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BLACKENING IN GREEN PEPPER BERRIES

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Key Word Index—*Piper nigrum*; Piperaceae; green pepper; enzymic blackening; *o*-diphenol oxidase; 3,4-dihydroxy phenylethanol glycoside; 2-(3,4-dihydroxyphenyl) ethylalcohol.

Abstract—The blackening of fresh green pepper (*Piper nigrum*) berries was found to be due to the enzyme—catalysed oxidation of 3,4-dihydroxy phenylethanol glycoside by an *o*-diphenol oxidase present in the fruit. The enzyme was also found to be active towards the aglycone of 3,4-dihydroxy phenylethanol glycoside.

INTRODUCTION

Pepper (*Piper nigrum* L.) is an important commercial spice valued since early times for its pungency and flavour. Black, white and green pepper are three different forms of pepper products available in the market. Whole, unripe but mature berries in the dried form constitute black pepper, while fully ripe dried fruit devoid of pericarp form the commercial white pepper. Green pepper obtained from unripe, but fully developed berries has of recent years become an important product of commerce valued in the western market for its delicacy. Unlike black and white pepper, however, it has a limited market as it undergoes blackening on storage within a short time unless preserved in brine, acetic acid or citric acid [1]. The cause of blackening in green pepper berries has not been studied except for a preliminary report by Lewis *et al.* [2] who suggested it was due to enzymic oxidation of polyphenols present in the skin. The present paper reports on the elucidation of the nature of the enzyme and its major substrate involved in the blackening of green pepper berries.

RESULTS AND DISCUSSION

Properties of pepper polyphenol oxidase (PPO)

The nature and substrate specificity of the enzyme preparation obtained from green pepper was determined by measuring its activity towards mono- and di-phenolic substrates at pH 7. It catalysed the oxidation of 4-methyl catechol (334 μmol O_2 consumed/min/mg protein) and catechin (117 μmol O_2 consumed/min/mg protein) but not of quinol, *p*-cresol, chlorogenic acid or quercetin (each tested at 0.5 mM). Its activity was inhibited by phenyl hydrazine and diethyldithiocarbamate indicating it to be an *o*-diphenoloxidase.

The oxidase was active over the pH range 3.0 to 8.5 (4-methyl catechol as substrate) with an optimum activity at pH 7.0. Its activity at pH 3.0 and 8.5 were 30 and 60% respectively of the maximum activity. Interestingly, pepper PPO although inactive towards common plant *o*-dihydroxyphenolic acids such as caffeic acid and chlorogenic acid at pH 7.0 showed low activity towards these acids at low pH values (3.0 to 6.0) with an optimum activity at pH 4.9 accounting for 20% of that shown towards 4-methyl catechol at the same pH. The enzyme was also ineffective at all pH values tested towards quercetin inspite of the presence in this compound of an *o*-dihydroxy group.

The levels of phenolase activity with 4-methyl catechol (0.5 mM) as substrate were measured in whole and different parts of the green pepper berry. The results showed that the specific activity of the enzyme in the skin was some five-fold higher than its specific activity in the flesh (whole pepper 340 μmol O_2 consumed/min/mg protein; skin 240; flesh 49).

Nature of the natural substrate

The phenolase was most active with a crude acetone extract and its butanol-soluble component (f_1). On TLC (solvent system, 2), f_1 was resolved into five components having R_f values of 0.56, 0.44, 0.34, 0.17 and 0.0 respectively. After acid treatment of f_1 the spots at R_f 0.56 and 0.34 were absent and a few new spots appeared at R_f values above 0.8. The products obtained on acid treatment were fractionated by successive extractions with ether and ethyl acetate. The most active compound(s) resided in the ethyl acetate fraction (f_2). On TLC separation using solvent system (1), all the components of f_1 remained at the base while f_2 showed a clearly defined spot (R_f 0.52). It gave a single spot on TLC using solvent systems of varying polarity.

