Properties of Diphenolase from *Vanilla planifolia* (Andr.) Shoot Primordia Cultured in Vitro

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Properties of diphenolase (PPO, EC1.10.3.1) from vanilla (*Vanilla planifolia* Andr.) shoot primordia culture were investigated. Two pH optima of the enzyme extraction at pH 6 and 8 were found. Nevertheless, the enzymes shared the same optimum pH of activity—between pH 3 and 4. Sodium dodecyl sulfate slightly improved diphenolase extraction but caused a 3-fold increase in its specific activity. The extracts of pH 6 and 8.0 revealed three isozyme bands after polyacrylamide gel electrophoresis—two of them were similar in both extracts and two distinct. The enzyme showed high thermal stability—no loss was observed after 120 min at 50 °C. Diethyldithiocarbamic acid, ethylenediaminetetracetic acid disodium salt, ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid, L-ascorbic acid, dithiothreitol, glutathione (reduced), and β-mercaptoethanol were found to be potent inhibitors of the diphenolase studied. The enzyme showed also monophenolase activity. K_m and V_{max} were calculated with monophenols [*p*-coumaric acid, 3-(*p*-hydroxyphenyl)-propionic acid, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid] and with diphenols (caffeic acid, hydrocaffeic acid, chlorogenic acid, 4-methylcatechol, protocatechuic aldehyde and acid, and 3,4-dihydroxyphenylalanine). The highest V_{max} was found with 4-hydroxybenzyl alcohol and the greatest affinity to protocatechuic acid, respectively—the most abundant monophenol and one of the least abundant *o*-diphenols in the studied *Vanilla* tissue.

Keywords: *Vanilla planifolia; Orchidaceae; monocots; tissue culture; metabolism; phenols; polyphenol oxidase*

INTRODUCTION

Polyphenol oxidase (PPO) in plants is a nuclear-encoded copper protein, which (utilizing molecular oxygen) catalyzes two reactions: ortho-hydroxylation of monophenols (monophenolase, monophenol monooxygenase, or tyrosinase being referred to as EC 1.14.18.1) and oxidation of 1,2-dihydroxybenzenes to *o*-quinones (diphenolase, catechol oxidase, diphenol:oxygen oxidoreductase EC 1.10.3.1). The products of the second step can be cyclized, which, after further oxidation, polymerize to melanin pigments or react with amino acid residues of cellular proteins.

The role of PPO in plants is not clear yet, but it has been proposed that it may be involved in necrosis development around damaged leaf surface and in defense mechanisms against insects and plant pathogen attack (*1*). Phenolic compounds may function by inhibiting bacterial growth or serve as the precursors in the formation of physical polyphenolic barriers limiting pathogen translocation. PPO-generated quinones modify plant proteins, decreasing the plant's nutritive availability to herbivores or invaders. Polymeric polyphenols seem to be more toxic to potential phytopathogens than are the phenolic monomers (*2*).

PPOs occur in inactive or latent forms (broad bean, pea, lettuce, and tobacco), which can be activated by a variety of treatments or agents (*1*). Arnon (*4*) first reported the existence of the latent form of PPO in plant thylakoid membranes.

The studied culture of *Vanilla planifolia* contains numerous C_6-C_3 and C_6-C_1 phenolics with the highest content of 4-hydroxybenzyl alcohol (*3*), a potential precursor of vanillin alcohol and vanillin. It would be very desirable to employ a *Vanilla* native enzymatic system capable of transforming this monophenol into protocatechuic aldehyde—the immediate vanillin precursor. Such transformation, besides oxidation of alcohol substituent, must involve ortho-hydroxylation, which could be achieved with the appropriate monophenolase, but it raises a problem of *o*-diphenol stability in the presence of diphenolase. Therefore, characteristics of diphenolase seemed to us important in order to gain an understanding of particular *Vanilla o*-diphenols.

The aim of this work was to characterize PPO capable of diminishing the biotechnological value of the culture as a source of vanilla aroma precursors and gain an insight into the involvement of PPO in *Vanilla o*-diphenol turnover. Despite numerous publications describing plant PPOs, there are only a few papers concerning PPO properties from monocots and even fewer from monocot vegetative tissues. This work describes properties of the PPO (condition of extraction, optimum pH, K_m, V_{max}, number of isozymes, thermal stability, and an effect of inhibitors) in crude extracts from *V. planifolia* shoot primordia culture.

