0273-2289/93/4302—0153 $03.00

Ascorbate Oxidase

Specificity and Analytical Application

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Received January 18, 1993; Accepted May 3, 1993

ABSTRACT

Ascorbate oxidase from *Cucurbita sp.* was isolated by ammonium sulfate precipitation and DEAE-dextran-silochrome column chromatography. The thermal and pH stabilities of the purified enzyme were investigated. The K_M for L-ascorbic acid (1.5 m*M*) and chlorohydroquinone (0.37 m*M*) was determined. Substrate specificity of ascorbate oxidase was investigated and compared with those of laccases from *Coriolus hirsutus* and *Cerrena maxima*. Ascorbate oxidase was covalently bound to a polymeric membrane and used in an enzyme electrode for ascorbic acid.

Index Entries: Ascorbate oxidase; substrate specificity; ascorbic acid; immobilization; enzyme electrode.

INTRODUCTION

A variety of methods are used to measure ascorbic acid (*1*). Enzymatic methods using immobilized ascorbate oxidase appear highly specific and particularly attractive (*2–5*). Looking for a suitable source of ascorbate oxidase, we carried out screening of local species of cucumbers and gourds. From the best choice, ascorbate oxidase was isolated, purified, characterized, and used in enzyme electrode for determination of ascorbic acid.

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MATERIALS AND METHODS

Materials

DEAE-dextran-silochrome was produced in the Institute of Biotechnology "Fermentas" (Vilnius, Lithuania). Fluoro-, cyano-, ethyl-, and acetylhydroquinones were kindly provided by N. Cénas of the Institute of Biochemistry (Vilnius, Lithuania). All other chemicals were of analytical grade.

Purification of Enzyme

Ascorbate oxidase was isolated from the peel of *Cucurbita sp.* fruit. The peel was separated, homogenized, and pressed through cheesecloth. The crude juice was centrifuged at 10,000*g* for 25 min. Solid ammonium sulfate was added to the supernatant, up to 70% saturation. The resulting precipitate was collected by centrifugation at 12,000*g* for 25 min, dissolved in a minimum volume of 10 m*M* phosphate buffer, pH 7.4, and dialyzed against the same buffer. The dialyzed solution (15 mL) was charged onto a DEAE-dextran-silochrome column (1.5 × 20 cm) previously equilibrated with 10 m*M* phosphate buffer, pH 7.4. The column was run at flow rate of 32 mL/h. Fractions of approx 5 mL were collected. The protein was eluted with 300 mL of a linear phosphate buffer, pH 7.4, gradient (10–200 m*M*). The elution profile was obtained by determination of ascorbate oxidase activity and optical density at 280 nm. Laccases of basidial fungus were obtained as described earlier (*6*).

Analytical Methods

Protein was determined according to Lowry (*7*) with bovine serum albumin as standard. Sodium dodecyl-sulfate electrophoresis was carried out according to ref. (*8*). The activity of native and immobilized enzyme was determined by measuring, with a Clark oxygen electrode, the decrease of oxygen concentration in the reaction mixture, containing 2 m*M* ascorbic acid and 50 m*M* phosphate buffer, pH 6.5. One unit of ascorbate oxidase activity was defined as the amount of enzyme that consumes 0.5 μmol O_2/min at 30°C and pH 6.5 (i.e., that causes the oxidation of 1 μmol of ascorbic acid).

Effect of pH

Determination of ascorbate oxidase activity at different pH values was carried out in 30 m*M* acetate-phosphate-borate buffer (pH 4–9) under the assay conditions described above.

Stability Experiments

For determination of thermal stability, ascorbate oxidase was incubated at 55–70°C in 100 m*M* phosphate buffer, pH 6.5. Samples were withdrawn at definite time intervals for determination of enzyme activity. For determination of pH stability, enzyme was incubated in 150 m*M* citrate phosphate buffer (pH 3–7) or 150 m*M* glycine–NaOH buffer (pH 8.5–12) for 60 min at 30°C, and then the remaining enzyme activity was determined.

