Ascorbate Oxidase

FURTHER STUDIES ON THE PURIFICATION OF THE ENZYME*

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SUMMARY

A new procedure has been described for the isolation and purification of ascorbate oxidase from green zucchini squash (Cucurbita pepo medullosa). The new purification method achieves a more than 200-fold purification with about a 14% recovery of the total activity present in the crude juice. The purified enzyme preparations have been found to be homogeneous by the criteria of ultracentrifugal analysis and electrophoresis on polyacrylamide gels. The sedimentation constant has been found to be \( s_{20, w} = 7.52 \) S and a molecular weight of 140,000 has been estimated. The preparations exhibit a specific activity in the range of 3800 to 4250 units per mg, and contain 0.46 to 0.52% copper corresponding to 10 to 12 atoms of copper per enzyme molecule. These specific activity and copper content values are significantly higher than those of homogeneous ascorbate oxidase preparations previously described. The amino acid composition of the new ascorbate oxidase preparation is similar to that of an earlier homogeneous preparation of the same molecular weight but having a significantly lower copper content and specific activity. The marked increase in specific activity of the new preparation appears to be due to the higher content of prosthetic copper rather than to a difference in the protein moiety.

The isolation and purification of ascorbate oxidase (L-ascorbate:O\(_2\) oxidoreductase, EC 1.10.3.3) from plant sources (mainly the yellow crook-neck squash, Cucurbita pepo condensae, and the green zucchini squash Cucurbita pepo medullosa) have occupied the attention of workers in this laboratory for over 25 years. During this period, a number of "purified" specimens of the enzyme have been described, and progressive increase in specific activity and copper content has been observed (1–5). For this purpose there was only one experimental approach possible, i.e. a further examination of the over-all purification process with the introduction of modifications to improve (cumulatively) the specific activity and copper content values of the isolated enzyme.

The present investigation (see also the accompanying article (6)) has resulted in a modified chromatographic procedure for the purification of ascorbate oxidase from green zucchini squash. The new procedure results in improved yields of enzyme specimens judged to be homogeneous by ultracentrifugal criteria and by the highly sensitive criterion of polyacrylamide gel electrophoresis. It is particularly noteworthy that the enzyme specimens thus prepared have been found to possess significantly higher specific activities and copper contents than reported by Tokuyama et al. (5).
Chemicals—L-Ascorbic acid was obtained from Eastman Organic Chemicals Company. Standard protein solutions (10 mg of protein nitrogen per ml of crystalline bovine albumin) were obtained from the Armour Pharmaceutical Company. Phosphocellulose, TEAE-3-cellulose, and Chelex-100 were purchased from Bio-Rad Laboratories. Copper-free water was prepared by deionization of tap water through a Culligan deionizer (Nelson, Phillips and Company, Inc.) and was used throughout this work except when otherwise specified.

Buffers—Phosphate buffers were prepared by mixing suitable aliquots of analytical grade KH2PO4 and K2HPO4; McIlvaine's buffer was prepared with analytical grades of Na2HP04 and citric acid.

Protein Determination—The protein concentration of all enzyme solutions was determined by the method of Lowry et al. (7) with crystalline bovine albumin as a standard. The protein value of ascorbic oxidase so obtained was found to be in satisfactory agreement with the result obtained by Tokuyama et al. who used the method of Sutherland et al. (8).

Copper Determination—The method of Stark and Dawson (9) was used. Prior to each determination, the enzyme specimens were mixed with frequent stirring over a period of at least 3 hours at 4° with 15 to 150 volumes of Chelex-100 resin to remove all extraneous "free" ionic copper. The resin was removed by centrifugation. The resin had been previously washed successively with 0.1 N NaOH, 0.1 N HCl, deionized water, and suspended in 0.01 M phosphate buffer, pH 7.6.