MATERIALS AND METHODS

Plant Material. *V. planifolia* (Andr.) shoot primordia culture was derived from callus obtained according to the method of Havkin-Frenkel et al. (*5*). The tissue was grown in sterile, liquid Gamborg B-5 medium with macro- and micronutrients and vitamins (Sigma) containing 2% sucrose in white light (PPF, 12 μmol m^{-2} s^{-1}), at room temperature in 500 mL Erlenmeyer flasks on a rotary shaker set at 100 rpm. The plant

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10.1021/jf001180z CCC: $20.00
Published on Web 06/14/2001

material was subcultured every 3 weeks in proportions of 5 g of tissue/100 mL of the medium in 500 mL Erlenmeyer flasks.

Enzyme Extraction. Fresh tissue (2 g) was homogenized, in an ice bath, with 10 mL of 50 mM NaPi buffer containing 1.7 mM SDS for 2 min in a Polytron homogenizer. The resulting slurry was filtered through Miracloth and centrifuged (20 min at 12000*g* at 4°C), and 5 mL of the supernatant was applied to a Sephadex G-25 column (bed volume = 40 mL) equilibrated with the same buffer to remove low molecular weight phenolics. The column was eluted with equilibrating buffer, and 7 mL following the void volume was collected. The Sephadex G-25 eluate, called the crude enzyme, was used as the enzyme source throughout the experiments. Parallel samples were subjected to measurements of residual phenolics content according to the Folin method (*6*) with *p*-coumaric acid (Sigma) as a standard. Only a trace amount of phenolics (<1 μg/g of fresh weight) was estimated in the eluate studied.

Assay of PPO Activity. PPO activity was measured with two spectrophotometric methods.

(a) Formation of o-Quinones Measured at $\lambda = 410$ nm. The standard reaction mixture (final volume = 1 mL) contained 50 mM protocatechuic acid, 0.5 mL of 0.5 M NaOAc buffer (pH 4.0), 100 units of catalase (Sigma), 1.7 mM SDS, and 0.5 mL of the crude enzyme.

(b) Using Yellow 2-Nitro-5-thiobenzoic Acid (TNB) Coupled with the Quinones (7). The reaction mixture contained 5 mM phenolic substrate, 0.5 M NaOAc buffer (pH 4.0), 1.7 mM SDS, 0.25 mM TNB, and 50 μL of the crude enzyme in a final volume of 1 mL. This method was used for estimation of enzyme kinetics, substrate specificity, effect of inhibitors, and thermal stability of the enzyme. It allowed avoiding the estimation of λ_{max} for different quinones formed from the substrates used. Absorbance changes were taken against the blank samples without substrate at room temperature for 5 min (during a linear course of the reaction) at 0.5 or 1 min intervals. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per minute. Presented data are means (±SD) of three to six independent experiments, each in three replicates.

Effect of pH. The activity of the enzyme (method a) was determined in the following 0.5 M buffers (containing 1.7 mM SDS): in the pH range 3−5 using NaOAc buffer, at pH 5.5−7.0 using NaPi, and at pH 7.0−9.5 using Tris-HCl buffers. The optimum pH for the PPO activity was measured with 50 mM protocatechuic acid and 4-methylcatechol. The crude enzyme (0.5 mL) in 50 mM NaPi buffer was added to 0.5 mL of the substrate dissolved in 0.5 M of the particular buffer and the final pH measured.

Activation by SDS. Activation studies were carried out in NaOAc buffer (pH 4.0) with 50 mM protocatechuic acid in a final volume of 1 mL (method a). The concentration of SDS in the medium varied from 0.34 to 34 mM.

Substrate Specificity and Enzyme Kinetics. Michaelis constants (K_m) and maximum velocities (V_{max}) were determined (method b) using 12 substrates [*p*-coumaric acid, 3-(*p*-hydroxyphenyl)propionic acid, caffeic acid, hydrocaffeic acid, protocatechuic acid, 4-hydroxybenzoic acid, chlorogenic acid, 4-hydroxybenzaldehyde, protocatechuic aldehyde, 4-hydroxybenzyl alcohol, 4-methylcatechol, and 3,4-dihydroxyphenylalanine] at 12 concentrations (0.05, 0.075, 0.125, 0.25, 0.417, 0.625, 1.25, 1.88, 2.5, 5, 7.5, and 10 mM). Stock solution of each substrate (prepared daily) was made by dissolving the substrate in 100 μL of EtOH, and then 0.5 M NaOAc buffer (pH 4.0) containing 1.7 mM SDS was added to make a final 10 mM solution. If necessary, the samples were gently heated to complete solubilization. Presented data were plotted according to the method of Lineweaver and Burk (*8*). The effect of enzyme concentration on the reaction rate was measured with protocatechuic acid as a substrate.