Substrate Specificity

For investigation of substrate specificity of ascorbate oxidase, the initial rate of oxygen consumption was measured in the reaction mixture composed of 50 m*M* phosphate buffer, pH 6.5, and 10 m*M* potential substrate. Substrate specificity of laccases was investigated by measuring, with a Clark oxygen electrode, the decrease of oxygen concentration in the reaction mixture containing 50 m*M* acetate buffer, pH 4.5, and 10 m*M* potential substrate.

Immobilization of Ascorbate Oxidase

Ascorbate oxidase was immobilized on a nylon membrane by the procedure described earlier (*9*). The polymer surface was activated by successive action of lithium aluminum hydride, 2,4,6-trichloro-*s*-triazine and polyethylenpolyamine. The enzyme was bound by crosslinking with glutaraldehyde.

Characterization of the Enzyme Membrane

The membrane containing immobilized ascorbate oxidase was placed onto a Clark oxygen electrode. The enzyme electrode for ascorbic acid was investigated as a biosensor for enzyme analyzers PLAG-11 (an analog of enzyme analyzer YSI Model 27, Yellow Springs Instrument Co.) and PLAG-31.

RESULTS

The purification of ascorbate oxidase from *Cucurbita sp.* consists of two steps: ammonium sulfate precipitation and DEAE–dextran–silochrome column chromatography. A typical elution pattern of ascorbate oxidase from DEAE–dextran–silochrome is presented in Fig. 1. Table 1 summarizes the results of ascorbate oxidase purification, including purification factor, specific activity, and yield. The two-step procedure increases the enzyme's specific activity by a factor of 11. Gel scanning after electrophoresis showed that 85% of the total protein was in the band containing ascorbate oxidase activity.

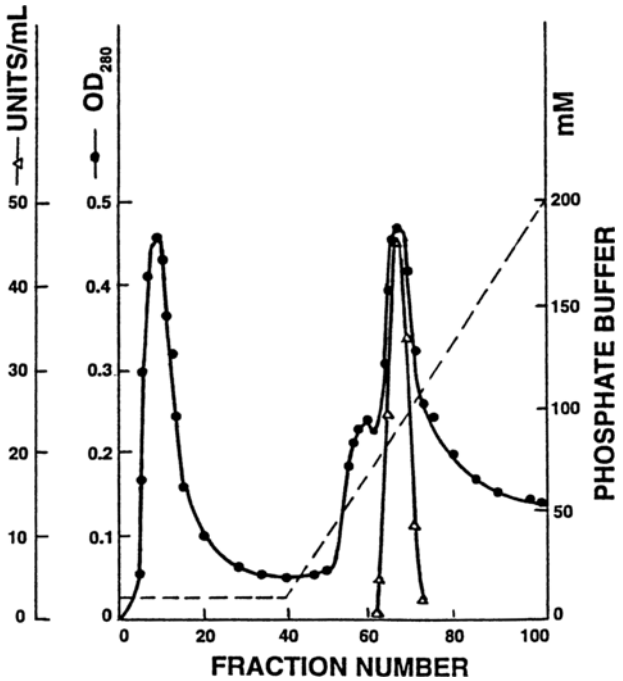

Fig. 1. DEAE-dextran-silochrome chromatography of ascorbate oxidase. Column, 1.5 × 20 cm; flow rate, 32 mL/h; fraction volume, approx 5 mL. Symbols: —•—, protein (OD_{280}); —△—, ascorbate oxidase activity (U/mL).

Table 1
Purification of Ascorbate Oxidase from *Cucurbita sp.*

Step	Total protein, mg	Total units	Specific activity, U/mg	Purification, fold	Recovery, %
Crude juice	180	3600	20	1	100
Ammonium sulfate precipitation	74	2664	36	1.8	74
DEAE-dextran-silochrome	10	2200	220	11	61

The enzyme showed optimum activity at pH 6.5 with L-ascorbic acid as the substrate (Fig. 2). Ascorbate oxidase was stable in the broad pH interval (pH 5–10), but rapidly lost its catalytic activity at pH < 4 and pH > 12 (Fig. 3). Thermal stability of the enzyme is presented in Fig. 4. It is high enough for analytical applications.

