Phytochemistry, 1968, Vol. 7, pp. 199 to 204. Pergamon Press. Printed in England

INTERCONVERSION OF SUB-UNITS OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS

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(*Received* 28 *June* 1967)

Abstract—The three fractions of catechol oxidase previously reported from apple chloroplasts were shown to result from various degrees of aggregation of sub-units of the same enzyme. This was demonstrated by gel filtration on columns and by thin layer gel filtration in Sephadex. Interconversion between the fractions takes place during storage at 2–4° and is enhanced by various treatments. The possible existence of such interconversions *in vivo* and their physiological significance are discussed.

INTRODUCTION

CATECHOL oxidase (o-diphenol: O_2 oxidoreductase, EC 1.10.3.1) can oxidize a great variety of natural and synthetic o-diphenols. Vast differences exist, however, between enzymes obtained from different sources.[1–6] Many workers have tried to establish whether more than one enzyme is involved in the oxidation of the variety of phenols in one organism or tissue. Information in the literature ranges from reports on differences in the properties of catechol oxidases in different parts of an organism[7] or in different subcellular particles[8,9] to separation of several forms of the enzyme by electrophoresis or column chromatography.[10–17] Multiple forms of catechol oxidase have also been reported for apples, where differences exist between enzyme preparations from different subcellular fractions.[18,19] Extracts of apple chloroplasts contain several fractions of catechol oxidase separable by electrophoresis and column chromatography.[19,20] These fractions differ in the relative rates at which they oxidize various

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substrates as well as in their sensitivity to inhibitors and their affinity for phenolic substrates and for oxygen.[19]

It was decided to try to establish whether the fractions of catechol oxidase from apple chloroplasts represent different enzymes of whether they are different forms of the same enzyme, either originally existing in the tissue or formed during the extraction of the enzyme.

RESULTS AND DISCUSSION

Extracts of apple chloroplasts obtained using various surface active agents were always separated by starch gel electrophoresis into three bands having catechol oxidase activity.[19] The extracts were also resolved into three fractions by chromatography on various columns (DEAE-cellulose, TEAE-cellulose, Celite-535, hydroxyl apatite). The electrophoretic

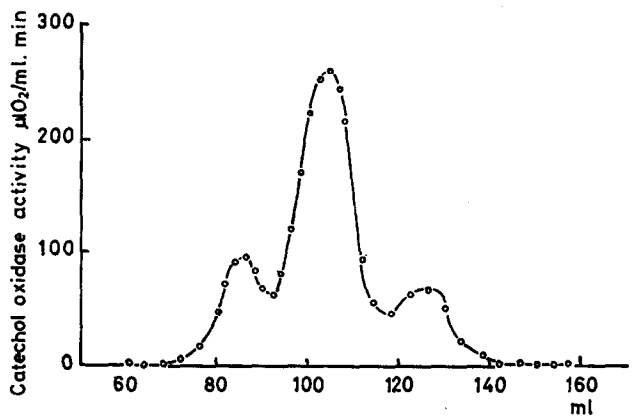

FIG. 1. GEL FILTRATION OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS ON A COLUMN OF SEPHADEX G-100.

Dimensions of column: 1·8 cm diameter × 113 cm height.
Elution with 0·02 M phosphate buffer pH 6·2.
Flow rate: 20 ml/hr.
Enzyme activity determined in phosphate-citrate buffer pH 5·1 using oxygen electrode.
Substrate: 4-methylcatechol 5 mM.

mobilities and the ionic strengths at which the fractions were eluted from a certain type of column were constant in all the preparations examined. However, appreciable differences were noticed in the relative activities of the fractions, depending on the concentration of protein, age of the fruit, the conditions of extraction and length of storage of the enzyme preparation before analysis was performed. Furthermore, while on electrophoresis of fractions immediately after their elution from a column only one band of catechol oxidase was usually detected, the same fractions were resolved into two or three bands after standing for several days at 2–4°.

When a Triton X-100 extract of chloroplasts was subjected to gel filtration on a Sephadex G-100 column, it was resolved into three fractions showing catechol oxidase activity (Fig. 1). Estimates for the molecular weights of the three fractions, as obtained by calibration of the column with proteins of known molecular weights, were 30,000–40,000, 60,000–70,000 and 120,000–130,000 (Fig. 2). Electrophoresis of the fractions from the peaks of catechol oxidase activity immediately after their elution showed that the fraction with the lowest molecular

