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o-DIPHENOL:OXYGEN OXIDOREDUCTASE FROM LEAVES OF SUGAR CANE

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Abstract—A non-particulate *o*-diphenol O_2 oxidoreductase (phenolase) has been isolated from leaves of sugar cane Gel filtration produced two fractions MW 32000 and 130000 The preferred substrate was chlorogenic acid Other *o*-diphenols (caffeic acid, catechol, pyrogallol, dihydroxyphenylalanine) all of which were slowly oxidized when tested alone, increased the rates of O_2 consumption obtained with catalytic amounts of chlorogenic acid Both enzyme fractions were inhibited by thiols, thioglycollate, which acted in a non-competitive manner, was most effective

INTRODUCTION

OUR PREVIOUS observations[1] indicate that lack of photochemical activity in chloroplasts isolated from sugar cane leaves is associated with high concentrations of chlorogenic acid and high activities of phenol oxidase (E.C. 1.10.3.1) in the leaf tissue. Addition of thiols to isolation media improves the efficiency with which some enzymes can be extracted from cane leaves.[2] However, chloroplasts isolated in the presence of such compounds show only limited photochemical activity.[3] Addition of thiols may inhibit specific enzymes[2] or change their apparent properties[2,4] or intercellular distribution.[5] In particular, the use of thiols may be deleterious when isolating enzymes with allosteric properties from C4- plant material.[4]

It appears that chloroplasts and enzymes with full biosynthetic and regulatory properties will not be isolated from tissues containing high levels of phenol oxidase until effects of phenol oxidation products can be overcome without the need to use reagents which may produce alternative, deleterious effects. As part of our investigations, we have isolated *o*-diphenol oxidase from leaves of sugar cane. Details of the properties, substrate specificities and response of the enzyme to treatment with thiols are reported.

RESULTS

Isolation

The use of standard methods for the isolation and purification of phenol oxidase from cane leaf tissue met with little success. Difficulties arose from the rapid precipitation of

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[3] BALDRY, C W , BUCKE, C and COOMBS, J (1969) *Biochem Biophys Res Commun* **37,** 828
[4] COOMBS, J and BALDRY, C W (1972) *Nature, New Biol* **238,** 268
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all protein, including phenol oxidase, by phenolic oxidation products. Use of thiols in the isolation medium, ammonium sulphate precipitation or the preparation of acetone powders led either to irreversible loss of activity or to the formation of an insoluble enzyme Furthermore, the crude phenol oxidase was found to be bound irreversibly to ion-exchange celluloses. Two active fractions with phenol oxidase activity were obtained by blending the tissue in buffer under N_2, separating the plastids by centrifugation, rapidly freezing and freeze drying, removing low molecular weight compounds by filtration through Sephadex G-25 and fractioning the enzyme mixture on Sephadex G-200.

During each stage of purification, all solutions were centrifuged at 62000 *g* for 30 min and only the supernatants used in further stages of purification Assay of the insoluble material indicated that less than 5% of the total activity was precipitated in this way The total activity finally recovered was equivalent to about 60% of that in the original leaf extract.

Polyacrylamide gel electrophoretic separation of the highly purified enzyme preparations gave a single band of protein which coincided with phenol oxidase activity Aqueous solutions of these preparations were colourless with a single maximum at about 280 nm Some samples differed in that two (or more) bands were obtained following gel electrophoresis. These consisted of a slower moving colourless protein band with high phenol oxidase activity and faster-moving, brown bands with lower enzymic activity Solutions of such samples were slightly coloured and showed an additional absorption band between 300 and 340 nm The average MW of these samples (determined by gel filtration) was slightly greater than that of the colourless samples. Repeated passage through Sephadex G-50 columns removed a low-molecular weight, brown moiety. When highly purified samples were applied to ion-exchange columns or precipitated with ammonium sulphate most of the activity could be recovered in a soluble form These results suggest that a fraction of the protein was complexed with phenol-oxidation products which increased the charge to weight ratio of the polymer Increased charge would account for both the increased mobility on polyacrylamide gels and the irreversible binding to ion exchange celluloses.