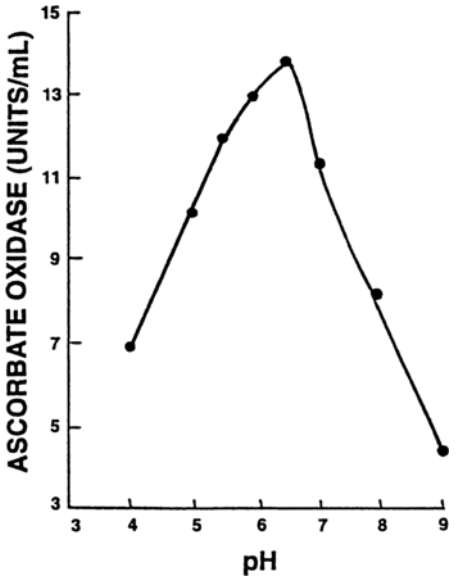

Fig. 2. pH optimum of ascorbate oxidase. Enzyme activity was determined in 30 m*M* acetate–phosphate–borate buffer (pH 4–9).

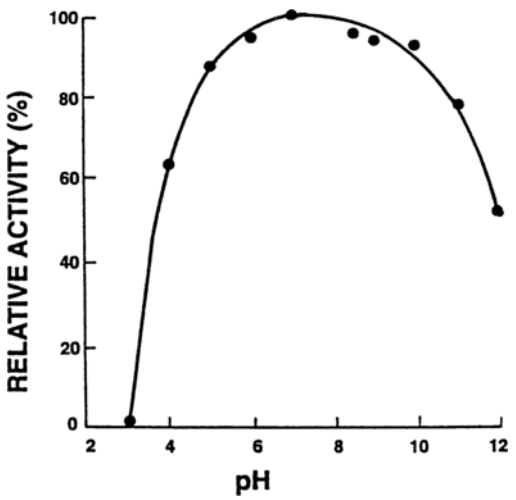

Fig. 3. pH stability of ascorbate oxidase. Enzyme solutions were incubated in 150 m*M* citrate phosphate buffer (pH 3–7) or 150 m*M* glycine–NaOH buffer (pH 8.5–12) for 60 min at 30°C, and then residual enzyme activity was determined under standard conditions. It was plotted as an activity percentage of the sample incubated at pH 7.0.

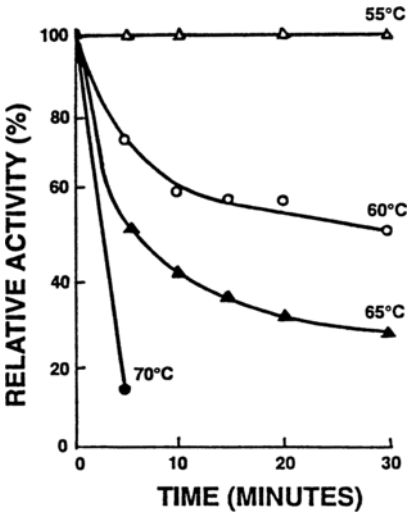

Fig. 4. Thermal stability of ascorbate oxidase. Enzyme solutions were incubated at indicated temperatures in 100 m*M* phosphate buffer pH 6.5. Samples were withdrawn at different time intervals for determination of activity under standard conditions. Residual activity was expressed as a percentage of untreated control.

The substrate specificity of native ascorbate oxidase was carefully investigated. Results are presented in Table 2. In addition to substrates given in Table 2, the following compounds were tested: hydroquinone, ethylhydroquinone, acetylhydroquinone, catechol, phloroglucinol, *o*-phenylenediamine, N-acetyl-*p*-phenylendiamine. None of them was notably oxidized. The K_M values were determined for two substrates: 1.5 m*M* for ascorbic acid and 0.37 m*M* for chlorohydroquinone. Substrate inhibition at high ascorbic acid concentrations (over 4 m*M*) was apparent.