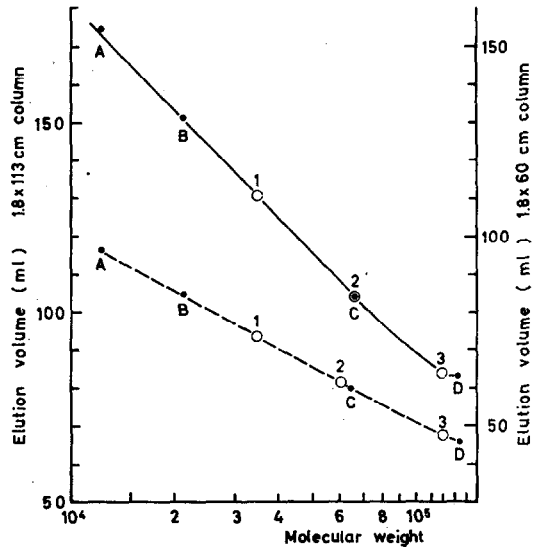

FIG. 2. ESTIMATION OF THE APPARENT MOLECULAR WEIGHTS OF THE THREE FRACTIONS OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS BY CALIBRATION OF SEPHADEX G-100 COLUMNS WITH PROTEINS OF KNOWN MOLECULAR WEIGHTS.

A—Cytochrome c. B—Trypsin inhibitor from soybean. C—Bovine serum albumin (monomer). D—Bovine serum albumin (dimer). 1,2,3-Catechol oxidase fractions.

—— 113 cm column
--- 60 cm column

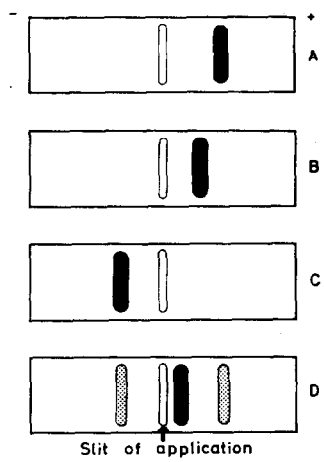

FIG. 3. STARCH GEL ELECTROPHORESIS OF THE THREE FRACTIONS OF CATECHOL OXIDASE FROM APPLE CHLOROPLASTS AFTER THEIR SEPARATION BY GEL FILTRATION ON SEPHADEX G-100. CONDITIONS OF GEL FILTRATION AS IN FIG. 1.

(a) Fraction III from column (elution volume 128–134 ml)
(b) Fraction II (elution volume 100–108 ml).
(c) Fraction I (elution volume 80–88 ml).
(d) Original preparation, before gel filtration (partially purified by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose).

Electrophoresis—in 0·04 M phosphate buffer pH 7·3. Other conditions, including the detection of catechol oxidase in the gels, as previously described, the plates being developed by spraying with 4-methylcatechol + paraphenylene diamine (19).

weight (fraction III) migrated farthest towards the anode (Fig. 3), corresponding thus to the fraction eluted from DEAE-cellulose at 0·14 M phosphate buffer pH 7·2.[19] The fraction with a molecular weight of 60,000–70,000 (fraction II) migrated more slowly towards the anode and corresponded to the fraction eluted at 0·09 M phosphate buffer, while the fraction with the highest molecular weight migrated to the cathode (fraction I) and corresponded to that eluted from DEAE-cellulose at 0·04 M phosphate buffer.

Similar results were obtained by gel filtration on columns of Sephadex G-75, Sephadex G-200 and Bio-gel P-150, though separation varied depending on the type of gel used. Results obtained by thin layer gel filtration of Triton X-100 extracts and of purified fractions after gel filtration or chromatography on columns were also in agreement with the above observations.

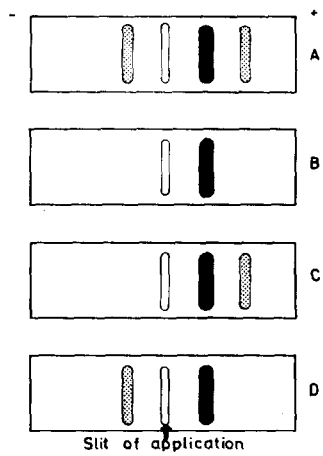

FIG. 4. STARCH GEL ELECTROPHORESIS OF A PURIFIED FRACTIONS OF CATECHOL OXIDASE FROM APPLES AFTER VARIOUS TREATMENTS. CONDITIONS FOR ELECTROPHORESIS AS PREVIOUSLY DESCRIBED (FIG. 3).

A—Crude Triton X-100 extract. B—Purified Fraction II stored 48 hr at 2–4°. C—Purified Fraction II stored 48 hr in the presence of 1 per cent Triton X-100 at 2–4°. D—Fraction II stored 48 hr in the presence of 1 N NaCl at 2–4°.

When the fractions separated by gel filtration were subjected to starch gel electrophoresis after being kept for several days at 2–4° new bands appeared suggesting that the protein underwent structural transformation during storage (Fig. 4). When fraction II was treated with 1 N NaCl for 48 hr at 2–4° it was partially converted to fraction I, while fraction II was partially converted to fraction III when treated with 1 per cent Triton X-100 (Fig. 4). No clear-cut changes in the electrophoretic pattern were caused by starch gel electrophoresis in the presence of 5 M urea.

