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Spinach Thylakoid Polyphenol Oxidase[1]

ISOLATION, ACTIVATION, AND PROPERTIES OF THE NATIVE CHLOROPLAST ENZYME

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ABSTRACT

Polyphenol oxidase activity (E.C. 1.14.18.1) has been found in two enzyme species isolated from thylakoid membranes of spinach chloroplasts. The proteins were released from the membrane by sonication and purified >900-fold by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. The enzymes appear to be the tetramer and monomer of a subunit with a molecular weight of 42,500 as determined by lithium dodecyl sulfate gel electrophoresis. The higher molecular weight enzyme is the predominant form in freshly isolated preparations but on aging or further purification, the amount of lower molecular weight enzyme increases at the expense of the higher.

Sonication releases polyphenol oxidase from the membrane largely in the latent state. C_{18} fatty acids, especially linolenic acid, are potent activators of the enzymic activity. In the absence of added fatty acids, the isolated enzyme spontaneously, but slowly, activates with time.

Purified polyphenol oxidase utilizes *o*-diphenols as substrates and shows no detectable levels of monophenol or *p*-diphenol oxidase activities. The K_m values for 3,4-dihydroxyphenylalanine and O_2 are 6.5 and 0.065 millimolar, respectively. Suitable substrates include chlorogenic acid, catechol, caffeic acid, pyrogallol, and dopamine; however, the enzyme is substrate-inhibited by the last four at concentrations near their K_m. A large seasonal variation in polyphenol oxidase activity may result from a decrease in enzyme content rather than inhibition of the enzyme present.

Polyphenol oxidase (*o*-diphenol: O_2 oxidoreductase)[2] has been found in chloroplasts of nearly a dozen higher plants (2, 3, 9, 14, 16, 27). The enzyme exists bound to the thylakoid membrane (9, 16, 18, 27), although there are reports of a soluble form in the stroma (24). The thylakoid-bound enzyme from spinach has not been isolated or extensively characterized. A polypeptide with polyphenol oxidase activity has been isolated from thylakoids of sugar beet chloroplasts (19), but the limited proteolysis necessary to release the enzyme from the membrane results in dissociation into numerous peptide fragments. Recently, a protein with polyphenol oxidase activity was isolated from the stroma of spinach chloroplasts (24). The enzyme consists of two forms, one with high activity and low molecular weight (protein A), the other with low activity and high molecular weight (protein B). The protein forms were reported to be interconvertible. At present, it is uncertain whether or not the thylakoid-bound and stromal forms of the enzyme are related.

The enzymic activity of plant polyphenol oxidase is latent. Activation can be achieved by treating extracts or membranes with detergents (16), acid or alkali (12), denaturing agents (22), or with proteolytic enzymes such as trypsin (27) or trypsin plus carboxypeptidase a (19). In many cases, the enzyme is activated upon release from the thylakoid membrane, but there is no indication that solubilization and activation are part of the normal function of the enzyme in the chloroplast. Indeed, the only physiological activator known is the process of aging.

In this paper, we report the isolation, purification, and several properties of thylakoid-bound polyphenol oxidase from spinach chloroplasts. We demonstrate that the enzyme can be liberated from the thylakoid membrane largely in the latent state, and that activity can be initiated in the bound and released forms by a group of physiologically important molecules, the fatty acids.

MATERIALS AND METHODS

Reagents and Chemicals. Linolenic acid was purchased from Aldrich; coumaric acid from Calbiochem; lithium dodecyl sulfate from BHD Chemicals; and trypsin from Nutritional Biochemicals Corporation. The protein standards ovalbumin, chymotrypsinogen, and ribonuclease A were supplied by Pharmacia, and human IgG was supplied by Miles Laboratories. Protein standards for SDS gel electrophoresis were obtained from Bio-Rad Laboratories. The remaining reagents and buffers were purchased from Sigma.

Protein Determination and Enzyme Assays. Protein was determined according to the method of Bradford (4) using bovine plasma gamma globulin as primary standard. Chl was determined in 80% acetone (2). Polyphenol oxidase activity was assayed by measuring O_2 uptake coupled to the oxidation of DL-DOPA using a Clark-type electrode. Unless otherwise stated, the electrode chamber contained 50 mM Hepes buffer (pH 7.5) and 12.5 mM DL-DOPA[3] in a final volume of 1.0 ml. After the system had equilibrated, 10 µl of linolenic acid (from a 1% stock solution in 95% ethanol) was added (as activator) followed by injection of a 25-µl aliquot of polyphenol oxidase-containing sample through a small hole in the vessel cap. The sample consisted of chloroplasts, partially purified extracts, or purified enzyme. Only the small aliquot of sample being used for assay was subject to activation, not the entire preparation. The electrode was calibrated daily with air-saturated water at 23 C. One unit of phenolase activity is defined as the amount of enzyme responsible for the uptake of 1

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[2] The commission on enzymes has recently revised the nomenclature to place *p*-diphenol: O_2 oxidoreductase (EC 1.10.3.2) and *o*-diphenol: O_2 oxidoreductase (EC 1.10.3.1) in the general category of monophenol monooxygenase (EC 1.14.18.1). Other names for the enzyme include phenolase, catecholase, catechol oxidase, and chlorogenic acid oxidase.

[3] Abbreviations: PPO, polyphenol oxidase; DOPA, DL-3,4-dihydroxyphenylalanine; LA, linolenic acid; dopamine, 3-hydroxytyramine; K_{AV}, elution constant for gel filtration; STN 0.4 M sucrose, 0.05 M Tris (pH 7.45), 0.01 M NaCl.

µmol O_2/min under the stated conditions. Over the range of enzyme concentrations used, there was a linear relationship between enzyme concentration and activity.

Substrate Studies. Kinetic constants of various substrates were determined by measuring the initial velocity as a function of substrate and O_2 concentrations. K_m were calculated by least-squares analysis of Lineweaver-Burk plots. O_2 concentration was adjusted by bubbling an appropriate mixture of O_2 and Ar through the reaction mixture prior to assay. Absolute O_2 concentration was determined with an O_2 electrode.