Measurement of Enzyme Activity—The ascorbic oxidase activity was determined manometrically by measuring the initial rate of oxygen uptake during the enzyme-catalyzed oxidation of L-ascorbic acid in a conventional Warburg apparatus at 25°. The activity was determined under the optimal conditions of reaction volume, pH, and substrate concentration prescribed in the assay method of Dawson and Magee (4), except that the positions of the enzyme and substrate during incubation in the Warburg flask were interchanged (10). One unit of ascorbic oxidase activity has been defined as the amount of enzyme that causes an initial rate of oxygen uptake of 10 μl per min under the prescribed conditions. Specific activity is expressed in units per mg of protein, or alternatively, as units per μg of copper.

Measurement of Enzyme Blue Color—A measurement of the optical density at 610 nm with a Beckman DU spectrophotometer was used as an index of the intensity of blue color of each enzyme preparation.

Polyacrylamide Gel Electrophoresis—The acrylamide gel electrophoresis experiments were carried out at 4° according to the method of Ornstein and Davis (11).

Column Chromatography—Prior to use the phosphocellulose was suspended in deionized water several times to remove the fine particles by decantation. The washed phosphocellulose was then equilibrated with 0.02 M potassium phosphate buffer, pH 5.5, overnight at 4°. The buffer was then decanted and the sedimented phosphocellulose was suspended in 0.01 M potassium phosphate buffer, pH 5.5, poured into the column, and again equilibrated with the same buffer. The TEAE-cellulose, previously and successively washed with 0.5 N NaOH and deionized water, was equilibrated with 0.02 M potassium phosphate buffer, pH 7.6. Just before use, the 0.02 M buffer was decanted and replaced by 50 volumes of 0.01 M phosphate buffer, pH 7.6, to make a suspension. The columns were packed with

1 The abbreviation used is: TEAE, triethylaminoethyl.

the equilibrated TEAE-cellulose suspension under a constant air pressure of 20 cm Hg and the material was then further equilibrated by passing more than 10 volumes of the same buffer through the column. Regeneration of the phosphocellulose and the TEAE-cellulose was carried out by washing each successively with 0.5 N HCl, water, 0.5 N NaOH, and finally with deionized water. The amounts of phosphocellulose and TEAE cellulose used and the dimensions of the columns are given in the legends of the figures.

Ultracentrifugal Analyses—Sedimentation analyses were performed in a Spinco model E ultracentrifuge equipped with schlieren optics and also equipped with a rotor temperature indicator and control unit. Values of the sedimentation coefficients were corrected to 20°c in the conventional way. All procedures and calculations for the ultracentrifuge runs were carried out according to the method of Schachman (12).

Amino Acid Analyses—Analyses were carried out according to the procedure of Spackman et al. (13) with a Beckman/Spinco model 120B amino acid analyzer equipped with a 6.6-mm optical path. Cysteine and cystine residues were determined as cysteic acid following performic acid oxidation (14). The tyrosine to tryptophan ratio was obtained according to the procedure of Benezet and Schmid (15). The tryptophan content was then calculated from the tyrosine residue value obtained in the amino acid analysis. The presence of amino sugars in the hydrolysates was established, and their identification made, by comparing the chromatogram peak positions of the enzyme hydrolysates with chromatograms obtained from the simulated hydrolysis of a standard amino acid mixture prepared so as to contain known amounts of authentic samples of the amino sugars. The ninhydrin constant for histidine was used for the estimation of amino sugars.2

RESULTS

Purification of Enzyme

Several of the purification operations described below (Steps 2, 5, and 7) were carried out at temperatures of 0-4° in the cold room or the refrigerator, and others, particularly the column chromatography experiments, were more conveniently performed under room temperature conditions. However, in all cases of work at room temperature, efforts were made to keep the enzyme systems at all times below 10°. Thus, all reagent solutions were precooled to 0-4° and all of the ion exchange cellulose columns were equilibrated with ice-cold buffer solutions before use. All elution buffers were similarly precooled and columns were thereby maintained continuously at temperatures below 10°.