Effect of Inhibitors. PPO activity was determined (method b) in the presence of the following inhibitors: L-ascorbic acid, glutathione (reduced), dithiotreithol (DTT), β-mercaptoethanol, diethyldithiocarbamic acid (DETC), NaEDTA, and EGTA at the concentrations indicated. Inhibitors were dissolved in 0.5 M NaOAc buffer (pH 4.0) containing 1.7 mM SDS.

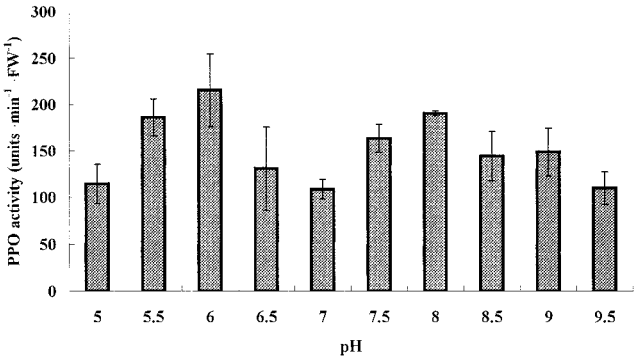

Figure 1. Optimum pH of extraction of *V. planifolia* diphenolase. The tissue was extracted with 50 mM NaOAc (pH from 3.0 to 5.0), NaPi (pH from 5.5 to 7.0), and Tris-HCl (pH from 7.0 to 9.5) buffers containing 1.7 mM SDS. Activity of the enzyme (after removal of low molecular weight phenolics on a Sephadex G-25 column) was measured in NaOAc buffer (pH 4.0) (containing 1.7 mM SDS) with 50 mM protocatechuic acid (method a).

Thermal Stability. Samples of the crude enzyme (1 mL) were kept in a water bath at various temperatures between 20 and 100 °C (at 10° C intervals) for 20 min. Fifty microliter aliquots of the incubated enzyme were removed and added to the reaction mixture kept at room temperature, and the activity was estimated (method b).

Protein Determination. Protein concentration in the Sephadex G-25 filtered enzyme extract (crude enzyme) was determined according to the Bradford (*9*) method, with bovine serum albumin (Sigma) as the standard.

Polyacrylamide Gel Electrophoresis (PAGE). Electrophoresis was carried out in 8% polyacrylamide gel (without SDS) according to the method of Angleton and Flurkey (*10*). Each pocket was loaded with the crude enzyme sample (which was not denaturated) containing 5 or 10 μg of protein, 1.7 mM SDS, 4% glycerol, and 1 μg of bromophenol blue. Electrophoresis was run for 1.5 h, at 100 V, at 25 °C in a Mighty Small II SE 259 apparatus (Hoefer Scientific Instruments). For diphenolase activity detection, the gels after electrophoresis were immersed in a solution of 30 mM protocatechuic acid and 90 mM *p*-phenylenediamine in 0.1 M NaPi (pH 7.0) for 5−10 min at room temperature. Next, they were thoroughly washed with distilled water and soaked in 10% citric acid for 2 min to destain the background (*11*). PPO activity products appeared as brown bands.

RESULTS AND DISCUSSION

Optimum pH and Effect of SDS. Two pH optima of the diphenolase extraction have been found—one at pH 6 and the other at pH 8 (Figure 1). Both groups of extracted enzymes exhibited maximal activity at acidic pH depending on the substrate: at pH 4 with protocatechuic acid and between pH 3 and pH 4 with 4-methylcatechol (Figure 2). This may suggest that these PPOs participate in after-injury reactions when the pH of the cell content drops significantly due to cell disruption and low pH activates domains of the PPOs' structure responsible for their enzymatic activity. The existence of a regulatory domain where pH controls the PPO activity has been suggested for PPO from apple and broad bean leaves (*12*, *13*). The acidic optimum activity of PPO from *Vanilla* was similar to that found in pear (*14*) and a suspension culture of *Vitis vinifera* (*15*) but significantly differed from that of PPO of mushroom (*16*), sweet potato roots, and *Lithospermum erythorhizon* (*17*, *18*).