Chromatography and Molecular Weight Estimation. Enzyme purity and molecular weights were determined by polyacrylamide slab gel electrophoresis using the method of Laemmli (13), except that lithium dodecyl sulfate was used in place of SDS. The gels were stained with Coomassie brilliant blue. Preparative gel chromatography was performed concurrently with molecular weight estimation using columns (2.5 × 100 cm) of Sephacryl S-200, Sephacryl S-300, and Sephadex G-100. Molecular weight standards and sample were equilibrated and run in 25 mM Hepes buffer (pH 7.0), and 0.25 M KCl. Ion-exchange chromatography was carried out on a 1.5- × 10-cm column of DEAE Bio-Gel A equilibrated with 25 mM Mes buffer (pH 6.0). The final chromatographic step was carried out on a 2.5- × 50-cm column of Sephadex G-150. In all cases, the enzyme was dialyzed against the appropriate buffer before chromatography.

RESULTS

Activation by Long Chain Fatty Acids. Polyphenol oxidase is normally latent in the chloroplast. Trypsin is the most widely used activator (27), although detergents (16) and aging are also effective. The rate of activation with detergents or trypsin is slow, and there is considerable uncertainty whether or not the entire preparation has been fully activated. We sought a method to activate the thylakoid bound form of the enzyme rapidly and quantitatively before proceeding with purification.

In the course of other work, we noticed that phenol oxidation was greatly accelerated in chloroplasts that had been exposed briefly to fatty acids. To examine this effect, we compared the relative abilities of linolenic acid and trypsin to activate the bound form of the enzyme in freshly prepared chloroplasts. Phenolase activity increased 4–5 fold in chloroplasts incubated 2 min with trypsin and the effect appeared to saturate (Table IA). Linolenic acid, on the other hand, activated several times more polyphenol oxidase than trypsin, and the activity did not appear to saturate. The trypsin-activated sample continued to activate with time (up to 2 h), whereas the linolenic acid-activated sample remained stable with time. BSA protected the linolenic acid-treated sample against activation, most likely because of its ability to sequester fatty acids.

The effect of substitution and chain length on the ability of various fatty acids to activate membrane-bound polyphenol oxidase is shown in Table IB. Long chain fatty acids, especially those with a high degree of unsaturation, were the most effective activators. Lipids and methyl esters of fatty acids were ineffective. In establishing a physiological activation mechanism, it may be significant that C_{18} unsaturated fatty acids are the major constituents of chloroplast membrane lipids (1).

Since the effectiveness of linolenic acid may, in part, be related to its ability to disrupt thylakoid structure and grana stacking (21), chloroplasts were sonicated to determine whether activation could be achieved with lower concentrations of linolenic acid or trypsin. In intact chloroplasts, linolenic acid-induced activation did not saturate below 350 µM, whereas in sonically disrupted chloroplasts, saturation occurred at linolenic acid concentrations near 250 µM (Fig. 1). The effect was even more pronounced in the supernatant of sonicated chloroplasts after centrifugation: saturation occurred at linolenic acid concentrations less than 150 µM. Presumably, the absence of Chl and membrane lipids in the supernatant leads to a higher concentration of free linolenic acid. Prolonged sonication resulted in a small amount of activation of the enzyme possibly due to the release of endogenous fatty acids from the membrane. Trypsin was also a more effective activator at low concentrations in sonicated chloroplasts (data not shown). We conclude that the effect of linolenic acid is two-fold: a) its disruptive effect on thylakoid structure (21) provides the membrane-bound enzyme greater accessibility to the medium, and b) it rapidly and completely activates the latent phenolase in sonically-disrupted chloroplasts (see below).

Table I. *Activation of Thylakoid-Bound Polyphenol Oxidase*

Conditions were 12.5 mM DL-DOPA in STN buffer and 40 µg/ml Chl.

A. Effectiveness of Linolenic Acid *versus* Trypsin as Activator[a]

Conditions	PPO activity
	µmol O_2/mg Chl·h
Control	15.2
8 µg/ml Trypsin	24.6
80 µg/ml Trypsin	59.2
800 µg/ml Trypsin	60.9
50 µM Linolenate	75
100 µM Linolenate	103
200 µM Linolenate	125
400 µM Linolenate	204
400 µM Linolenate + 80 µg/ml trypsin	240.0
250 µM Linolenate + 1% BSA	10.0
100 µg/ml Trypsin + 1% BSA	52.0

B. Effectiveness of Various Fatty Acids and Derivatives as Activators[b]

Activator	No. Carbons	No. Double Bonds	Activation[b]
			%
Butyric	4		0
Lauric	12		13
Myristic	14		37
Palmitic	16		41
Palmitoleic	16	1	103
Stearic	18		57
Oleic	18	1	56
Linoleic	18	2	82
Linolenic	18	3	100
Arachidonic	20	4	107
Methyl oleate		1	8
Methyl linoleate		2	9
Triolean			0
Trilinolean			0
Trilinolenin			0

[a] Activators were prepared in ethanol and added to the reaction mix to a final concentration of 250 µM. Stearic acid was prepared as a saturated solution in water.

[b] Relative to linolenic acid.

Isolation and Purification of Thylakoid-Bound Polyphenol Oxidase. The sonication experiments indicated that the phenolase enzyme remained in the supernatant following centrifugation which removed most of the Chl-containing membranes (Fig. 1). Also, osmotically-shocked chloroplasts were found to release the soluble (stromal) proteins, but retain most of the polyphenol oxidase activity. We therefore osmotically shocked chloroplasts prior to sonication, which ensured that only the thylakoid-bound form of the enzyme would be isolated.