The stepwise operations described below are the result of numerous exploratory experiments carried out during the period of this investigation. Because of the limited season of commercially available fresh raw material in the early summer months, the first two steps were developed for preparing a crude form of the enzyme of suitable stability for storage. Several 40-bushel batches of the zucchini squash were thus processed to provide crude ammonium sulfate precipitates (Step 2) which were stored under deep freeze conditions (-15°) until subsequently used in about 1-kg amounts for the purification operation described in Steps 3, 4, etc.

Step 1: Preparation of Crude Juice—A 40-bushel batch of green squash was hand-peeled and the pulp was discarded. The

Details on these and other studies are presented in the Ph.D. dissertation of M. H. Lee, Columbia University, New York, New York, 1968.
peelings (about 1/4 inch thick) were minced with a power-driven meat grinder. A small amount of solid sodium borate (Na₂B₁₀O₁₄·10 H₂O) was periodically added to the fluid mince from the grinder in order to raise the pH of the crude juice (pH 5.9) to near neutrality (pH 6.8). The mince was placed in canvas bags and subjected to hydraulic pressure, with the use of a hand wine press. The juice was then immediately transferred to the cold room and treated with ammonium sulfate as described below. In the usual case, a 40-bushel batch of squash yielded about 110 liters of juice possessing a total oxidase activity of 7 to 10 x 10⁶ units. The juice at this stage usually had a specific activity of about 20 units per mg of protein and 50 units per μg of copper.

Step 2: Ammonium Sulfate Precipitation—Solid ammonium sulfate was added slowly to the crude juice in amounts corresponding to 65% saturation at 4°C. The addition was carried out in the cold room with continuous hand stirring for about 30 min after all of the ammonium sulfate had dissolved. The proteinaceous greenish precipitate that developed was allowed to settle overnight and most of the clear supernatant fluid was then removed by siphon and discarded. The green precipitate was then collected by batch-wise gravity filtration through 12 to 15 fluted Eaton Dyckman No. 617 papers (40-cm diameter). A total of about 4 kg of moist green precipitate was usually obtained possessing about 90% of the original enzyme activity.

Step 3: Fractionation with Acetone—A suitable quantity (usually about 1 kg of the frozen ammonium sulfate precipitate from Step 2) was weighed and suspended in about 3 liters of ice-cold distilled water to extract the soluble enzyme. The suspension was stirred for about 30 min and the insoluble residue was removed by centrifugation (11,700 x g for 10 min) in the cold. The precipitate was discarded and the supernatant solution was divided into seven or eight 400-ml aliquots. To each aliquot was added 1.3% NaCl and the material was chilled to about -3°C by immersion in a Dry Ice acetone bath. By slowly revolving the beaker in the bath (or by stirring) the frozen material was deposited on the wall of the beaker to a thickness of about 1/4 inch. The beaker was then removed from the bath and the solid allowed to thaw and soften until it could be broken up by a glass rod into a semisolid mass of snow-like consistency. A 0.9 volume of chilled acetone (−15°C) was then added slowly in increments to each beaker to precipitate the enzyme while the suspension was stirred vigorously. The resulting precipitate was allowed to settle for about 10 min and the clear supernatant fluid was decanted. The precipitates were collected separately on filter paper (Whatman No. 2) by suction filtration, combined, and then suspended in about 800 ml of ice-cold 0.002 M phosphate buffer, pH 7.6, phosphate buffer. The material was then dialyzed against cold running tap water for 4 to 6 hours. Insoluble matter was removed by suction filtration and discarded. At this point the filtrate usually had a specific activity of about 185 units per mg and 205 units per μg of copper. The enzyme at this stage contained about 0.09 μg copper and about 79% of the total activity of the original ammonium sulfate precipitate.