Diphenolase extraction without detergent followed by activity measurement in the absence of SDS was very poor and barely sufficient for activity determination

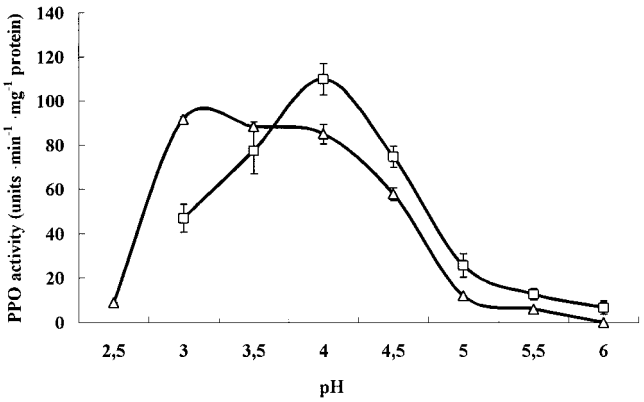

Figure 2. Optimum pH of activity of *V. planifolia* diphenolase. The tissue was extracted with 50 mM NaPi buffer (pH 6.0) containing 1.7 mM SDS. Activity of the extracted enzyme (after removal of low molecular weight phenolics on a Sephadex G-25 column) was measured with 50 mM protocatechuic acid (squares) and 4-methylcatechol (triangles) in NaOAc buffer (pH from 3.0 to 5.0), NaPi (pH from 5.5 to 7.0), and Tris-HCl (pH from 7.0 to 9.5) containing 1.7 mM SDS at room temperature (method a).

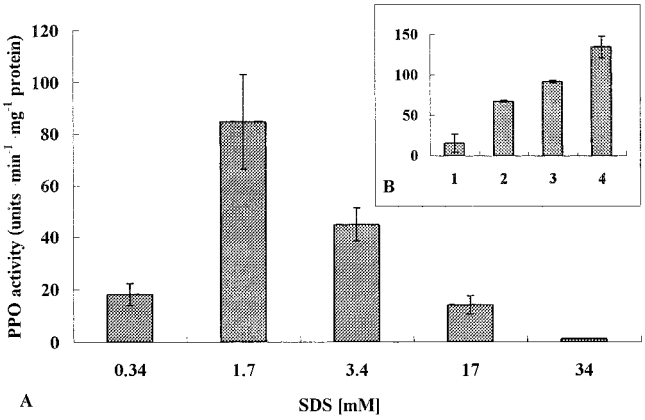

Figure 3. Effect of SDS concentration on the extraction and activity of *V. planifolia* diphenolase: (A) activity of the enzyme (method a) was measured with 50 mM protocatechuic acid in NaOAc buffer (pH 4.0) in the presence of indicated SDS concentrations, at room temperature; (B) (1) extraction and activity without SDS, (2) extraction without SDS and activity with 1.7 mM SDS, (3) extraction without SDS and activity measured after 20 min of incubation with 1.7 mM SDS, (4) extraction and activity with 1.7 mM SDS.

(Figure 3B, column 1). After the addition of 1.7 mM SDS into the reaction mixture or after incubation of the crude enzyme with 1.7 mM SDS (or CTAB, data not shown) at room temperature for 20 min, the activity of PPO increased by about 3 and 4 times, respectively (Figure 3B, columns 2 and 3), and showed an optimum at pH 3. These indicate that SDS significantly enhances the activity of the enzyme and only slightly the enzyme extraction (Figure 3B, columns 3 and 4). A similar effect of SDS on PPO activity was observed in banana (*19*, *20*), broad bean (*21*, *22*), and table beet leaves (*23*).

In contrast to our results, PPO from *Vicia faba* (*21*) and iceberg lettuce (*24*) was active at acidic pH (pH 3−4) in the absence of SDS, and the presence of the detergent in the reaction medium shifted the optimum activity of PPO from these sources by >1 pH unit toward alkaline conditions.