Step 1. Chloroplast Preparation. Chloroplasts were prepared by

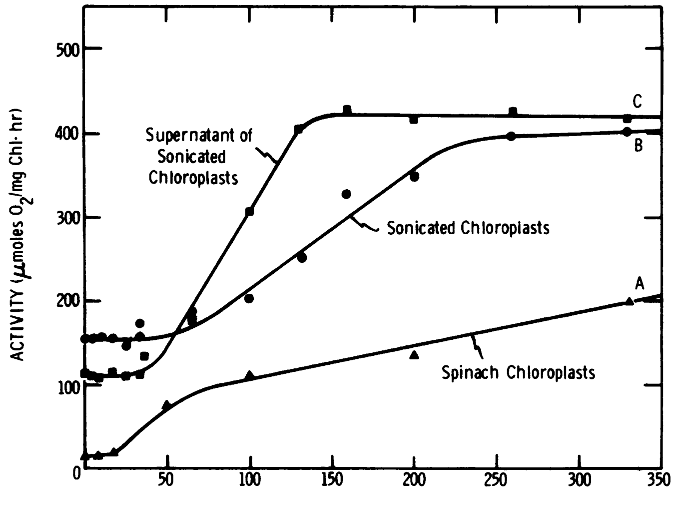

FIG. 1. Linolenic acid activation of polyphenol oxidase in spinach chloroplasts, sonicated spinach chloroplasts, and supernatant of the sonicated chloroplasts after 150,000g centrifugation. All samples were in 25 mM Tricine buffer, pH 7.0. Activity was followed by measuring O_2 uptake coupled to the oxidation of 12.5 mM DL-DOPA.

homogenizing 1 kg of depetiolated spinach leaves with 1.5 liters of cold STN buffer for 45 s in a Waring Blendor. The homogenate was filtered through two layers of Miracloth and centrifuged at 1000g for 2 min. The pellet was discarded and the supernatant was recentrifuged at 3,000g for 25 min. Chloroplasts were osmotically shocked by resuspension of the pellet in 500 ml of 25 mM Tricine buffer (pH 7.35) for 20 min and collected by centrifugation at 20,000g for 20 min.

Step 2. Sonication. Pelleted chloroplasts were resuspended in 120 ml of 25 mM Tricine buffer (pH 7.35), and sonicated for 150 s (ten 15-s intervals) at the highest setting of a Branson sonifier. Broken membrane fragments were collected by centrifugation at 150,000g for 135 min.

Step 3. Ammonium Sulfate Fractionation. The supernatant was carefully decanted from the soft membrane pellet and fractionated with 35 to 65% ammonium sulfate. The precipitate was collected by centrifugation and resuspended in 2.5 ml of 25 mM Hepes buffer (pH 7.0) and 0.25 M KCl.

Step 4. S200 Chromatography. The straw-colored solution was centrifuged at 50,000g to remove insoluble material and applied to a Sephacryl S-200 column equilibrated with 25 mM Hepes buffer (pH 7.0) and 0.25 M KCl. Most of the polyphenol oxidase appeared in a high molecular weight fraction (Fig. 2a) labeled polyphenol oxidase I (PPO I), but a small amount of activity eluted in a lower molecular weight fraction labeled polyphenol oxidase II (PPO II).

Step 5. Ion-Exchange Chromatography. The fractions containing PPO I were pooled and dialyzed against 25 mM Mes buffer (pH 6.0) for 12 h and applied to a DEAE Bio-Gel A column. The column was washed with two volumes of buffer, and the sample eluted with a 0 → 0.400 M linear KCl (400 ml) gradient. Polyphenol oxidase activity appeared in two fractions (Fig. 2b): a small amount at approximately 0.15 M KCl (DEAE Peak A) and a larger amount near 0.25 M KCl (DEAE Peak B).

Step 6. G-150 Chromatography. DEAE Peak B was concentrated to ~3 ml on an Amicon ultrafiltration membrane (PM-30) and applied to a Sephadex G-150 column. The protein was eluted with 25 mM Hepes buffer (pH 7.0) and 0.25 M KCl. The majority of activity appeared in a high molecular weight fraction labeled G-150 Peak A and a lesser amount in a lower molecular weight fraction labeled G-150 Peak B. DEAE Peak A was also concentrated by ultrafiltration and applied to the G-150 column. The activity appeared with the same K_{AV} values as DEAE peak B, except there was greater activity in the lower molecular weight peak (Fig. 2c).

Comments on Purification Procedure. Table II summarizes the results of purifying lamellar-bound polyphenol oxidase. Chloroplasts prepared from summer grown spinach had specific activities ranging from 1 to 3 units of polyphenol oxidase per mg protein. After centrifugation, the supernatant of the osmotically shocked, sonicated chloroplasts showed a 10-fold increase in specific activity over intact chloroplasts. The supernatant was light-green and contained 86% of the lamellar enzyme. The 35 to 65% ammonium sulfate fractionation step removed the remaining Chl and purified the enzyme ~2-fold over the preceding step. The first activity peak on Sephacryl S-200 (PPO I) contained ~70% of the applied enzyme with a specific activity exceeding 200 units per mg. The second activity peak (PPO II) was usually the minor constituent (Fig. 2a), but the ratio of PPO II/PPO I increased as the preparation aged. When PPO I was eluted from DEAE-Agarose, the activity separated into two fractions, DEAE Peak A and DEAE Peak B. The specific activity of the larger peak (DEAE Peak B) exceeded 500 units per mg protein, and overall recovery was 26%. The similar elution patterns of DEAE Peaks A and B on Sephadex G-150 indicated that the two protein species on DEAE were equivalent and probably related through monomer-multimer interconversions. The proteins were homogeneous by gel electrophoresis in the presence of lithium dodecyl sulfate.

These procedures resulted in a >900-fold purification of polyphenol oxidase with a specific activity in excess of 1000 units per mg protein. The yield averaged ~10%.

