Phytochemistry, 1973, Vol. 12, pp. 2127 to 2132. Pergamon Press. Printed in England.

PROPERTIES OF ASCORBATE OXIDASE ISOZYMES*

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(*Revised Received* 15 *February* 1973. *Accepted* 16 *April* 1973)

Key Word Index—*Cucurbita pepo*; *Cucumis salivus*; Cucurbitaceae; squash; cucumber; ascorbate oxidase; isozymes; molecular weight determination; gel filtration; gel electrophoresis.

Abstract—The ascorbate oxidase of two squash cultivars was resolved into five molecular forms by gel electrophoresis; that of cucumber was resolved into three forms. Molecular weight estimates by Sephadex gel filtration and interconversions of these forms strongly suggest the presence of a monomeric form of MW 30 000 for the cucumber enzyme and 35 000 for that of the squashes. The other two forms in the cucumber appear to be a dimer and a tetramer, whilst a tetramer, an octamer, a dodecamer, and a polymer of MW between 670 000 and 2 000 000 are likely to be the other four forms present in the squashes. The monomer was the most abundant form in the cucumber and the tetramer in the two squashes. The peel of these fruits was higher in activity than the flesh, but the isozyme pattern was the same in peel and flesh. The tetramer of the squashes and the dimer of cucumbers were the most resistant forms to heat inactivation. The enzyme is soluble and not associated with subcellular particles.

INTRODUCTION

IN A PREVIOUS communication[1] we reported the separation of ascorbic acid oxidase (AAO), also known as L-ascorbate:O_2 oxidoreductase, E.C.1.10.3.3, present in yellow summer squash (*Curcurbita pepo*, L. var. *condensa*), into five different molecular forms (isozymes) by means of polyacrylamide gel electrophoresis.

In the present paper, the separation into isozymes of the AAO obtained from two other sources, green zucchini squash (*C. pepo*, L. var. *medullosa*) and cucumber (*Cucumis sativus* L.), is demonstrated, and some physical and chemical properties of the isozymes of all three plant sources are described.

RESULTS AND DISCUSSION

A typical pattern of polyacrylamide disc gel electrophoresis of AAO from yellow summer squash skin consisted of five bands, numbered 1, 4, 8, 12 and *X* (previously indicated as *A* to *E*). The numbers denote molecular multiplicity of basic units and are justified later. The concentration of the polyacrylamide gel played a very important role in the resolution of the molecular forms. The best resolution was obtained with the 8% gel.

With this method it was found that there is no difference in the AAO isozyme pattern between yellow summer squash and green zucchini squash. There were only three molecular forms of AAO, designated as 1, 2 and 4, present in the cucumber, in comparison to the five forms of the squashes. When the electrophoretic pattern of all proteins present in the supernatant of the three plant sources were compared, it was found that all three differed from each other.

* Michigan Agricultural Experiment Station Journal Article Number 6033.

[1] AMON, A. and MARKAKIS, P. (1969) *Phytochemistry* **8**, 997.

The recovered enzyme activity from all five zones from yellow summer squash was 80% of that applied to the column; of the recovered activity 70% was associated with form 4 and 30% with the other forms combined. The same results were obtained with green zucchini squash. In the cucumber, 50% of the recovered activity was attributed to form 1, 40% to form 2, and 10% to form 4. In another experiment, each of the forms was eluted from several columns and the eluates placed on new gels for electrophoresis. Each form appeared alone in the position expected from the mixed forms. This indicates that the multiple forms separated by acrylamide gel electrophoresis cannot be considered artifacts of the electrophoretic treatment.

Tissue Specificity

The peel and flesh of yellow summer squash, green zucchini squash and cucumber were tested with regard to AAO form multiplicity. In all species no difference in isozyme pattern between peel and flesh was apparent, but the peel had higher enzyme activity than the flesh (Table 1). Yellow summer squash displayed the highest activity among the three commodities in both peel and flesh, while cucumber showed the lowest activity.

