Phenolics of Green Pepper Berries (*Piper nigrum* L.)

Chiranjib Bandyopadhyay,* Vaduvatha S. Narayan, and Prasad S. Variyar

Food Technology and Enzyme Engineering Division, Bhabha Atomic Research Centre, Bombay 400 085, India

The nature of phenolic compounds and their involvement in enzymatic blackening of green pepper berries during conventional sun-drying were investigated. Among the five commercial varieties of pepper studied, a great variation in the total phenolics including *o*-diphenol oxidase oxidizable phenolics was noted. However, no correlation was observed between the total or *o*-diphenol oxidase oxidizable phenolic content of a particular variety and the extent of its blackening. Conversion of green pepper to black pepper by the drying process was accompanied by a 75% decrease in total phenolic content and a complete loss of *o*-diphenol oxidase oxidizable phenolic fraction that suggested a major role for the enzymatic phenolic oxidation during the pepper blackening. A compound that served as an efficient substrate for pepper *o*-diphenol oxidase was isolated for the first time from green pepper berries and was characterized as 3,4-dihydroxy-6-(*N*-ethylamino)benzamide on the basis of chromatographic and spectrometric methods.

INTRODUCTION

Pepper (*Piper nigrum* L.) is the largest commodity in the international spice trade of which India is the major producer and exporter. Black, white, and green peppers are three different forms of pepper products available in the market. Black pepper is produced by conventional sun-drying of mature green pepper berries. Green pepper obtained from unripe fully matured berries is becoming an important commercial product valued in the Western market for its delicacy. However, unlike black pepper, green pepper has limited market as it blackens quickly on storage unless preserved in brine or vinegar.

Apart from the major quality attributes such as pungency and aroma, the appearance with respect to brownness/blackness is of importance for direct use of black pepper as a spice in the whole or ground form (Purseglove et al., 1981). Since phenolics are known to contribute to browning/blackening and thus to the appearance of finished pepper corns, the nature and distribution of phenolic compounds in different varieties of green pepper decide the quality of pepper marketed. Phenolics also contribute to the astringent taste and thus to the overall flavor of foods. We reported earlier that the blackening of fresh green pepper (Panniyur variety) was due to enzymatic oxidation of (3,4-dihydroxy phenyl)-ethanol glycoside by an *o*-diphenol oxidase (PPO) present in the fresh fruit (Variyar et al., 1988). Except for a preliminary report (Regional Research Laboratory, 1986) on the total phenolic content of green pepper berries, there is no information available at present on the nature of phenolic constituents in commercial varieties of green pepper berries. The present study relates to the nature of phenolics and their role in blackening of five important commercial varieties of Indian green pepper berries.

MATERIALS AND METHODS

Materials. Fresh green pepper berries of five commercial varieties, namely Karimunda, Panniyur, Balankotta, Chetan, and Utharinkotta, were procured from Kerala, India, by air and stored at 0–2 °C. The berries were used within 2 days after arrival. All solvents and reagents were of highest analytical grade. The solvents were redistilled before use.

Preparation of Pepper Extract Concentrate. Berries (50 g) of each variety were added in small lots through a reflux condenser to a flask containing boiling 80% aqueous methanol (100 mL) in about 15 min, and the refluxing was continued for another 45 min. The flask was then cooled to room temperature, and the contents were homogenized in a Waring blender at high speed for 3 min. The homogenate was filtered under suction, and the residue was reextracted with 80% aqueous methanol as above (4 × 100 mL) until the filtrate was pale yellow. The pooled filtrate (500 mL) was concentrated in vacuo at 40 °C to ca. 20 mL. An aliquot (10 mL) of the concentrated solution was kept aside, the remaining portion was evaporated to dryness, and the residue was dissolved in distilled water to give a 10% stock solution. The phenolic content of these solutions, after appropriate dilution, was estimated by the method of Swain and Hillis (1959) using Folin–Danis reagent with the help of Shimadzu UV–vis spectrophotometer, UV-240 at 725 nm and catechin as standard.

Solvent Extraction. The original concentrate (10 mL) that was kept aside was successively extracted with diethyl ether (5 × 20 mL), ethyl acetate (5 × 20 mL), and *n*-butanol (10 × 20 mL). Each extract, after removal of solvent, was tested for its ability to act as substrate toward fresh pepper PPO. Since most of the PPO reactive phenolics were retained in the *n*-butanol extracts, these fractions were further analyzed. *n*-Butanol extracts were subjected to acid hydrolysis (1 N HCl, 100 °C, 1 h), and the hydrolysates were successively extracted with diethyl ether, ethyl acetate, and *n*-butanol exactly as above. Pepper PPO activity toward each of these extracts was tested. High activity toward ethyl acetate and *n*-butanol fractions was recorded.