Latency and Stability of Isolated PPO I. Although sonication released lamellar-bound polyphenol oxidase from the membrane largely (>75%) in the latent state, we found that the enzyme spontaneously activated upon aging or further purification. To investigate this process further, we monitored the activity of the enzyme (± linolenic acid) during different stages of the preparation. In this experiment, an aliquot of sample was removed for immediate measurement of phenolase activity and an equivalent sample was stored in liquid N_2 for measurement 8 days later. The enzyme was ~9% activated in freshly sonicated chloroplasts and in the ammonium-sulfate fraction, but 50–60% became activated after the freeze-thaw process (Table III). When the ammonium sulfate-precipitated enzyme was stored at 4 C for two days, however, over 50% of the enzyme became activated. Over 40% of the enzymic activity was expressed following chromatography of the fresh enzyme on Sephacryl S-200. The spontaneous activation may have been due to a conformational change which occurred in the enzyme after release from the thylakoid membrane. It is not due to enzyme dissociation since both PPO I and PPO II show approximately the same degree of latency. The crude ammonium sulfate fraction is therefore the material of choice for study of the activation process.

Polyphenol oxidases I and II kept at 4 C were most stable between pH 5 and 7 (Fig. 3). After 10 days of storage at pH 6.1, 88% of the activity was retained compared with 29% and 45% at pH 4.6 and 7.25, respectively. The enzyme could be stored in liquid N_2 without appreciable loss of activity (Table III). The enzyme was most stable at low ionic strength. Activity degraded an additional 25% at pH 8.1 after 24 h at 4 C in 0.5 M KCl.

Interconversion of Enzyme Forms. Both DEAE Peaks A and B fractionated on G-150 to produce identical higher and lower molecular weight species (Fig. 2c). To investigate further this interconversion, we placed PPO II from the S-200 column on DEAE-Agarose and eluted with a 0 → 0.400 M KCl gradient. The activity eluted in two peaks, similar to those shown in Figure 2b for PPO I. In this instance, however, greater activity was present in DEAE Peak A. When each component was concentrated and

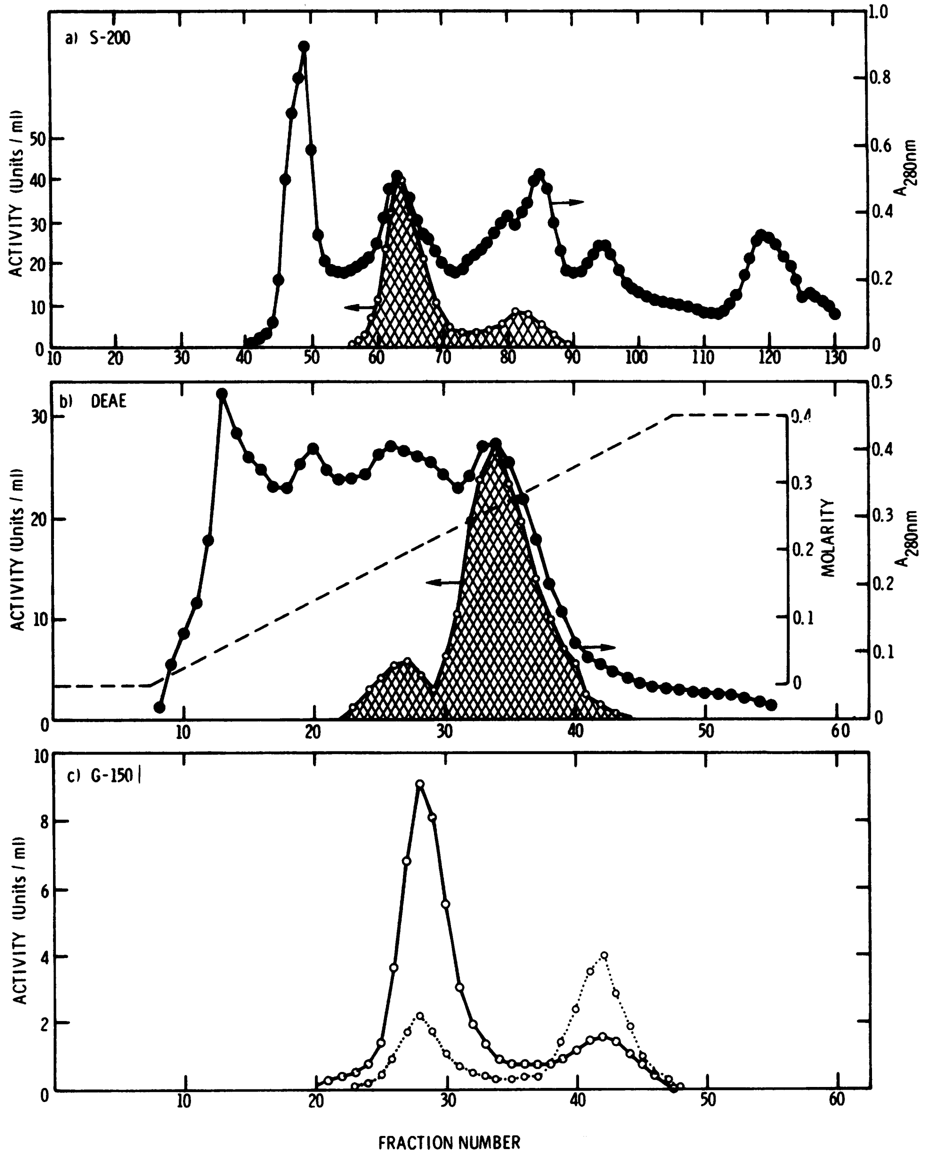

FIG. 2. (a) Sephacryl S-200 chromatography of the 35 to 65% ammonium sulfate fraction in 25 mM Hepes (pH 7.0, and 0.25 M KCl. A at 280 nm (●) and phenolase activity (○) were monitored in each 3.5-ml fraction. The activity was measured as DL-DOPA-mediated O_2 uptake after activation with 300 μM linolenic acid. Tubes 58–69 constitute PPO I; tubes 78–88 constitute PPO II. (b) DEAE BioGel A chromatography of PPO I after dialysis against 25 mM Hepes buffer, pH 7.0. The loaded column was washed with 25 mM Hepes buffer and eluted with a 0 → 0.400 M KCl gradient. A at 280 nm (●) and phenolase activity after activation (○) were monitored in 6-ml fractions. Tubes 22–28 and 31–40 constitute Peaks A and B, respectively. (c) Sephadex G-150 chromatography of DEAE Peak A (····) and DEAE Peak B (——). The samples were concentrated by ultrafiltration, applied to an equilibrated G-150 column, and eluted with 25 mM Hepes buffer, pH 7.0, and 0.25 M KCl. The phenolase activity after activation was monitored in each 3.5-ml fraction.

placed on G-150, the activity eluted in a pattern similar to that found for PPO I. Thus, the individual protein species of polyphenol oxidase are interconvertible.