Properties

The enzyme was eluted from a G-200 column as two fractions which are referred to as PPO I (MW 130000) and PPO II (MW 32000) (Table 1) Control samples gave 60–70% of total activity in PPO I This form could be converted quantitatively to PPO II by eluting the columns with buffer of high ionic strength, using aged preparations (left in a refrigerator at 4° under N_2 for 1 month), or eluting with sodium dodecyl sulphate or urea

A broad pH optimum was observed using both fractions with little change in activity between pH 4·5 and 7·5 Most experiments were carried out at slightly acid pH (6·3) as this reduced the rate of non-enzymic oxidation Both forms of enzymes were denatured fairly rapidly at 55° The halftime for loss of activity ($t_{\frac{1}{2}}$ 55°) for PPO II was about twice that for PPO I (Table 1)

The apparent Michaelis constant (K_m) values for various possible substrates were calculated by least squares analysis of data obtained using the double reciprocal (Lineweaver–Burk) form of the Michaelis–Menten equation. (Since we are interested in the reactions which occur in leaf brei during the isolation of chloroplasts and enzymes from leaf material the reactions were carried out at concentrations of O_2 which occur in air-saturated water.) The values of maximum velocity (V_{max}) with various substrates are reported relative to the rates observed with chlorogenic and for each enzyme fraction A number of other phenols

were also investigated. Catechol and pyrogallol supported low rates of enzyme catalysed O_2 consumption. However, protocatechuic and gallic acids, vanillin, *p*-coumaric acid, tyrosine, cinnamic acid and phenylalanine had no effect.

TABLE 1 PROPERTIES OF *o*-DIPHENOL OXIDASE ELUTED FROM SEPHADEX G-200

Fraction	1	2
MW	130000	32000
Sp act (μmoles O_2/mg protein/min)	5·7	1500
pH optimum	4·5–7·5	4·5–7·5
$t_{\frac{1}{2}}$ (55°C)	1·6	3·0
K_m (mM) for		
chlorogenic acid	2·4	1·1
caffeic acid	6·0	8·7
dihydroxyphenylalanine	10·1	45·0
V_{max}(Relative)		
chlorogenic acid	1·00	1·00
caffeic acid	0·39	0·32
dihydroxyphenylalanine	0·11	0·15

Reaction with mixed phenols

At concentrations similar to those which occur in the leaf brei during preparation of enzymes (0·1 to 1·0 mM), only chlorogenic acid supported oxygen consumption at significant rates by either PPO I or PPO II. No oxygen uptake was observed with 1·0 mM *p*-coumaric acid, purpurogallin, caffeic acid, catechol, pyrogallol or dihydroxyphenylalanine (DOPA). However, when these compounds were added, at this concentration, to reaction mixtures containing 0·1 mM chlorogenic acid an increase in enzyme catalysed O_2 consumption was observed with catechol, caffeic acid, pyrogallol and DOPA (Table 2). The products of such reactions were separated by high voltage paper electrophoresis and the amount of chlorogenic acid oxidized estimated. It was found that in control samples most of the chlorogenic acid had been used. When other phenolic compounds were included in reaction mixtures the amount of chlorogenic acid consumed was decreased. This substrate-sparing effect was most pronounced with compounds which caused the greatest increase in rate of oxygen consumption. These results suggest that a primary enzyme-catalysed oxidation of chlorogenic acid is followed by the chemical reduction of the resulting quinone by the secondary *o*-diphenol.

These reactions were used to develop the modified histochemical technique by which phenol oxidase was detected on the polyacrylamide gels. After separation of proteins, they were incubated in a solution containing both chlorogenic acid and DOPA. This resulted in the formation of the characteristic, black melanin pigments from DOPA, which were not produced if DOPA alone was used as substrate.

Inhibition

Previous observations with crude leaf brei[2] suggested that phenol oxidase in sugar cane is more sensitive to inhibition by thioglycollate than by other thiols. β-Mercaptoethanol was also fairly effective. These results were confirmed using the purified enzyme fractions (Fig. 1). PPO I was more sensitive to inhibition by thioglycollate than was PPO II. With

other thiols there was little difference in the response of the two forms of enzyme. With glutathione, cysteine, dithiothreitol and dithioerythritol concentrations of 1.0 mM or higher were required to produce 50% inhibition

TABLE 2 EFFECTS OF PHENOLIC COMPOUNDS ON RELATIVE RATES OF OXYGEN CONSUMPTION IN PRESENCE OF CATALYTIC AMOUNTS OF CHLOROGENIC ACID