The major component of fraction f_1 (R_f 0.56), isolated by preparative TLC (solvent 2) after acid treatment followed by ethyl acetate extraction, showed a single spot (R_f 0.52) on TLC (solvent 1) corresponding to f_2, while only glucose was detected in the remaining aqueous solution. Thus the major active compound (f_2) was originally present in the berry as a glucoside.

Purified f_2 showed a molecular ion peak at m/z 154 which corresponded to a molecular formula of $C_8H_{10}O_3$. The presence of a catechol moiety was indicated by a strong ferric reaction, enzymatic assay and a fragment at m/z 124 (base peak) in the mass spectrum of the compound. The dimethyl ether of fraction f_2 showed a

prominent band in the IR spectrum at 3450 cm^{-1} suggesting the presence of an alcoholic hydroxyl group. This was supported by the mass spectrum and the presence of three acetate methyl signals in the NMR spectrum of f_2 acetate. Two triplets at δ2.94 (2H, *t*, $J=7.0$ Hz) and 4.3 (2H, *t*, $J=7.0$ Hz) were assigned to $-CH_2-CH_2-OH$. From the above evidence f_2 was assigned the structure 2-(3,4-dihydroxyphenyl) ethyl alcohol.

The high activity of PPO towards both the glycoside and its aglycone suggested that the glucose moiety was attached to the alcoholic hydroxyl group.

3,4-Dihydroxyphenyl ethanol occurs widely in Nature. It has been isolated as a glycoside from *Coptidis rhizoma* [3], fruits of *Ligustrum obtisifolium* [4] and olive juice [5] and as an aglycone from olive oil [6] and leaves of *L. obtisifolium* [7].

From the above results it is suggested that the phenolase isolated from green pepper berries is an *o*-diphenol-oxidase having high specificity towards 3,4-dihydroxyphenyl ethyl alcohol and its glycoside with low activity towards phenolic acids at acidic pH values. Thus damage of skin tissues results in an instantaneous reaction of the above enzyme and substrate yielding black oxidized products.

EXPERIMENTAL

Plant material. Freshly plucked home-grown green pepper berries (Panniyur variety) were airlifted from Kerala (South India) and stored at 0–2°. The berries were used within 2 days of storage.

Me_2CO *powder.* Fresh green pepper berries (25 g) were frozen in liquid N_2 and then homogenized in a pre-cooled Waring Blendor with chilled Me_2CO (−30°, 5 ml/g fruit) for 2–3 min. The slurry was quickly filtered under suction. The wet cake was blended again with Me_2CO (−30°) and this was repeated until the filtrate was pale yellow in colour. Finally, the cake was dried free of Me_2CO at 0–4° and stored at −30° until use.

For the separation of skin and flesh, the green pepper berries were pre-cooled at 0–4°. The skin was carefully peeled off manually and both the skin and the flesh were chilled immediately in liquid N_2. Me_2CO powders of skin and flesh were prepared as described above.

Enzyme preparation. Preparation of crude enzyme was essentially carried out according to the method of ref. [8]. The Me_2CO powder (1 g) was stirred with 15 ml cold 0.02 M NaOAc buffer, pH 5.7 (buffer A) for 45 min. The residue was removed by filtration through a double layer cheese cloth and the filtrate centrifuged at 15000 *g* for 10 min. To the clear supernatant, 75 ml of chilled Me_2CO was added and the ppt. quickly separated by centrifugation. The ppt. was dissolved in 3 ml of buffer A. This was used as enzyme preparation for assay.