Our results suggest that the three fractions of catechol oxidase from apple chloroplasts represent various degrees of aggregation of sub-units of the same enzyme. Reports in the literature on the molecular weight of catechol oxidases are quite scarce. Kertesz and Zito[21] reported a molecular weight of 130,000 for catechol oxidase purified from *Psalliota*. In an earlier report,[22] however, the molecular weight of a catechol oxidase purified by these authors from the same source and by essentially the same procedure was reported to be about

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32,500. Other works on mushroom catechol oxidase reveal the existence of a number of enzyme fractions[10,15] several of which can undergo interconversion.[15] A similar situation seems to exist in *Neurospora*. The molecular weight of the purified enzyme is about 130,000 but conversion to other fractions occurs during column chromatography.[23] Interconversion between different forms of catechol oxidase from *Neurospora* during incubation at 5–25° has also been reported.[24] Heterogeneity of form and molecular weight has also been reported for the latent and active forms of tyrosinase from *Vicia*[25] and polyphenol oxidase from tea.[26] It seems likely from our results that the sub-units of the enzyme are identical, since all methods of separation yielded only three fractions. However, this is not absolutely certain. In order to establish this unequivocally, large amounts of the various fractions in a high state of purity are required. This is difficult to achieve both because of the nature of the starting material and the apparent interconversion between the fractions. The purest preparation we have been able to obtain contained two peaks in the ultracentrifuge. The sedimentation coefficient of the major peak (corresponding to about 90 per cent of the protein in the preparation), was $S_{20}=2{\cdot}70$. This value is very close to the value reported by Kertesz and Zito[22] ($S_{20}=2{\cdot}71$) for the purified enzyme from mushroom with a molecular weight of 32,500.

It is at present impossible to assess whether the multiplicity or heterogeneity of the catechol oxidase as reported here, also exists in the intact tissue. Such multiplicity may equally well be the result of the extraction and purification procedure. Nevertheless, it is tempting to speculate about the possible physiological significance of the differences in properties observed in the various fractions.[19] If such differences also occur *in vivo*, and are also caused by interconversions, this might be of very great significance in the tissue. An example would be changes in affinity for oxygen. The failure to detect such differences in properties among various fractions, as reported by Walker and Hulme[20] may well be due to interconversion reactions which occurred between the time of separation and of analysis. A further observation indicating a possible physiological significance of such interconversion is the fact that in extracts of chloroplasts from young fruits (6–10 weeks after fruit set) the predominant fraction was fraction I (molecular weight of about 130,000). In ripe fruit the amounts of this fraction are always smaller than fractions II and III. It is not clear whether this is due to the higher levels of catechol oxidase in young fruit[27] and hence in the extracts, or whether it reflects the normal situation in the tissue.

EXPERIMENTAL

Apples, variety Grand Alexander, were either purchased at a local supermarket or picked from selected trees in an orchard near Jerusalem and stored at 2–4° in the laboratory for short periods before use.

Chloroplasts fractions and their extracts with Triton X-100 and other surface active agents were prepared as previously described.[19]

Column chromatography, starch gel electrophoresis and partial purification of the enzyme by ammonium sulphate fractionation and chromatography on DEAE-cellulose were performed as previously described.[19]

Catechol oxidase activity was determined in phosphate-citrate buffer pH 5·1, using a polarographic oxygen electrode[18] with 5 mM 4-methylcatechol as a substrate.

Hydroxyl apatite was prepared according to Tiselius *et al.*[28]

Preparation and equilibration of columns for gel filtration, application of enzymes or protein markers and the determination of elution volumes were carried out according to Andrews.[29] Columns were eluted with

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0·02 M phosphate buffer pH 6·2 and 2 or 4 ml fractions were collected using a "Shandon" automatic fraction collector AF/260. Cytochrome c was determined spectrophotometrically at 550 mμ after reduction with sodium dithionite. Other proteins were determined according to Lowry *et al.*[30]

Thin layer gel filtration and the detection of catechol oxidase and protein markers in the gels were performed as previously described.[31]

Sedimentation velocity studies were carried out with a Spinco model E ultracentrifuge equipped with Schlieren phase plate optics, at a protein concentration of 2·5 mg/ml in 0·005 M phosphate buffer pH 7·2.

The various types of Sephadex were supplied by Pharmacia. Celite-535 was obtained from Johns-Manville Co. and Bio-gel P-150 from Calbiochem.

Bovine serum albumin (crystalline), trypsin inhibitor from soybean (type I-S 3X crystallized) and cytochrome c (type II from horse heart) were supplied by Sigma.

Acknowledgement—This work has been supported in part by Grant FG-Is-140 of the United States Department of Agriculture to AMM.

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