Ascorbate oxidase immobilized on a nylon membrane was tested in an enzyme electrode for quantitative determination of ascorbic acid. The enzyme analyzer gave linear response in the range of ascorbic acid concentrations up to 4 m*M*. The lowest detectable concentration was approx 0.3 m*M* (50 mg/mL). The sensitivity of the analyzer was high enough for most analytical applications. We successfully measured the content of ascorbic acid in some kinds of fruits, juices, and vitaminized soft drinks.

Operational stability of the membrane was found to be not very high. After 1 wk of work, sensitivity of the analyzer was about 25% of its initial value. There was no change in membrane activity after storage for 2 mo at 4°C in 50 m*M* phosphate buffer, pH 6.5. Except for ascorbic acid, none of the substrates listed in Table 2 or any other from the numerous compounds tested gave noticeable signals of analyzer at concentrations up to 100 m*M*.

Table 2
Substrate Specificity of Ascorbate Oxidase and Laccases

	Relative activity, %		
	Ascorbate oxidase	Laccase	
Substrate	*Cucurbita sp.*	*Coriolus hirsutus*	*Cezzena maxima*
L-Ascorbic acid	100	100	100
Fluorohydroquinone	1	–	–
Chlorohydroquinone	6	–	23
Cyanohydroquinone	5	–	–
Hydroxyhydroquinone	3	–	34
Catechol	0	143	30
p-Methylaminophenol	2	–	26
o-Phenylenediamine	0	100	27

DISCUSSION

We proposed a very simple and rapid method for purification of ascorbate oxidase consisting of two steps: precipitation by ammonium sulfate and ion-exchange chromatography. It allowed us to attain ascorbate oxidase with specific activity over 200 U/mg, i.e., considerably better than minimal requirements for enzyme electrode, in two to three working days. The key step of purification was chromatography on ion-exchanger DEAE-dextran-silochrome. This ion exchanger was designed and produced in the Institute of Biotechnology "Fermentas." It consists of macroporous inorganic carrier covered by DEAE-dextran. Worse results were obtained when such ion exchangers as SP-Sephadex and CM-Cellulose were used. CM-Cellulose gave a lower purification factor. In the case of SP-Sephadex, low recovery (15%) was obtained.

Ascorbate oxidase isolated from *Cucurbita sp.* has high thermostability and broad pH tolerance. Traditional sources of ascorbate oxidase isolation have been *Cucurbita pepo medullosa* (*10*), *Cucurbita pepo condensa* (*11*), and *Cucumis sativus* (*12*). Such characteristics of the enzyme purified by us as pH optimum and pH stability are similar to those described in refs. (*10–12*), but there are some differences in substrate specificity of the ascorbate oxidase obtained in this study and the one described earlier. The K_M value for ascorbic acid of our ascorbate oxidase is about eight times higher that from *Cucurbita pepo medullosa* (*13*) and similar to that of the cucumber's ascorbate oxidase (2).

Ascorbic acid is readily oxidized by a number of nonspecific oxidases (laccases) found in microorganisms and plants, but many other organic compounds (aromatic amines, phenols) are oxidized at a similar rate or even better (*14*). For example, specificity of laccases from *Coriolus hirsutus* and *Cerrena maxima* is presented in Table 2. The ascorbate oxidase purified by us does not catalyze the oxidation of catechol and *o*-phenylenediamine. Therefore, it does not contain significant amounts of laccase.

It can be concluded from the results of our specificity studies and from examination of molecular models that two centers on substrate molecules may be essential for binding to the active site of ascorbate oxidase: (1) a hydroxyl group (6-OH of ascorbic acid, 4-OH of hydroquinone derivatives) and (2) ionized hydroxyl group at approx 6 Å distance (3-OH of ascorbic acid, 1-OH of hydroquinone derivatives). The second center also may serve as a route for electron transfer. Electron withdrawing substituents at the 2-position of hydroquinone ring (F, Cl, OH, CN) may help in binding by increasing acidity of the 1-OH group. In the case of 4-methylaminophenol, the methylamino group may be the second binding center.

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