The optimum concentration of SDS for diphenolase activation from *V. planifolia* tissue was ∼1.7 mM (Figure 3A). In SDS concentrations >3.4 mM a significant decrease in PPO activity was observed. In this respect *Vanilla* PPO was similar to banana PPOs. Sojo et al. (*19*) showed that 2 mM SDS was optimal for the activation of banana pulp latent PPO. Also, similar results were obtained with broad bean (*25*), for which activation of PPO by SDS occurred only up to the critical point of micelle formation by the detergent. Stimulation of PPO activity by detergents in vitro can be interpreted as a result of conformational change of the enzyme protein. Rarely was SDS not effective (*22*) or PPO partly inhibited (*26*). However, in most of the plants studied cationic, anionic, and nonionic detergents (*27*) activated PPO.

Table 1. Kinetic Data of Vanilla PPO[a]

substrate	K_m (mM)	V_{max} (units/mL/min)
monophenols		
p-coumaric acid	0.42	478
3-(*p*-hydroxyphenyl)propionic	2.89	618
4-hydroxybenzyl alcohol	14.77	823
4-hydroxybenzaldehyde	6.37	56
4-hydroxybenzoic acid	6.33	120
diphenols		
caffeic acid	0.42	123
hydrocaffeic acid	0.67	159
3,4-dihydroxyphenylalanine	1.96	89
chlorogenic acid	0.49	347
4-methylcatechol	1.15	125
protocatechuic aldehyde	0.59	301
protocatechuic acid	0.37	216

[a] Activity of the enzyme was measured at room temperature with phenolic substrates and 0.25 mM TNB in acetate buffer (pH 4.0) containing 1.7 mM SDS (method b).

Substrate Specificity and Enzyme Kinetics. *Vanilla* diphenolase activity was affected by the enzyme concentration in the reaction medium and its steady-state rate increased linearly, a behavior that has been widely described in other PPOs (*19*, *23*, *28*).

The investigated PPO was active with all of the phenolic substrates used in this study. The enzyme exhibited diphenolase and also monophenolase activity. The highest PPO activity was found with monophenols—4-hydroxybenzyl alcohol (the most abundant compound in the studied *Vanilla* tissue), 3-(*p*-hydroxyphenyl)-propionic and *p*-coumaric acids (Table 1). Among six substrates of C_6-C_1 type 4-hydroxybenzyl alcohol and protocatechuic aldehyde and acid were the best. PPO-oxidizing monophenols showed a typical lag phase varying with the substrate used (Figure 4).

K_m values calculated from the Lineweaver−Burk graphs are shown in Table 1. As shown, the PPO has the highest affinity for protocatechuic acid among the diphenols and for *p*-coumaric acid among the monophenols. The lowest K_m was found with 4-hydroxybenzyl alcohol, although the V_{max} with this substrate was the highest among all tested phenolics.

Diphenolases from different sources display different substrate specificities. Plant PPOs, including *Vanilla*, have low activity toward L-DOPA (*11*, *29*, *30*), which is the typical substrate for mammalian enzymes (*31*). In contrast to our studies, PPO from *Solanum berthaultii* trichome (*11*) did not accept *p*-coumaric acid and PPO from mung bean leaves (*30*) and papaya fruits (*29*) were the most active with catechol or 4-methylcatechol.

As shown in Figure 4 and Table 1 the studied tissue contains diphenolase and monophenolase activities. Our preliminary results (data not presented) showed that monophenolase is capable, in vitro (in the presence of

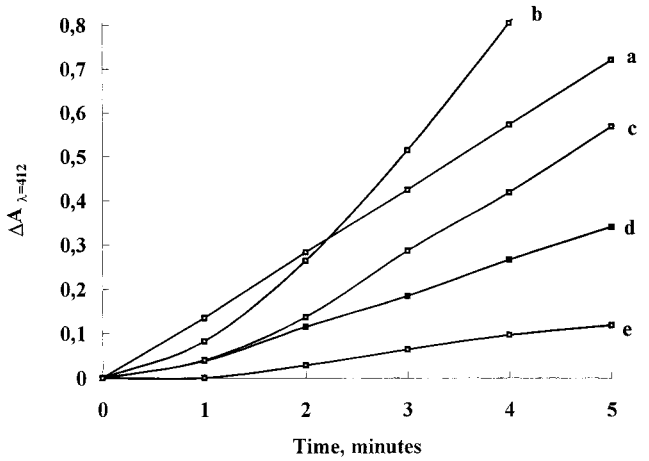

Figure 4. Enzymatic activities of *Vanilla* PPO: (a) diphenolase activity at room temperature [the reaction medium (1 mL) contained 50 μL of crude enzyme, 0.25 mM TNB, 5 mM protocatechuic acid, and 1.7 mM SDS in 0.5 M NaOAc buffer (pH 4.0)]; (b−e) monophenolase activity at room temperature [(b) with *p*-coumaric acid, (c) with 4-hydroxybenzyl alcohol, (d) with 4-hydroxybenzoic acid, (e) with 4-hydroxybenzaldehyde; each substrate was at 5 mM concentration in reaction medium as in (a)].