Step 4: Phosphocellulose Chromatography—The dialyzed enzyme solution obtained in Step 3 was further dialyzed against 3 volumes of 0.01 M phosphate buffer, pH 5.5, for a period of 24 hours (the buffer was changed after each 8-hour period). The small amount of protein material that precipitated was removed by centrifugation and the enzyme solution was then directly added to the phosphocellulose column (see "Experimental Procedures"). Usually it was most convenient to use several small columns (3.3 x 11 cm) rather than a single larger column. It was observed that a blue enzymatically active component in the solution was absorbed in a band at the top of the column, while yellowish components (inactive) passed through. The loading of the column with enzyme was therefore controlled by adding enzyme solution until the blue band was dispersed throughout most of the column. The column was then washed with 0.01 M phosphate buffer, pH 5.5, until no further yellow color was observable in the eluent. With this concentration of phosphate there was essentially no movement of the blue band. The column was then washed with about 3 bed volumes of 0.03 M phosphate buffer, pH 5.5, which removed colorless nonenzymatic protein components. Finally the enzyme was eluted with 0.1 M phosphate buffer, pH 5.5, and fractions were collected by use of a fraction collector. The elution of the enzyme was easily followed by observing the movement of the blue-green band down the column. A typical elution pattern is seen in Fig. 1. Only the distinctly greenish blue fractions (for example 16 through 22 in Fig. 1) were combined for further purification. The specific activity of the enzyme in the combined fractions was usually in the range of 1500 to 1800 units per mg with about 70% recovery of the total activity applied to the column. Lower yields of enzyme having specific activities as high as 2260 units per mg were obtainable in a single chromatographic operation by appropriate selection and pooling of the elution fractions.

Step 5: Dialysis I—The blue enzyme solutions combined from the several phosphocellulose columns (usually about 80 ml) were dialyzed against 1-liter volume of 0.01 M phosphate buffer, pH 5.5, for 24 hours in the refrigerator. The buffer was exchanged at the end of each 8-hour period. During the dialysis about one-half of the activity precipitated in the form of a blue flocculent sediment which was separated by centrifugation (17,300 x g for 10 min at 4°C). The resulting blue pellet was...
activity of the blue enzyme solution at this stage was usually about 3000 units per mg. Carried out with 0.02 M and 0.06 M phosphate buffer, pH 7.6, to give an intensely blue solution. A small amount of cold enzyme (66D-I) from Step 5 (Dialysis I) containing 2.31 mg per ml of protein (3130 units per mg) in 0.01 M phosphate buffer, pH 7.6, was applied to the column. Elution of the enzyme was carried out with 0.02 M and 0.06 M phosphate buffer, pH 7.6, as indicated. Fraction volume, 2.5 ml; flow rate 0.6 ml per min.

then suspended in about 40 ml of 0.01 M phosphate buffer, pH 7.6, to give an intensely blue solution. A small amount of colorless, inactive, and insoluble protein was removed by re-centrifugation under the same conditions. The specific activity of the blue enzyme solution at this stage was usually about 3000 units per mg.

Step 6: TEAE-cellulose Chromatography—The blue enzyme solution from Step 5 was applied to a TEAE-cellulose column (see “Experimental Procedures”) under a constant air pressure of 20 cm Hg. When the blue band had developed to about one-half of the length of the column the application of enzyme was stopped. The column was then treated with an additional 5 volumes of 0.01 M phosphate buffer, pH 7.6, and then with 2 volumes of 0.02 M phosphate buffer, pH 7.6. During the latter treatment (0.02 M phosphate) the blue band moved down the column somewhat. The blue enzyme was then eluted with 0.06 M phosphate buffer, pH 7.6, and collected in small fractions with the use of a fraction collector. The movement of the blue band down and off the column showed the progress of the enzyme elution. A typical elution pattern for the TEAE-cellulose chromatography is shown in Fig. 2.