Molecular Weight and Subunit Structure. G-150 Peaks A and B showed a single band on SDS polyacrylamide gels, indicating that the enzyme was homogeneous. In both cases, the protein band corresponded to a molecular weight of 42,500 (Fig. 4). Molecular weights of PPO I and PPO II were also estimated by gel filtration with Sephadex G-100 and Sephacryl S-200 and S-300. A comparison of the K_{AV} values with those of standard proteins (Fig. 5) indicated a mol wt of 158,000 ± 7,000 for PPO I and 42,500 ± 1,500 for PPO II.

From these data, we infer that PPO I is a tetramer of PPO II. We suggest that the breakdown of the 158,000 dalton tetramer following S-200 and DEAE chromatography leads to formation of the 42,500 dalton monomer. The rate of tetramer to monomer dissociation, however, appears to be slow. A concentrated 1-day-old $(NH_4)_2SO_4$ preparation yielded about 75% PPO I and 25% PPO II when chromatographed on Sephacryl S-200, while a 3-day-old preparation yielded equal activity peaks for PPO I and PPO II.

Kinetics and Substrate Specificity of Polyphenol Oxidase I. Plant polyphenol oxidase is assumed to catalyze a reaction between two molecules of *o*-diphenol and one of O_2 to yield two molecules of *o*-quinone and water (20). Multiple, interconvertible enzyme forms make determination of reaction constants difficult since each species of enzyme (including the membrane-bound form) may show different kinetics (11). To determine the K_m, we chose freshly isolated PPO I from Sephacryl S-200 since it was the major enzyme species found after release from the thylakoid membrane.

Double reciprocal plots of initial velocity *versus* substrate con-

Table II. *Purification of Spinach Thylakoid Polyphenol Oxidase*

Fraction	Total Protein	Total Activity	Specific Activity	Purification Factor	Recovery
	mg	*units*	*units/mg*	*fold*	*%*[a]
Sonicated chloroplasts	1024	1719	1.68	1.0	100
Supernatant of sonicated, osmotically shocked chloroplasts	83.8	1486	17.7	10.7	86
Ammonium sulfate 35–65% cut	34.0	1140	33.5	20.3	66
S-200 Chromatography					
Peak A (PPO I)	2.97	638	215.0	130.3	37
Peak B (PPO II)	7.17	319	44.5	26.5	19
DEAE ion exchange[b]					
Chromatography of PPO I					
Peak A		180			10.4
Peak B	0.64	450	541	328	26.1
G-150 Chromatography[c]					
Peak A	<0.17	171	~1000	>900	10.0
Peak B	<0.08	85	~1000	>900	4.9

[a] Percent of total activity of sonicated chloroplasts.

[b] Represents only PPO I on DEAE.

[c] Represents only DEAE Peak B on G-150.

Table III. *Stability and Activation of PPO I During Preparation and Storage*

Fraction	Before Freezing			8 Days in Liquid N_2		
	Activity −LA	Activity[a] +LA	Activated	Activity −LA	Activity[a] +LA	Activated
	units		*%*	*units*		*%*
Sonicated chloroplasts	299	3289	9.1	2169	3498	62.0
Ammonium sulfate 35–65% fraction	242	2852	8.5[b]	1396	2909	48.0
S-200 Chromatography Peak A	965	2389	40.4	1980	2200	90.0

[a] After activation with 300 μM linolenic acid.

[b] 52.2% becomes activated when aged 2 days at 4 C.

centration yield a series of converging lines that intersect near the horizontal axis left of the vertical axis (Fig. 6). The linear reciprocal plots indicate that there exists no reversible connection between the points of combination of the substrate that adds twice during the reaction sequence (6). These data may support a mechanism whereby DL-DOPA and O_2 sequentially bind to the enzyme (in either an ordered or random mechanism) followed by a release of product and subsequent binding of the second molecule of DL-DOPA to the enzyme. A similar mechanism has been proposed for polyphenol oxidase from tea (8) and grape (15), although in the former instance the enzyme was blue and could oxidize *p*-phenylenediamine; and in the latter case, the enzyme had cresolase activity. If we assume the first segment of the reaction to be rate-limiting, the kinetic constants can be determined by analyzing the data according to a two-substrate reaction mechanism. Accordingly, the vertical intercept represents the reciprocal velocity when the variable substrate is at infinite concentration (5). K_m were determined by replotting intercepts of the reciprocal plots *versus* reciprocal concentrations of the fixed substrate (Fig. 6, insert). The resulting K_m, determined by the least-squares method, were 0.065 mM for O_2 and 6.5 mM for DL-DOPA. Under normal atmospheric conditions, the enzyme is therefore >80% saturated with O_2.

Kinetic constants for the other substrates were determined with air-saturated buffer (Table IV). The optimum pH for all substrates was between 7.0 and 8.0. Of all physiologically significant substrates tested, the enzyme had the highest affinity for dopamine, with a K_m 10 times lower than that of DL-DOPA and 4 times lower than that of catechol. There was considerable substrate inhibition at dopamine concentrations slightly greater than its K_m and it was not as useful for kinetic studies as DL-DOPA. Pyrogallol, catechol, and caffeic acid were also effective substrates, but showed substrate inhibition at concentrations near 10, 25, and 25 mM, respectively.

The purified enzyme was inactive towards *m*- or *p*-diphenols, ascorbic acid, phenylenediamine (both ortho and para), and 2,5-dihydroxy-*p*-benzoquinone.