TABLE 1. AAO ACTIVITY IN THE PEEL AND FLESH OF YELLOW SUMMER SQUASH, GREEN ZUCCHINI SQUASH AND CUCUMBER

Commodity	ml final supernatant		Units per g wet tissue		mg protein	
	Peel	Flesh	Peel	Flesh	Peel	Flesh
Yellow summer squash	6·5	4·2	19·5	12·6	0·73	0·25
Green zucchini squash	6·0	3·4	18·0	10·2	0·68	0·21
Cucumber	3·0	0·5	9·0	1·5	0·28	0·04

Reaction mixture in Warburg flask: 1·5 ml of 0·2 M Na_2HPO_4 –0·1 M citric acid buffer, pH 5·7, 0·5 ml gelatin solution (750 mg gelatin in 150 ml H_2O), 0·5 ml of 0·028 M ascorbic acid and 0·5 ml enzyme solution. One activity unit is the amount of enzyme catalyzing an initial rate of 10 μl of O_2 uptake per min.

Estimation of MWs of AAO isozymes

On the basis of at least triplicate gel filtrations on Sephadex G100 and Sephadex G200, estimates of MW were made (Table 2). The MW of form *X* could not be established with any degree of accuracy higher than that indicated because the elution volume of blue dextran 2000 did not fit in the semilogarithmic relationship existing between protein markers and elution volumes. When the fractions, eluted from the gel filtration, were individually resubjected to polyacrylamide gel electrophoresis, they displayed the same mobility as during the first electrophoresis. The results suggest that the five AAO forms of yellow summer and green zucchini squashes and the three AAO forms of cucumber represent various degrees of aggregation of their monomers.

The MW of highly purified AAO reported in the literature, 134 000–140 000 for the AAO of yellow summer squash,[2] 140 000 for the AAO of green zucchini squash,[3] and 132 000 for the AAO of cucumber[4] are in excellent agreement with what in this work is

[2] STARK, G. R. and DAWSON, C. R. (1962) *J. Biol. Chem.* **237**, 712.
[3] TOKUYAMA, K., CLARK, E. E. and DAWSON, C. R. (1968) *Biochem. J.* **64**, 189.
[4] NAMAKURA, T., MAKINO, N. and OGURA, Y. (1968) *Biochem. J.* **64**, 189.

designated as tetrameric forms of AAO. It is possible that the other forms were lost during purification in previous work. Tokuyama *et al.*[3] suggested the possibility of the presence of a small amount of a higher MW species of AAO from yellow summer and green zucchini squashes. Molecular species of AAO from cucumber above 200000 MW, as reported by Porath *et al.*,[5] could not be detected in this study. However, this does not exclude the possibility of formation of aggregates of that size under certain conditions and treatments.

TABLE 2. MW ESTIMATES ($\times 10^{-3}$) OF ASCORBATE OXIDASE ISOZYMES

Band	Yellow summer squash	Green zucchini squash	Cucumber
1	30–42	30–35	29–32
2	—	—	62–66
4	135–155	125–150	120–135
8	260–310	240–280	—
12	390–460	370–440	—
X	670–2000	670–2000	—

Effect of Temperature

Enzyme-containing extracts were kept at 5, 25, 40, 50, 60, 70 and 100° for periods up to 72 hr at the lowest temperature and 1 min at the highest temperature. Periodic electrophoretic separations during the holding times (details not presented) show, (a) that the different molecular forms of AAO vary in their resistance to heat inactivation, the tetramer of the squashes and the dimer of cucumber being the most heat resistant; (b) mild heat (40–50°) converts the octamer and heavier forms present in squashes to a dimer; and (c) at 100° for 1 min all AAO forms are inactivated.

Effect of Different Chemicals

(a) A urea concentration of 5 M in the polyacrylamide gel did not alter the total activity of AAO nor the characteristic isozyme pattern of green zucchini squash. When the urea concentration was increased to 7 M, the forms 8, 12 and *X* disappeared, the activity of the tetramer decreased while that of the monomer increased. In an 8 M urea gel only the monomer remained with approximately 75% of the total activity. The origin of the monomer after treatment with 8 M urea was explored. The tetramer was isolated from a gel and put on a new gel containing 8 M urea. Electrophoresis on the new gel resulted in approximately 80% conversion of the tetramer to the monomer and 20% of the activity was lost. In the same way it was shown that 8 M urea converted the 8-, 12- and *X*-forms to the monomer with a 50% loss in total activity.