Step 7: Dialysis II—The fractions from the TEAE-cellulose column possessing specific activities higher than about 3400 units per mg were combined for further purification by precipitation dialysis. The blue enzyme solution was dialyzed for 24 hours in the refrigerator against a 0.002 M phosphate buffer, pH 6.4. The buffer was changed twice during the dialysis as described earlier. Under these conditions of pH and ionic strength about 90% of the activity precipitated during the dialysis process. The blue precipitate was collected by centrifugation and then resuspended in 0.01 M phosphate buffer, pH 7.6, as described in Step 5. Enzyme specimens thus obtained had specific activities in the range of 3800 to 4250 units per mg and copper contents in the vicinity of 0.46 to 0.52%.

The over-all purification results achievable by the stepwise procedures described above are summarized in Table I for a typical enzyme purification involving a 1.5-kg sample of frozen raw material (see Step 2) dissolved in about 4 liters of distilled water.

Criteria of Homogeneity

Ultracentrifugal Analysis—The sedimentation analyses of purified ascorbate oxidase specimens having specific activities in the range of 3800 to 4250 units per mg displayed a single symmetrical boundary in the schlieren diagram at all observation times (Fig. 3, upper curve). Enzyme preparations having specific activities in the vicinity of 3500 units per mg were judged to be contaminated with a trace amount of a faster sedimenting material (Fig. 3, lower curve).

Polyacrylamide Gel Electrophoresis—At each stage in the purification procedure described above the resulting enzyme solution was examined by polyacrylamide gel electrophoresis. As shown in Fig. 4 there was a progressive decrease in the number of observable components with each step in the purification process. The final preparations, having specific activities in the region of 4200 units per mg, showed only a single protein component. Preparations having a specific activity of about 3500 units per mg (Step 6) appeared to be contaminated with a small amount of a slower moving component.

Characteristics of Purified Enzyme

Stability—Incubation of the purified enzyme (freshly prepared) at 80° for 10 min resulted in complete loss of activity. However, concentrated solutions of the purified enzyme have been found in this and earlier (5) investigations to be quite stable for long periods of time when stored at low temperatures. Such enzyme solutions containing in the order of 10,000 units per ml or higher (3 mg or higher of enzyme protein per ml) have been found in this investigation to retain about 80% of their activity after storage for 3 months at 4°C.

Specific Activity and Copper Content—The preparation of ascorbate oxidase having the highest specific activity obtained by use of the new purification procedure (see Step 7, Table I) had a specific activity of 4250 units per mg and a copper content of 0.46% (corresponding to an activity of 918 units per µg of copper). On the basis of a molecular weight of 140,000 (estimated as described below on the same preparation) a copper content of 0.46% corresponds closely to 10 atoms of copper per enzyme molecule. It should be noted that in several of the purification experiments, the last step of the new procedure (Step 7) yielded homogeneous ascorbate oxidase preparations having somewhat higher copper contents (as high as 0.52%). In all cases, however, the specific activity values ranged between 3800 and 4250 units per mg. A 0.52% copper content value corresponds to 12 atoms of copper per enzyme molecule of 140,000 molecular weight.

Sedimentation Constant—Three different samples of purified enzyme, when centrifuged in 0.01 M potassium phosphate buffer, pH 7.6, containing 0.1 M KCl, gave the same observed sedimentation constant. The constant was found to be independent of the enzyme protein concentration up to at least 5 mg per ml. After correction for temperature and density, the extrapolated zero concentration value was 6.88 ml/g-m. S. Estimation of Molecular Weight—On the basis of the above sedimentation constant a molecular weight of 140,000 was
TABLE 1
Purification summary for ascorbate oxidase (66D-I)* from green zucchini squash (Cucurbita pepo medullosa)

