at 26° and their activities established by polarography.

Determination of energy of activation. The enzymatic activity was polarographically estimated at temps ranging from 10 to 40°.

Inhibition studies. Samples of enzyme were pre-incubated for 30 min with Cu-complexing reagents in the reaction chamber of the O_2 electrode. Ascorbate (2 mM) was then added and O_2 uptake recorded.

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