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SHORT COMMUNICATION

ASCORBATE OXIDASE ISOZYMES

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Abstract—The ascorbate oxidase of yellow summer squash (*Cucurbita pepo condensa*) was separated into five isozymes by polyacrylamide gel electrophoresis. One of the forms accounted for 70 per cent of the recovered total enzymic activity. A reversible association–dissociation occurs in the isozymes as a result of changes in ionic concentration.

ASCORBATE oxidase (L-ascorbate:O_2 Oxidoreductase, E.C. 1.10.3.3) is a copper-containing protein, present in plant tissues and catalyzing the aerobic oxidation of vitamin C. Very highly purified preparations of this enzyme have been prepared from the yellow summer squash (*Cucurbita pepo condensa*) and the green zucchini squash (*C. pepo medullosa*). These preparations were found to be 100 per cent homogeneous "in electrophoresis and in ultracentrifugation" and to represent a protein of 140,000 molecular weight and 0·34 per cent copper content.[1] Recently,[2] on the basis of gel filtration, the ascorbate oxidase of cucumbers was separated into two large size (MW 200,000–900,000 assuming globular shape) and one small size (of the order of MW 10,000) molecular forms.

In this investigation, polyacrylamide gel electrophoresis was used to demonstrate the presence and study some properties of the multiple molecular forms of the ascorbate oxidase of yellow summer squash.

In a typical electrophoresis experiment, 60 g of squash peel was homogenized in a Waring blendor for 1 min at low speed with 60 ml of 0·05 M phosphate buffer, pH 7·0, containing sucrose at the concentration of 0·25 M. The homogenate was centrifuged at 20,000 × *g* for 20 min at 2°, and the supernatant was centrifuged a second time at 100,000 × *g* for 2 hr at 2°, and then subjected to polyacrylamide gel electrophoresis according to Davis.[3] The gel tubes were 7·5 cm long and 0·5 cm in o.d. The best separation was obtained with a 4·5 cm high, 8 per cent lower gel, and 0·5 cm high, 3 per cent upper gel. About 0·3 ml of the dialyzed preparation, to which sucrose was added to reach the concentration of 2 per cent, was layered on top of the upper gel. The tube was filled with Tris–glycine buffer, pH 8·3, which was first deprived of oxygen by bubbling nitrogen through it and then made 10^{-3} M in ascorbic acid. After anodic migration of the front for about 4·5 cm (1·5 hr), during which the current was kept constant at 2·75 mA per tube at 2°, the gel was extruded and placed in a solution of 25 mg of 2,6-dichlorobenzenoneindophenol (dye) per 100 ml of demineralized water. At

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[2] J. PORATH, G. B. SAMORODOVA-BIANKI and S. HJERTEN, *Biokhimiya* **32**, 578 (1967).
[3] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).

the loci of ascorbate oxidase activity, the ascorbic acid, which had penetrated the gel during electrophoresis, was oxidized and the dye remained blue. At all other loci the ascorbic acid decolorized the dye. The colored columns were rinsed with water and photographed on high contrast film in transmitted light; five forms, A–E, were obtained by this separation.

Subsequently, the sections of several columns corresponding to forms A, C, D, and E were cut off with a razor blade, triturated with 0·5 ml of 0·05 M phosphate buffer, pH 7·0, and the mixture was tested for ascorbate oxidase activity by Warburg manometry.[4] Similarly form B was tested alone since, by the width of the zone, this form appeared to have more activity than the others. It was found that the recovered enzyme activity from all five zones was 80 per cent of that applied on the column, and of the recovered activity 70 per cent was associated with form B and 30 per cent with the other four forms combined.

When the supernatant of the second centrifugation of the squash extract was dialyzed against demineralized water, rather than phosphate buffer, only one zone appeared on the electropherogram. This zone occupied position B and had the same activity as zones A–E together. Upon elution of this composite zone with 0·05 M phosphate buffer, pH 7·0, and dialysis against the same buffer all five forms reappeared. When these five forms were again eluted with 0·05 M phosphate buffer, pH 7·0, from several columns and the combined eluates dialyzed against demineralized water, the single composite form reappeared on the gel. It is clear that a reversible association–dissociation occurs in the isozymes of ascorbate oxidase as a result of change in the ionic strength. A similar effect of the ionic strength has been demonstrated with tyrosinase[5] and soluble hexokinase.[6]

In another experiment, each of the five forms was eluted with 0·05 M phosphate buffer, pH 7·0, from several columns and the eluates placed on new gels for electrophoresis. Each form appeared alone in the position expected from the mixed forms.

In order to investigate the reason for the lack of electrophoretic inhomogeneity reported earlier,[1] the yellow squash ascorbate oxidase was purified according to Dawson and Magee.[7] 12 lb of peel were used as starting material and the filtrate of the second ammonium sulfate precipitation, instead of being discarded, was dialyzed against 0·05 M phosphate buffer, pH 7·0, and subjected to the polyacrylamide gel electrophoresis. Zones B, C, and D appeared with B again being the dominant one. The effort of tracing forms A and E of the crude extract among the purification steps is still in progress, in parallel with work on the molecular size and other properties of the separated forms of ascorbate oxidase.

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