| Purification steps       | Total volume | Total activity | Total protein | Specific activity | Enzyme yield | Enzyme purification (factor) | Copper data |
|--------------------------|--------------|----------------|---------------|-------------------|--------------|-------------------------------|------------|
|                          | ml           | units          | mg            | units/mg          | %            |                               | ug/mg Cu   |
| 1. Crude juice           | 28,300       | 1,989,600      | 99,480        | 20                | 100          | 1                            | 34,818     |
| 2. Ammonium sulfate      | 4,490        | 1,715,180      | 24,156        | 71                | 86           | 3                            | 15,701     |
| 3. Fractionation with acetone | 3,000     | 1,354,000      | 7,319         | 34                | 68           | 9                            | 6,806      |
| 4. Phosphocellulose      | 183          | 688,400        | 385           | 1,790             | 34           | 89                           | 1,015      |
| 5. Dialysis              | 1            | 363,200        | 116           | 53,130             | 18           | 156                          | 445        |
| 6. TEAE-cellulose        | 15.4         | 320,270        | 92.1          | 3,550              | 16           | 177                          | 403        |
| 7. Dialysis II           | 9.7          | 284,510        | 66.9          | 4,250              | 14           | 213                          | 310        |

*These data for oxidase 66D-I are very similar to corresponding data obtained during the processing of six other preparations by M. H. L. during the period 1966-69. During the past 3 years the data have been repeatedly confirmed in these laboratories via six additional preparations.

†Dialysis in 0.01 M phosphate buffer (pH 5.5).

‡Dialysis in 0.002 M phosphate buffer (pH 6.4).

![Fig. 3 (left)]. Sedimentation patterns of Enzyme 66D-I (specific activity 4250 units per mg, Cu 0.46%, upper curve) and 67A-ID3 (specific activity 3500 units per mg, Cu 0.49%, lower curve) in 0.01 M phosphate buffer pH 7.6, containing 0.1 M KCl. The exposure was made 40 min after the maximum speed had been attained.

![Fig. 4 (right)]. Disc electrophoretic patterns of the enzyme at various steps of the purification procedure. The electrophoreses were carried out on 7.5% standard polyacrylamide gels at pH 9.5 for 90 min at 4°C. The gel columns were 0.9 x 8.5 cm, and a current of 5 ma per tube was applied.

**DISCUSSION**

Previous preparations of ascorbate oxidase having specific activities of about 2000 units per mg have been judged to be essentially homogeneous by ultracentrifugal and electrophoretic criteria (3, 5). Purified preparations of ascorbate oxidase having specific activities in the vicinity of 2000 units per mg have been found in this investigation (see Table I, Step 4) and in previous investigations (3, 5) to contain about 0.24 to 0.27% copper. The chromatographic purification procedures developed by Tokuyama et al. (5), and in this investigation, have resulted in ascorbate oxidase preparations of significantly higher specific activities and copper contents. The purification process results in increases in copper contents that correspond to a progressive increase in catalytic efficiency of the enzyme copper toward a maximal value. In the typical purification experiment indicated in Table I, the catalytic efficiency value (units per μg of copper) reached 918, a value which lies intermediate between the 740 value of Dunn (3) and the 1030 value of Tokuyama (5). In all of the purification experiments, in which the procedure outlined in Table I was used, the final homogeneous enzyme preparations had activity and copper values corresponding to 750 to 1000 units per μg of copper. A comparison of these homogeneous preparations is presented in Table II, and it is particularly noteworthy that no significant calculated by use of the Svedberg equation (16). In the calculation the following values were used for the diffusion constant, \( D_{\text{ca,m}} \), and the partial specific volume, \( \bar{v} \), of the enzyme: \( D_{\text{ca,m}} = 4.95 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1} \), as reported by Tokuyama et al. (5); \( \bar{v} = 0.732 \) as calculated from the specific volumes of the constituent amino acid residues (see below) according to Cohn and Edsall (17).

**Amino Acid Composition**—The results of the amino acid analysis of the purified enzyme 67A-ID3 (specific activity = 3500 units per mg, copper content = 0.49%) were very similar to those previously reported by Stark and Dawson (18) in 1962 involving a homogeneous ascorbate oxidase having a specific activity of 2280 units per mg and a copper content of 0.32%.