Isolation of natural substrate. The solvent was removed under red. pres. from the Me_2CO filtrate obtained above. The remaining aq. soln (A) was successively subjected to exhaustive extractions with Et_2O, EtOAc and *n*-BuOH. The solvent from the BuOH-extract was removed as above and the residue designated as f_1. A part of f_1 was treated with 1 M HCl for 1 hr at 100°, dild with H_2O and then extracted successively with Et_2O and EtOAc. The EtOAc extract was washed with H_2O, concd and the residue designated as f_2. Fraction f_2 was submitted to CC on silica gel (15 g) with Et_2O–MeOH of increasing MeOH content. The fractions eluting with Et_2O–MeOH (19:1) were combined and concd *in vacuo* to obtain a chromatographically pure compound which was identified by its MS and by IR and NMR of its methylated and acetylated derivatives. MS *m/z*; 155, 154 $[M]^+$, 124 [base peak] and 123.

Acetylated aglycone (15 *mg*). Ac_2O–C_5H_5N, room temp. overnight. ^1H NMR ($CDCl_3$): δ2.03 (3H, *s*, alcoholic OCOMe), 2.29 (6H, *s*, 2x phenolic OCOMe), 2.94 (2H, *t*, $J=7.0$ Hz, $ArCH_2CH_2O$–), 4.3 (2H, *t*, $J=7.0$ Hz, $ArCH_2CH_2O$–) 7.15–7.05 (3H, aromatic protons).

Methylated aglycone (15 *mg*). CH_2N_2–Et_2O, room temp. overnight. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 1600, 1520; ^1H NMR ($CDCl_3$): δ2.85 (2H, *t*, $J=7.0$ Hz, $ArCH_2CH_2O$–), 3.9 (2H, *t*, $ArCH_2CH_2O$–), 3.95 (6H, *s*, 2x phenolic OMe), 6.8–6.85 (3H, aromatic protons).

Enzyme activity was measured by the initial rate of O_2 uptake using a Clark electrode in a 3 ml cuvette at 30°. The reaction mixture consisted of 1.5 μmol substrate (0.15 ml), 0.1–0.2 mg enzyme proteins and freshly aerated 0.02 M Na-Pi buffer, pH 7.0, to a final vol. of 3 ml, and was stirred constantly. For the assay of natural substrates, 0.1 ml of A and each of its subsequent fractions (1% soln in 80% aq. MeOH) were used. The sp. act. is expressed as μmol O_2 consumed/min/mg protein. Protein was estimated by Miller's method [9].

pH optima. The effect of pH on the oxidation of 0.5 mM 4-methyl catechol including other standard substrates by pepper polyphenol oxidase at pH values ranging from 3 to 8.5 was studied using 20 mM of citrate (pH 3–6), phosphate (pH 6–7.8) and Tris–HCl (pH 7.8–8.5) buffers. The activity of the enzyme was unaffected at overlapping pH values of the above buffer systems indicating that the change in buffer had no effect on enzyme activity.

General techniques. ^1H NMR: 60 MHz; MS: 70 eV, EI; TLC: silica gel G (Merck, type 60) with the following solvent systems: (1) toluene–HCOOEt–HCOOH (5:4:1), (2) toluene–EtOH–HCOOH (5:4:1), (3) HCOOEt–HCOOH–H_2O (8:2:3), (4) *n*-BuOH–HOAc–H_2O (6:1:2), (5) $CHCl_3$–MeOH (4:1) and (6) Me_2CO–MeOH (1:4). The spots were visualized by exposing the plates to I_2 vapour and also by spraying the plates with a fresh mixture of equal vols of 0.5% $FeCl_3 \cdot 6H_2O$ and 0.5% $K_3Fe(CN)_6$.

f_1 was separated on prep. TLC (0.5 mm silica gel G plates) using solvent (ii). The major band (R_f 0.56) detected under UV (366 nm) was then scraped from the plate and the material recovered by elution with 80% aq. MeOH. Acid hydrolysis of the recovered glycoside (1M HCl, 1 hr, 100°), yielded the aglycone and sugar residue.

Sugars were analysed by PC (Whatman 3 MM) using *n*-BuOH–HOAc–H_2O (4:1:5) as solvent and a mixture of 0.45 g oxalic acid and 0.9 g aniline–HCl in 100 ml H_2O as spray reagent [10].

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