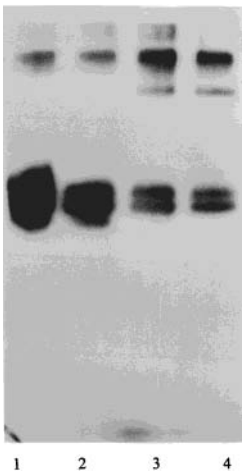

Figure 5. PAGE of diphenolase from *V. planifolia* crude extract: (lanes 1 and 2) extract pH 6.0 (10 and 5 μg of protein); (lanes 3 and 4) extract pH 8.0 (10 and 5 μg of protein). The enzyme activity was developed with 30 mM protocatechuic acid in 0.1 M NaPi (pH 7.0) containing 90 mM *p*-phenylenediamine.

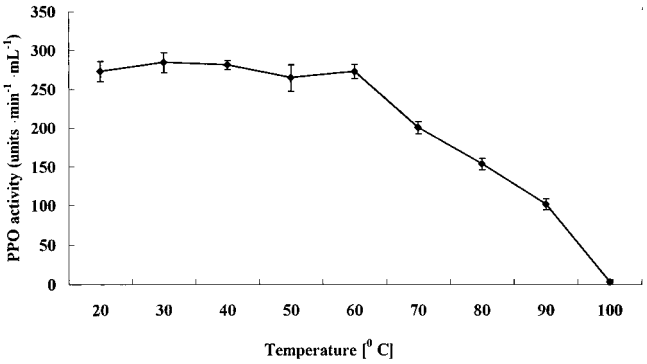

Figure 6. Heat inactivation of *Vanilla* PPO. The enzyme extract was kept for 20 min at indicated temperatures prior to activity estimation carried out at room temperature. The reaction medium contained 50 μL of crude enzyme, 0.25 mM TNB, 5 mM protocatechuic acid, and 1.7 mM SDS in 0.5 M NaOAc buffer (pH 4.0) in a final volume of 1 mL.

ascorbic acid), of synthesizing caffeic acid from *p*-coumaric acid. The monophenolase showed the highest V_{max} with 4-hydroxybenzyl alcohol (concentration in the tissue of ~11 mM), and the diphenolase had a high affinity to protocatechuic acid and aldehyde (concentration in the tissue of ~0.7 mM). Such a coincidence in the case of tissue damage will lead to significant loss of both compounds. These losses can be prevented by a low concentration of ascorbic acid inhibiting diphenolase and simultaneously allowing for accumulation of *o*-diphenols resulting from monophenolase activity. If monophenolase activity would be manifested also in vivo, it could participate in the biosynthesis of *o*-diphenol precursors of vanillin. However, the tissue studied accumulates very high quantities of 4-hydroxybenzyl alcohol and *p*-coumaric acid, but very small amounts of *o*-diphenols (*3*). This may result either because of different locations of monophenolase and its potential substrates or because the substrates in the conjugates are unacceptable to the enzyme. Verification of this hypothesis is the subject of our current studies.

Electrophoresis. The extracts of pH 6 and 8.0 revealed three isozyme bands after partial SDS-PAGE—two of them were similar in both extracts and two distinct (Figure 5). A dark-stained starting point over all of the lanes could be explained by the formation of the artifactual deposits formed by oxidized phenol−protein complexes, which did not enter the separating gel.

The partially purified banana pulp and iceberg lettuce PPOs appeared as a single band in PAGE, but two, fourm or nine bands were detected with other banana pulps (*19*, *20*, *24*). Also, Angleton and Flurkey (*10*) data showed that in a variety of plants PPO occurs in multiple isoenzyme forms.