There was no detectable cresolase (monophenol oxidase) activity in the isolated enzyme, even with addition of a small amount of catechol to eliminate the characteristic lag period in the hydroxylation reaction (7).

Seasonal Variation of Enzyme Content. In purifying the enzyme over the course of several seasons, we noticed that there was a loss of polyphenol oxidase activity in chloroplasts and an attendant decline in the yield of purified enzyme from summer to winter. In August through October the yield of enzyme after S-200 chromatography was 3500–4000 units/kg spinach leaves which decreased in mid-November to 1000–1800 units/kg and in mid-December to less than 350 units/kg. In each case, the drop in activity was accompanied by a decline in the 158,000 dalton peak on Sephacryl S-200. Sato and Hasegawa (24) reported a large seasonal variation in phenolase activity of spinach chloroplasts which they attributed to the existence of an inactive protein inhibitor complex. If the decline in activity of the membrane-bound enzyme were due solely to a low molecular weight inhibitor, there should have been no decline in the protein content of PPO I on S-200. Instead, the 158,000 and 42,500 dalton peaks declined through mid-December until both were lost within the envelopes of the co-purifying proteins. Obviously, the degree of purification

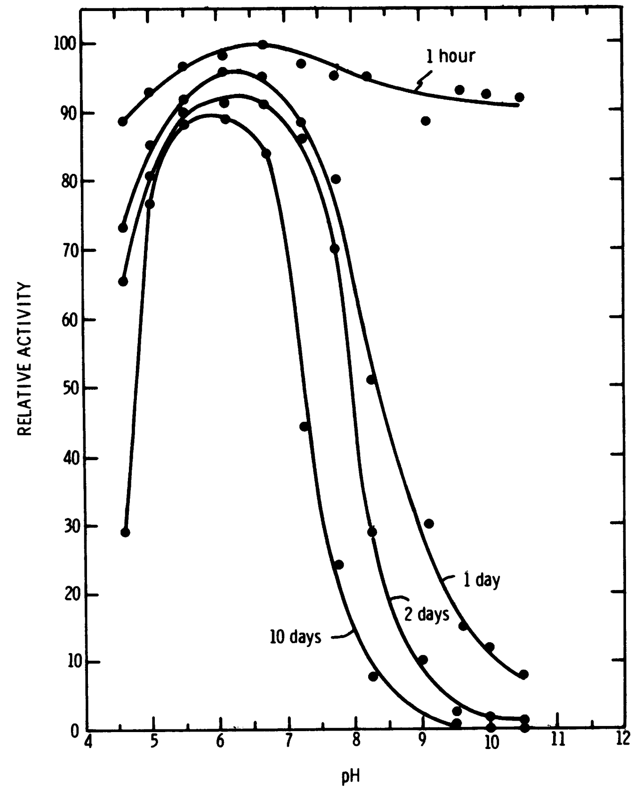

FIG. 3. pH stability of the isolated enzyme. PPO I from S-200 was stored in 50 mM buffer at 4 C for 10 days and assayed daily for activity. The buffers were: pH 4.6–5.7, succinate; 6.1–6.7, Mes; 7.3–7.8, Hepes; 8.3–9.1, Tris; and 9.6–10.5, glycine. In several instances, the sample was stored in two different buffers at the same pH; no significant differences in activity were found.

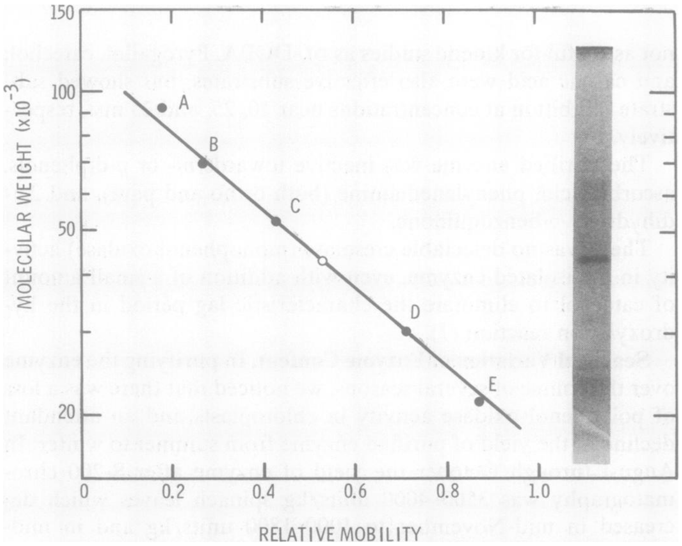

FIG. 4. Lithium dodecyl sulfate-polyacrylamide gel electrophoresis of G-150 Peak B. The calibration curve was determined using the following protein markers: A) phosphorylase b, B) BSA, C) ovalbumin, D) carbonic anhydrase, and E) soybean trypsin inhibitor. (O) indicates the protein band.

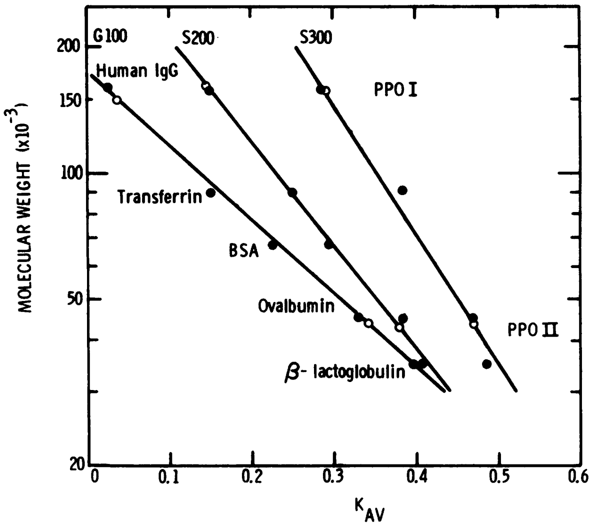

FIG. 5. Molecular weight estimation on Sephadex G-100, Sephacryl S-200, and Sephacryl S-300. The molecular weights of PPO I and PPO II were estimated using the protein standards: A) human IgG, B) transferrin, C) BSA, D) ovalbumin, and E) B-lactoglobulin. The void volume was determined with blue dextran (S-200 and S-300) or with thyroglobulin (G-100). The buffer was 25 mM Hepes, pH 7.0, and 0.25 M KCl. The average molecular weights (O) were determined four times with S-200, four times with S-300, and twice with G-100.

of the enzyme from spinach chloroplasts following the described protocol will depend upon the season grown.