Compound added* (mM)	Rate	% Chlorogenic acid used
Control†	1.0	89
Coumaric acid	1.0	74
Purpurogallin	1.0	86
Caffeic acid	1.4	57
Catechol	1.4	58
Pyrogallol	1.6	29
DOPA	1.8	13

* To chlorogenic acid (0.1 mM)
† No added phenols

Kinetics of inhibition were investigated for both PPO I and PPO II, using thioglycollate as inhibitor. The values for K_m in the presence of this compound obtained by statistical analysis of Lineweaver–Burk plots (2.3 mM for PPO I, 1.3 mM for PPO II) did not differ significantly from those obtained in control experiments. The values obtained for V_{max} were decreased. These results indicate that thioglycollate is a non-competitive inhibitor of both forms of the enzyme

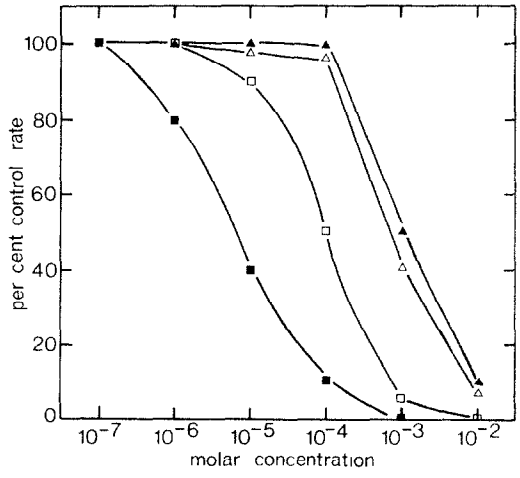

FIG 1 INHIBITION OF SUGAR CANE *o*-DIPHENOL OXIDASE BY THIOGLYCOLLATE [■ □] AND *β*-MERCAPTOETHANOL [▲, △] CLOSED SYMBOLS = PPO I, OPEN SYMBOLS = PPO II

DISCUSSION

The present extraction procedure was developed in order to obtain an active phenolase in a form similar to that which occurs *in vivo* Using these methods, the *o*-diphenol oxidase found in leaves of sugar cane appeared to occur in a cytoplasmic, soluble, non-particulate form Although insoluble, particulate and chloroplast forms have been reported from a

variety of plant species, the possibility that these represent tanned protein or protein co-precipitated with subcellular organelles cannot be excluded. This is suggested by the variation in properties which can occur with variations in separation techniques.[6,7]

In particular, it has been shown that activity of enzyme extractable from leaves of tea can be shifted from an insoluble to a soluble form by increasing the level of polycaprolactam powder used in the extraction media.[8] Unfortunately, neither the use of polyamide powder or of insoluble polyvinylpyrrolidone (PVP-Polyclar AT) produced a significant improvement in activity of enzyme from leaves of sugar cane. This result was consistent with the suggestion[9] that such compounds will be of little value in the case of tissues rich in chlorogenic acid. The low MW phenols were largely removed during the Sephadex G25 chromatographic step. This separation is facilitated by the high affinity of Sephadex for aromatic and hydroxylated substances which are retarded or in some cases irreversibly bound to the gel.

The sugar cane enzyme gave two fractions differing in MW. Multiple forms of phenol oxidase (often referred to as isozymes) have been reported from many sources including fungi,[10,11] animal tissues[12] and higher plants[13–19] In general these forms can be accounted for in terms of aggregates of monomers (MW 32000). The cane enzyme is similar with a tetrameric form (MW 130000) predominating when the enzyme was prepared under optimal conditions. In some preparations the multiple forms could not be accounted for in terms of aggregated subunits alone. These forms, which could be distinguished by electrophoresis, resulted from a variation in the ratio of charge to MW. The most probable cause for such multiple forms is complex formation between protein and phenol-oxidation products. The formation of such complexes may account for the apparent multiplicity of isozymes reported in a variety of tissues[16–19] Evidence of the existence of true isozymes (that is enzymes composed of subunits differing in primary amino acid structure, catalysing the same reaction) is slight.