Thermal Stability and Effect of Inhibitors. The thermal stability of diphenolase from *V. planifolia*, as shown in Figure 6, was very high. After 15 min at 70 °C PPO retained 73% and at 80 °C lost only 50% of activity. At 50 °C no loss of activity after 120 min was observed (data not shown). To the contrary, 50% temperature inhibition (after 10 min of incubation of the enzyme at 50 °C) was shown by an enzyme from iceberg lettuce (*24*). Şakrioğlu et al. (*32*) with a PPO from *Rosa dumalis* found a 20% decrease in PPO activity at 50 °C after 60 min. Our results indicate that the thermal stability of *Vanilla* PPO is very high and even higher than that found in another monocot—dates; the enzyme lost 60% of activity at 70 °C during 10 min (*33*). The storage of the *Vanilla* enzyme extract at −20 °C for 2 months resulted in no loss of PPO activity.

All of the reducing agents used in this study inhibited *o*-diphenolase activity with the extent of the inhibition being dependent on the concentration of the compound used (Table 2). Among the tested reducing agents L-ascorbic acid appeared to be the most effective (K_i = 13 μM) inhibitor, similarly as for PPO from banana, potato, and *R. dumalis* PPO (*20*, *34*, *32*). β-Mercaptoethanol at 50 mM concentration markedly inhibited PPO from *Vanilla* (Table 2), and it was also true with PPO from potato cells in suspension culture (*35*). However, in *Lithospermum* (*18*) already 0.1 mM concentration of this reducing agent completely inhibited the enzyme. It has to be emphasized that ascorbate cannot be regarded as a true inhibitor because it acts as an antioxidant and reduces the initial quinone formed by the PPO to the original diphenol. DTT (K_i = 1.2 mM), glutathione (K_i = 0.9 mM), β-mercaptoethanol

Table 2. Percent of Inhibition of Diphenolase Activity by Various Inhibitors[a]

	concentration of inhibitor						
inhibitor	1 μM	5 μM	10 μM	1 mM	5 mM	10 mM	50 mM
ascorbic acid	9.4	11.3	38.6	100	100	100	
glutathione		12.8	11.8	54.3	63.5	71.6	
DTT		8.8	8.7	46.4	94.1	97	
β-mercapto-ethanol			12.9	23	54.5	57.1	83.7
DETC			10.6	23.2	23.8	25.2	33.2
NaEDTA			21.3[b]	22.0[b]	31.9[b]	27.8	89.6
EGTA			14.9[b]	15.2[b]	12[b]	10.9	94.1

[a] Activity of the enzyme (units min^{-1} mg^{-1} of protein) was measured at room temperature with 5 mM protocatechuic acid and 0.25 mM TNB in acetate buffer (pH 4.0) containing 1.7 mM SDS (method b). [b] Percentage of enzyme activation.

(K_i = 4.8 mM), and DETC inhibit PPO by reacting with the copper of the active center as well as (except DETC) by reacting with quinone products. DETC in our study was found to be ~4 times less effective in the PPO inhibition compared to L-ascorbic acid. PPO from *Lithospermum erythrorhizon* (*18*) was completely inhibited by 0.5 mM DETC, but PPO from banana (*20*), similar to that from *Vanilla*, showed only a 20% inhibition by DETC at 1 mM concentration.

It was surprising that NaEDTA and EGTA acting as agents chelating copper atoms from the PPO active center inhibited *Vanilla* PPO only at concentrations >10 mM (Table 2). Lower concentrations of NaEDTA and EGTA had an opposite effect, and ~12−30% stimulation of PPO activity was observed. Similarly, Şakrioğlu et al. (*32*) found that among weak inhibitors of diphenolase from *R. dumalis* (NaCl, NaEDTA, ethylene glycol, thiodiglycol) NaCl at low concentration showed also some activator effect. Nevertheless, the mechanisms of these activations remain unclear.

ABBREVIATIONS USED

DETC, diethyldithiocarbamic acid; DTT, dithiotreitol; EGTA, ethylene glycol bis(β-aminoethyl eter) *N*,*N*,*N*′,*N*′-tetraacetic acid; NaEDTA, ethylenediaminetetraacetic disodium salt; PAGE, polyacrylamide gel electrophoresis; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; TNB, 2-nitro-5-thiobenzoic acid.

ACKNOWLEDGMENT

We are grateful to Dr. D. Havkin-Frenkel for the generous gift of a primordia culture of vanilla and Prof. St. Lewak for critical reading of the manuscript.

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Received for review September 27, 2000. Revised manuscript received May 2, 2001. Accepted May 7, 2001. This research was, in part, financially supported by Grants BW 1385/32 and 6 PO4C 047 20 from the State Committee for Scientific Research (Poland).

JF001180Z