DISCUSSION

The relationship between the lamellar polyphenol oxidase and the enzyme species isolated by Sato and Hasegawa (23, 24) remains unclear. In the latter case, proteins A and B were isolated from the supernatant of the chloroplast brei and had reported mol wt of 36,000 and 72,000 on Sephadex G-150 and G-200. The two enzyme species reported here were released from the lamellae by sonicating osmotically-shocked chloroplasts; their apparent mol wt were 42,500 and 158,000. In both instances, the enzyme forms appeared to be reversibly interconvertible. Although there is a discrepancy in the molecular weights, we think it possible that the stromal enzymes are the dimer and monomer of the dissociated high molecular weight lamellar enzyme. Accordingly, Sato's protein A would represent the 42,500 mol wt species reported in this paper. It remains unexplained why we were able to characterize the tetramer and monomer but not the dimer.

Because polyphenol oxidase can be released from the lamellae largely in the latent state, activation cannot be related to the solubilization of the native enzyme. Mayer (18) and Mayer and Friend (17) found that various detergents caused activation of membrane-bound catechol oxidase in sugar beet chloroplasts without causing solubilization. In other cases, solubilization and activation were achieved simultaneously, *e.g.*, with trypsin (11) or trypsin and carboxypeptidase (19). Activation also cannot be due to dissociation of the higher molecular weight species since both PPO I and PPO II show equivalent degrees of latency.

To our knowledge, linolenic acid represents the first physiologically important molecule identified as an activator of plant polyphenol oxidase. Spinach polyphenol oxidase can be activated with trypsin, but the nature of the activation process is unclear since it occurred even with denatured trypsin preparations (27). Activation of broad bean catechol oxidase by detergents, acid, alkali, chaotropic, or denaturing agents (12, 22, 26) has been attributed to conformational changes in the enzyme (22, see also Ref. 14). Since fatty acids have been shown to cause conformational changes in proteins (25), activation of the spinach enzyme by linolenic acid may share a similar mechanism.

Linolenic acid may also prove significant in the mechanism of ozone or other stress-induced injury to plant tissues. The pigmentation that results from ozone injury is caused by the reaction of oxidized *o*-diphenols with the amino and thiol groups of proteins to form reddish-brown polymerization products (10). Howell re-

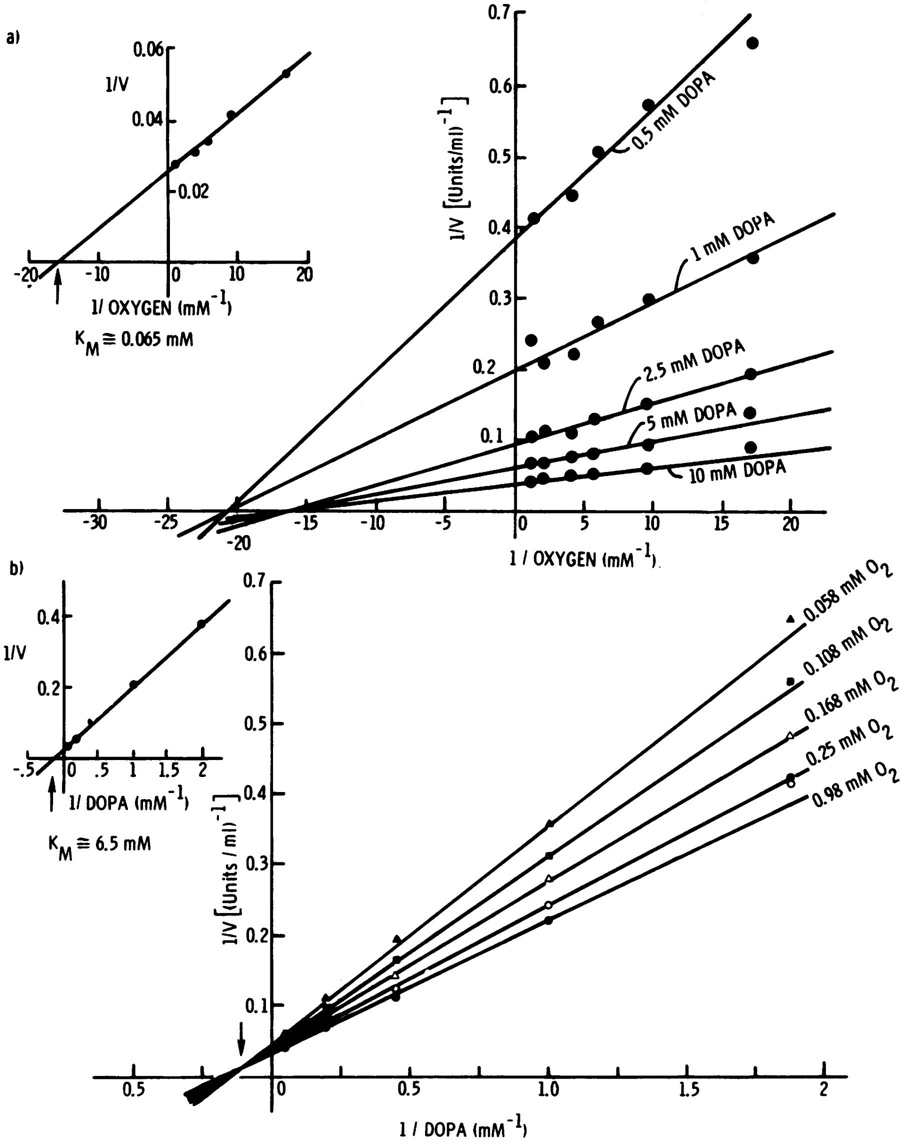

FIG. 6. Primary plots (main figure) and secondary plots (insert) of the effect of DL-DOPA and O_2 concentrations on the initial velocity of PPO I catalysis. The reaction mixture contained 25 mM Hepes buffer, pH 7.5, and various concentrations of O_2 and DL-DOPA in a final volume of 1.00 ml. After the system had equilibrated in the electrode chamber, linolenic acid (300 μM) was added as activator, and the reaction was initiated by injecting a 25-μl aliquot of enzyme through a small hole in the vessel cap. (a) O_2 concentrations: 0.98 mM, 0.25 mM, 0.168 mM, 0.108 mM, 0.058 mM. (b) DL-DOPA concentrations: 10 mM, 5 mM, 2.5 mM, 1 mM, 0.5 mM. Activities are expressed in units/ml enzyme.