In common with phenol oxidases isolated from a variety of higher plant tissues (tobacco,[7] banana,[13] sweet potato,[16] peach[18]), the sugar cane enzyme lacks monophenolase activity and has a high affinity for a specific *o*-diphenol (chlorogenic acid) which co-occurs in high concentrations in the leaf. This contrasts with the enzyme (tyrosinase) found in other higher plants such as sugar beet, beans and potatoes, which resembles the fungal and animal forms with high monophenolase activity. These two classes of enzyme (specific *o*-diphenol oxidase and tyrosinase) may differ in the form of their visible colour and UV absorption spectra. However, in these respects the sugar cane enzyme resembles the colourless tyrosinase rather than the blue *o*-diphenol oxidase. Once again, it is highly prob-

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able that these differences reflect variations in extraction procedure and more particularly the degree to which the protein is tanned.

The sugar cane enzyme appears highly specific for chlorogenic acid. Oxidation of other phenols may occur through secondary chemical reactions. Such a sequence would parallel reactions observed with enzyme isolated from peach fruits and leaves of tea. In the peach, the chemical oxidation of phloroglucinol and resorcinol follows the enzymic oxidation of 4-methyl catechol.[20] In tea, the oxidation of both gallic acid and chlorogenic acid can be accounted for in terms of chemical reaction with flavonoids oxidized by enzymes and bound to proteins.[7]

Although properties of the two forms PPO I and PPO II were essentially similar, the K_m for chlorogenic acid of PPO II (the purer of the two fractions) was lower than that of PPO I. On the other hand the K_m for other substrates (DOPA in particular) was higher. With other tissues the properties of multiple forms are usually similar. However, in some cases in addition to variations in electrophoretic and chromatographic behaviour a variation is seen in the substrate specificity e.g. the ratio of monophenolase to diphenol oxidase activity may vary.[21,22] Again this could reflect chemical reactions between bound phenolics and the apparent substrates.

The mechanism of inhibition by thiols has been discussed in detail by Anderson[9] who recognized two classes. First, those which reduce quinones back to the *o*-diphenol (including thioglycollate) and second, those which complex with the quinone to form a non-inhibitory product. In addition, thioglycollate will prevent non-enzymic chemical polymerization of phenolics.[23] With the cane enzyme, thioglycollate appears also to act as a specific inhibitor. This is suggested by the low concentration needed to inhibit PPO I and the fact that inhibition is immediate and irreversible.

EXPERIMENTAL

Plants of *Saccharum* sp. var. B 37/50 were grown from nodes in a greenhouse maintained above 20°. Young leaves were harvested, washed and deribbed. About 50 g tissue was blended under N_2 for 3 min in 0·05 m HEPES pH 7·5 (100 ml) at 4°, filtered through muslin and particulate matter removed by centrifugation at 62000 *g* for 30 min. The supernatant was rapidly frozen and freeze dried. The dried powder was dissolved in H_2O (30 ml), clarified by centrifugation and placed on a column of superfine Sephadex G-25 (V_T = 300 ml), pre-equilibrated with 0·05 m HEPES buffer. The totally excluded vol. was collected and placed on a G-200 Sephadex column (V_T = 220 ml) pre-equilibrated with HEPES buffer and calibrated using proteins of known MW. The MW of PPO I and PPO II were determined by the method of Andrews.[24] Protein by the method of Lowry.

Phenol oxidase activity was determined polarographically using an O_2 electrode. Chlorogenic acid and other substrates were routinely added to a final conc. of 1·0 mM in 0·05 M MES buffer pH 6·3. The K_m values were determined using a range of substrate from 0·5 to 20 mM as appropriate.

Samples were also resolved on 9% polyacrylamide gels. Protein was detected using Xylene Brilliant Cyanin G. Phenol oxidase activity was detected by incubating gels in a solution of 1% (w/v) EDTA plus 1% (w/v) chlorogenic acid at pH 7·0 in MOPS buffer at 30° for 30 min.

Chlorogenic acid consumption was estimated by spotting aliquots onto borate buffer (0·05 M pH 9·2) impregnated paper (Whatman 3 MM). Reaction products were separated by HV electrophoresis for 30 min and the papers photographed under U/V light using a Kodak Wratten 2B filter. This cut out fluorescent emission from all compounds, except chlorogenic acid. The amount of chlorogenic acid was estimated by scanning the U/V photographs using a Chromoscan and relating to standard amounts of chlorogenic acid resolved in the same way. Buffers: MES 2(N-morpholino)ethane sulphonic acid; BEPES N,N-bis(hydroxyethyl) piperazine-N-2 ethane sulphonic acid; MOPS 3(N-morpholino) propane sulphonic acid.

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