In order to further explore the differential resistance of the AAO variants to urea degradation, the tetramer and form-12 bands were cut off the gel, trituraed separately and an equal amount of activity of either form was applied on a 7 M urea gel. It was found that form-12 was completely converted to the monomer. These results indicate that form-12 is not simply composed of three tetramers. The loss in activity was probably due to the release of copper upon urea treatment.[2] The depolymerizing effect of urea may be interpreted on the basis of absence of intersubunit disulfide bonds.

[5] PORATH, J., SAMORODOVA-BIANKI, G. D. and HJERTEN, S. (1967) *Biokhimiya* **32**, 578.

(b) When the monomer of green zucchini squash was treated with 0·02 M 2-mercaptoethanol for 10 min, no change in the AAO isozyme pattern was observed. Under similar conditions disulfide bonds of peptide chains are split. It is concluded that the monomer is probably composed of one peptide chain.

(c) Approximately 20% of the total AAO activity was left after lowering the pH of the green zucchini squash supernatant to 3·6 by addition of HCl and incubating at 0° for 30 min. Upon gel electrophoresis only the tetramer was detected in the treated preparation. The acid inactivation of the AAO activity seemed to be irreversible, since no activity increase was detected after dialysis of the acid-treated sample and adjustment to the initial pH of 7·0.

(d) When the green zucchini squash supernatant was adjusted with NaOH to pH 11·0 and allowed to stand for 30 min at 0°, the total activity was greatly decreased, all the original forms of AAO disappeared and a new form appeared. Upon Sephadex G100 gel filtration the new form was shown to have a MW between 58 000–77 000, indicating it to be a dimer. When each of the original AAO bands was cut off from a gel, triturated with phosphate buffer, pH 7·0, treated for 30 min at pH 11·0 and again subjected to electrophoresis, forms 8, 12 and *X* were converted to the dimer, with a small loss (10–20%) of activity. The tetramer and the monomer were not converted to the dimer and apparently were destroyed by the treatment.

Clark *et al.*[6] reported that direct molecular weight determinations of the alkali-treated, purified enzyme from yellow summer squash revealed some heterogeneity of the sample. Two new components were found, the major one was calculated to have a MW of about 65 000; the second component (comprising less than 15% of the protein) had a MW of about 110 000. They suggested that the native enzyme (MW 135 000–140 000) is composed of more than one polypeptide chain, although no further evidence was provided. The 65 000 MW form of AAO seems to be identical with the dimer found in our investigation.

Isoelectric point (pI) of AAO isozymes

By plotting the electrophoretic mobilities of each AAO molecular form against the pH of the acrylamide gel, in the pH range of 4·5–8·0, which covers both anodic and cathodic migration, it was possible to estimate the pH at which the mobility was zero, that is the pI. Interestingly, it was found that all ten forms isolated from the two kinds of squash had the same pI, which was approximately 5·3. No appreciable difference was detected between the mobilities of the corresponding forms of the two squashes at any pH. Dunn and Dawson[7] estimated the pI of purified AAO from the crookneck squash to be between 5·0 and 5·5. The three forms of the cucumber AAO had also a common pI, approximately 6·7. Willis,[8] using a suramin inhibition technique, assigned a pI of 5·1 to the cucumber AAO, while Nakamura *et al.*[4] thought that it should be between 6·0 and 7·8, based on electrophoretic measurements.

K_m *and* V_{max} *of AAO*

Frieden and Maggiolo[9] using two different techniques reported two widely differing K_m values for the AAO of yellow summer squash: 5×10^{-3} M by manometry and

[6] CLARK, E. E., POILLON, W. N. and DAWSON, C. R. (1966) *Biochim. Biophys. Acta* **118,** 72.
[7] DUNN, F. J. and DAWSON, C. R. (1951) *J. Biol. Chem.* **189,** 485.
[8] WILLIS, E. D. (1952) *Biochem. J.* **50,** 421.
[9] FRIEDEN, E. and MAGGIOLO, I. W. (1957) *Biochim. Biophys. Acta* **24,** 42.