Table IV. *Substrate Specificity of Spinach Polyphenol Oxidase I*

All activities were determined at the optimal pH for the substrate using 50 mM Hepes buffer. The O_2 concentration was 0.265 mM. Linolenic acid (300 μM) was added to the assay mixture to ensure complete activation of the enzyme.

Substrate	K_m
	mM
Dopamine	0.74
Catechol	3.13
DL-DOPA	8.30
Chlorogenic acid	11.60
Pyrogallol	15.70
Caffeic acid	40.80

ports (10) that the primary site of ozone degradation is most likely in membranes. Under normal conditions, the enzyme is bound to the lamellae and latent, while the bulk of phenols are sequestered in the vacuole. Under stress conditions, membrane lipids hydrolyze to free fatty acids due to the activation of lipases. The free fatty acids could both activate the enzyme and disrupt membrane integrity, thus permitting interaction of enzyme and substrate.

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LITERATURE CITED

1. ALLEN CF, P GOOD, HF DAVIS, SD FOWLER 1964 Plant and chloroplast lipids. I. Separation and composition of major spinach lipids. Biochem Biophys Res Commun 15: 424–430
2. ARNON DI 1949 Copper enzymes in isolated chloroplasts; polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
3. BALDRY CW, C BUCKE, J COOMBS, D GROSS 1970 Phenols, phenoloxidase and photosynthetic activity of chloroplasts isolated from sugar cane and spinach. Planta 94: 107–123
4. BRADFORD M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Chem 72: 248–254
5. CLELAND WW 1963 The kinetics of enzyme catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. Biochim Biophys Acta 67: 188–196

6. CLELAND WW 1970 Steady state kinetics. *In* D Boyer, ed, The Enzymes, 3rd Edition, Vol. 2, Academic Press, New York pp 1-65
7. COLEMAN JE 1974 Structure and mechanism of copper oxidases *In* JR Whitaker, ed, Food Related Enzymes, Advances in Chemistry Ser. No. 136, American Chemical Society pp 267-303
8. GREGORY RPF, DS BENDALL 1966 The purification and some properties of polyphenol oxidase from tea (*Camellia sinensis* L) Biochem J 101: 569-581
9. HENRY EW 1976 The ultrastructural localization of polyphenol oxidase in chloroplasts of *Brassica napus* cv. Zephyr. Z Pflanzenphysiol 78S: 446-452
10. HOWELL RK 1974 Phenols, ozone and their involvement in pigmentation and physiology of plant injury. America Chemical Society Symposium Series, Air Pollution Effects on Plant Growth, Vol. 3 pp 94-105
11. KATZ Y, AM MAYER 1969 Changes in properties of catechol oxidase from chloroplasts following liberation from membranes. Israel J Bot 18: 11-19
12. KENTEN RH 1957 Latent phenolase in extracts of broad-bean (*Vicia faba*) leaves. I. Activation by acid and alkali. Biochem J 67: 300-307
13. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. Nature 227: 680-685
14. LERNER HR, AM MAYER, E HAREL 1972 Evidence for conformational changes in grape catechol oxidase. Phytochemistry 11: 2415-2421
15. LERNER HR, AM MAYER 1976 Reaction mechanism of grape catechol oxidase—a kinetic study. Phytochemistry 15: 57-60
16. LIEBEREI R, B BIEHL 1976 Friesetzung und Aktivierung von Polyphenoloxydasen aus Thylakiodmembranen der Spinat-Chloroplasten. Ber Dtsch Bot Ges 89: 663-676
17. MAYER AM, J FRIEND 1960 Properties and solubility of phenolase in isolated chloroplasts. Nature 185: 464-485
18. MAYER AM 1965 Factors controlling activity of phenolase in chloroplasts from sugar beets. Israel J Bot 13: 74-81
19. MAYER AM 1966 Catechol oxidase: enzymic liberation from sugar beet chloroplasts. Phytochemistry 5: 1297-1301
20. MAYER AM, E HAREL 1979 Polyphenol oxidases in plants. Phytochemistry 18: 193-215
21. OKAMOTO T, S KATOH, S MURAKAMI 1977 Effects of linolenic acid on spinach chloroplast structure. Plant Cell Physiol 18: 551-560
22. ROBB DA, LW MAPSON, T SWAIN 1964 Activation of the latent tyrosinase of broad bean. Nature 201: 503-504
23. SATO M 1976 Association by 2,3-dihydroxybenzaldehyde of monomeric phenolase in spinach chloroplasts. Phytochemistry 15: 1665-1667
24. SATO M, M HASEGAWA 1976 The latency of spinach chloroplast phenolase. Phytochemistry 15: 61-65
25. SCANU A, H POLLARD, R NIRZ, K KATHANY 1969 On the conformational instability of human-serum low-density lipoprotein: effect of temperature. Proc Natl Acad Sci USA 62: 171-178
26. SWAIN T, LW MAPSON, DA ROBB 1966 Activation of *Vicia faba* tyrosinase as effected by denaturing agents. Phytochemistry 5: 469-482
27. TOLBERT NE 1973 Activation of polyphenol oxidase of chloroplasts. Plant Physiol 51: 234-244