**Table No.**

| Purification step | I  | II | III | IV  | V  |
|-------------------|----|----|-----|-----|----|
| Protein (μg)      | 579| 500| 200 | 216 | 200|
| Specific activity (units per mg) | 71 | 185 | 1790 | 3550 | 4250 |
change in the molecular weight of the enzyme has been observed as the specific activity (units per mg of protein) has approximately doubled and the copper content has increased also by a factor of about two. Furthermore, as pointed out above, the amino acid compositions have been found to be very similar for two purified ascorbate oxidase preparations differing significantly in specific activity and copper content. These points of comparison are consistent with the view that the same protein moiety constitutes the apoenzyme of the homogeneous ascorbate oxidase preparations listed in Table II.

Previous studies have shown that the copper is tightly bonded to the protein moiety of ascorbate oxidase at physiological pH (10, 10–22). However, the copper bond is sensitive to hydrogen ion and a variety of agents and conditions. Thus, dialysis of the enzyme in systems more acidic than about pH 4 results in rapid loss of prosthetic copper, and dialysis against cyanide ion at pH 7.0 is used to prepare the apoenzyme (22–24). The copper is also freed from the protein by treatment with urea (24), sodium dodecyl sulfate (18), and by thermal inactivation (25). Consequently it is suggested that the marked increase in specific activity of the enzyme that has been achieved with the development of a new purification procedure is simply the result of a lower loss of prosthetic copper during the isolation process.

It now appears that ascorbate oxidase specimens having a specific activity in the range of 3800 to 4200 units per mg are composed of protein molecules that carry a near maximal amount of prosthetic copper (0.46 to 0.52%). The essentially homogeneous preparations of the enzyme, isolated by previous investigators and found to have specific activities at either the 2000 units per mg or 3000 units per mg levels, were very likely composed of the same protein moieties carrying lower amounts of prosthetic copper.

It seems likely that the variation in the catalytic efficiency of the prosthetic copper of the enzyme, from 140 to 1030 units per μg of copper, may be dependent on factors other than just the copper content. Thus, it has been proposed, based on an earlier investigation in these laboratories (20), that the prosthetic copper of ascorbate oxidase exists in both the cupric and cuprous oxidation states, and only the cupric copper is catalytically active (27). Similar observations have also been made recently concerning the copper of lactase (28–31) and cucumber ascorbate oxidase (32). Consequently variations in the ratio of Cu(II)/Cu(I) may account, at least in part, for the different levels of catalytic efficiency of the prosthetic copper observed in different ascorbate oxidase preparations.

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Table II
Comparison of homogeneous ascorbate oxidase preparations

| Year | Investigators         | Type squash | Specific activity | Copper content | Molecular weight |
|------|-----------------------|-------------|------------------|----------------|-----------------|
|      |                       |             | Units/mg of protein | Units/μg Cu | Percent | Atoms/molecule |
| 1951 | Dunn and Dawson       | Yellow      | 1925             | 740           | 0.26    | 6              | 146,000 ± 10% |
| 1965 | Tokuyama, Clark, and Dawson | Yellow-green   | 3600            | 1100          | 0.33    | 8              | 134,000-140,000 |
| 1968 | Lee and Dawson        | Green       | 3800-4200        | 750-1000      | 0.46-0.52| 10-12          | 140,000       |

a Tokuyama’s purification data (5) showed that, although there were some differences in the yield and quality of the enzyme obtained from yellow and green squash at comparable intermediate stages of the purification procedure, the preparations obtained in the last step were essentially the same in quality, i.e., specific activity, copper content, and blue color. The green zucchini squash was used in the present investigation because seasonal supplies were more conveniently available than in the case of the yellow squash.

b Protein content determined by dry weight method.

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