$3{\cdot}9 \times 10^{-5}$ M by spectrophotometry. They explained this discrepancy on the basis of large differences in enzyme substrate concentrations in the two test methods. Our values for K_m using the Warburg and spectrophotometric methods lie relatively close together (Table 3). Our data from the spectrophotometric method are 5–6 times higher than those from the Warburg method. The reason for the closer agreement between our Warburg and spectrophotometric values must be due to the identical concentrations used for the substrate in both methods, although the enzyme concentration in the Warburg method had to be higher for measurable oxygen uptake. Other reported K_m values for AAO are $2{\cdot}4 \times 10^{-4}$ M (25°), $4{\cdot}2 \times 10^{-4}$ M (20°) and 2×10^{-3} M (30°).

TABLE 3. K_m AND V_{max} VALUES OF THE AAO OF YELLOW SUMMER SQUASH, GREEN ZUCCHINI SQUASH AND CUCUMBER

Commodity	Method	K_m ($\times 10^4$ M)	V_{max} (M min^{-1}) ($\times 10^5$)
Yellow summer squash	Spectrophotometric	1·86	1·06
	Warburg	9·80	—
Green zucchini squash	Spectrophotometric	3·60	0·86
	Warburg	22·10	—
Cucumber	Spectrophotometric	1·81	1·28
	Warburg	11·25	—

Intracellular Distribution of AAO

The AAO activity associated with the four fractions obtained by differential centrifugation of the crude extract of the squash and cucumber fruits was determined as percentages of the total activity of the extract. The 120 *g* fraction (debris) had 8–10%; the 3000 *g* fraction (chloroplasts), 6–8%; the 35 000 *g* fraction (mitochondria), 3–6%; and the supernatant of the last centrifugation had 74–87% of the total activity. When the particles of the three first fractions were resuspended in phosphate buffer, pH 7·0, and recentrifuged at speeds identical with those of the first centrifugation the activity of the particles was reduced to 4, 2 and 2%, respectively. Upon a third similar centrifugation no activity was detected in the particulate fraction. It may therefore be concluded that ascorbic acid oxidase is a soluble enzyme not firmly associated with subcellular particles.

EXPERIMENTAL

Yellow summer squash, green zucchini squash and cucumber fruits were obtained from local farmers. Part of the material was used immediately and the rest was frozen and stored at −20°. When the peel was analyzed separately, fresh fruit was used and knife-peeling to a depth of about 1 mm was applied. Extraction was performed by disintegration in a Waring blendor at high speed for 1 min in the presence of phosphate buffer, pH 7·0, ionic strength 0·02. The polyacrylamide gel electrophoretic separation and visualization of AAO isozymes was conducted as described earlier.[1]

Estimation of molecular size by gel filtration. Two different columns were prepared as follows. Demineralized water was added to Sephadex G100 and the gel was allowed to swell on a boiling water bath for 5 hr. The hydrated gel was deaerated under vacuum and the column (1·5 × 84 cm) was filled at 4° and equilibrated with 0·05 M phosphate buffer, pH 7, ionic strength 0·1. The void volume was determined using blue dextran 2000 (MW 2×10^6). The column second (1·5 × 61 cm) was prepared in the same way as the first, with Sephadex G200. The flow rate of the Sephadex G100 column was 10 ml/hr, that of Sephadex G200 was 6 ml/hr. The Sephadex G100 column was calibrated with chymotrypsinogen A (beef pancreas) 6× cryst. salt free (MW 25 000), ovalbumin (2× cryst.) (MW 45 000), albumin (bovine) cryst. (MW 67 000), and gamma globulins (Human) (MW 160 000); the Sephadex G200 column was calibrated with gamma globulins (human) amorphous, salt free (MW 480 000) and thyroglobulin (bovine) (MW 670 000). The elution volume (V_e) of each of the markers was measured using an ISCO UV monitor with a recorder at 254 nm. V_e was

plotted against log MW; a straight line relationship was obtained. The elution vols of the multiple molecular forms were determined as follows. Eight replicate electrophoretic separations of the AAO isozymes were performed simultaneously in the same apparatus. One of the 8 gels was developed for visualization of the enzymatic bands and then all 8 gels were aligned with their ends at the same level to facilitate dissection of the gels with a razor blade at the loci of AAO activity. The corresponding loci of the seven unstained gels were combined, triturated in 3 ml phosphate buffer and an aliquot was applied on Sephadex G100 or Sephadex G200. The effluent was collected as 1·5 ml fractions. In testing each fraction for AAO activity 1·5 ml of effluent and 0·1 ml of 10^{-3} M ascorbic acid were shaken for 1 min and then 0·1 ml dye (25 mg 2,6-dichlorobenzenoneindophenol per 100 ml H_2O) was added. The presence of a blue color after one minute indicated a minimum of 10 μg of AAO in the reaction mixture.

Thermal treatments of AAO forms. The extract obtained from summer squash peel with phosphate buffer, pH 7·0, ionic strength 0·1, was centrifuged at 35 000 *g* for 20 min. 1 ml aliquots of the supernatant were maintained at different temperatures for different durations (cited in Results and Discussion). Immediately after the treatment the samples were cooled in ice water and subjected to polyacrylamide gel electrophoresis and staining.

Chemical treatments of AAO. Urea at the concentration of 5, 7 and 8 M was incorporated into the polyacrylamide gel and electrophoresis was conducted as usual. 2-Mercaptoethanol was added to the supernatant of the 35 000 *g* centrifugation at the final concentration of 0·02 M and incubated for 10 min at 0°; electrophoresis followed immediately after incubation.

The effect of pH on the enzyme was studied by adding 0·2 M NaOH or HCl to the supernatant until the desired pH level was reached. Electrophoresis was performed after 30 min of incubation at 0°.

Isoelectric points of AAO forms. After the first electrophoretic separation of the enzyme preparation containing all isozymic forms, the individual gel bands showing AAO activity were cut off with a razor blade and three identical bands were combined, triturated with 0·5 ml phosphate buffer and subjected to a second electrophoretic run. The electrophoretic mobility was expressed in mm per hour under the conditions of measurement. Acetate buffers were employed at pH 6·0, 6·5, 7·0, 7·5 and 8·0. In the pH range 4·5–8·0 both cathodic and anodic migration for all forms occurred. The quantity of TEMED solution necessaty to polymerize the separation gel in 30 min was 3·6, 3·0, 1·8, 0·8, 0·5, 0·4, 0·35 and 0·3 ml per 100 ml buffer for pH 4·5, 5·0, 5·5, 6·0, 6·5, 7·0, 7·5 and 8·0, respectively.

K_m *and* V_{max} *determination for AAO.* Peel tissue (60 g) was blended with 60 ml phosphate buffer, pH 7, ionic strength 0·1, in a Waring blender at high speed for 1 min. The homogenate was squeezed through four thicknesses of cheesecloth and the filtrate centrifuged at 35 000 *g* for 30 min at 2°. The supernatants obtained from yellow summer squash and green zucchini squash were diluted with H_2O 1:100, that from cucumber 1:50. Both the Warburg method and a spectrophotometric method were used in determining the AAO activity. In the latter method the components of the reaction mixture were transferred into a Beckman DU spectrophotometer cuvette (1 cm lightpath) in the following order: 0·1 ml ascorbic acid solution, 0·1 ml EDTA (3×10^{-5} M), 2·7 ml 0·01 M phosphate buffer, pH 7·2, and 0·1 ml of enzyme preparation. The change in A_{265} was measured for 3 min using a Ledland Log Converter and a Sargent Recorder. From the first straight line portion of each tracing the decrease in A_{265} per min (velocity) was calculated. The K_m and V_{max} values were determined from a Lineweaver–Burk plot.

Intracellular distribution of AAO isoenzymes. A published procedure was used for obtaining subcellular fractions.[10]

[10] TOLBERT, N. E., OESER, A., KIRAKI, T., HAGEMAN, R. H. and YAMAZAKI, R. H. (1968) *J. Biol. Chem.* **243**, 5179.