Thin-Layer Chromatography (TLC). Thin-layer chromatography of unhydrolyzed *n*-butanol extracts of pepper varieties was carried out on a 10% ammonium sulfate impregnated silica gel G plate (250 μm layer thickness) using toluene–ethanol–formic acid (5:4:1 v/v) as developing solvent. The spots were visualized either by heating the plate at 180 °C for 10 min or by spraying the plate with a freshly prepared mixture of equal volumes of 0.5% ferric chloride and 0.5% ferricyanide. The diethyl ether extracts of the hydrolyzed samples containing mostly phenolic acids were subjected to TLC on a cellulose–silica gel G (1:1 w/w) plate using dichloromethane–toluene–formic acid (5:4:1 by vol) as described by Schulz and Herrmann (1980b). A compound (B) with high reactivity toward pepper PPO was located in the *n*-butanol extracts of all five varieties of green pepper samples. This compound, having an R_f value of 0.44, was isolated from *n*-butanol extracts by preparative silica gel TLC. The isolated compound B and its acid hydrolyzed (1 N HCl, 100 °C, 1 h) product appeared at the same R_f when rechromatographed on silica gel TLC, indicating that compound B was un-

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affected by acid hydrolysis. The isolation and characterization of compound B were further pursued as follows.

The unhydrolyzed *n*-butanol extract (200 mg) of Karimunda variety was rich in compound B as revealed by TLC. This extract was acidified to pH 3.5 with 1 N HCl and then applied on a Dowex-50 (Na^+ form) column (15 × 1.6 cm). After the column was washed with 10 bed volumes of distilled water, the bound compound was eluted (50 mL) with 1 N HCl. The acidic eluate was placed in a boiling water bath for 1 h under nitrogen atmosphere, cooled, and successively extracted with diethyl ether (5 × 25 mL) and ethyl acetate (5 × 25 mL). The remaining aqueous solution containing compound B was neutralized to pH 5.0, extracted with *n*-BuOH (4 × 20 mL), and concentrated in vacuo. The band containing compound B (R_f = 0.44), separated by silica gel TLC, was scraped off and eluted with 80% aqueous MeOH. The dried residue left after removal of solvent in vacuo was treated with pyridine and acetic anhydride (2:1 v/v) and kept overnight at ambient temperature. After the usual workup, the acetylated products were dissolved in chloroform and purified on a short column (15 × 1.6 cm) of silica gel using chloroform–methanol as eluting solvent. Fractions eluting with $CHCl_3$:MeOH (99:1 v/v) were pooled and concentrated to obtain 15 mg of acetylated compound B that gave a single spot on TLC on solvent systems of varying polarity (Variyar et al., 1988).

Preparation of Pepper PPO. Partially purified PPO from pepper berries was obtained as described by Variar et al. (1988). Briefly, fresh green pepper berries (25 g) were frozen in liquid nitrogen and then homogenized in a Waring blender with chilled acetone (5 mL/g of fruit) for 2–3 min. The slurry was quickly filtered under suction. The cake was washed twice with chilled acetone and dried free of acetone at 0–4 °C. Acetone powder (1 g) thus obtained was stirred with 15 mL of 20 mM sodium acetate buffer, pH 5.7, for 45 min at 0–2 °C. The insoluble material was then removed by centrifugation at 15000*g* for 15 min. To the clear supernatant was added 75 mL of chilled acetone, and the precipitate obtained after centrifugation was dissolved in 3 mL of the above buffer and was used as the enzyme source. PPO was assayed by the initial rate of oxygen uptake by using a Clarke electrode in a 3-mL cuvette at 30 °C as described earlier (Variyar et al., 1988). In a final volume of 3 mL, the assay mixture contained freshly aerated 20 mM sodium phosphate buffer, pH 7.0, 1 mg of substrate, and 100 μg of enzyme. Specific activity of the enzyme was expressed as oxygen consumed min^{-1} (mg of protein)$^{-1}$ at 30 °C. Pepper PPO activity toward standard substrates, e.g., 3,4-dihydroxyphenylalanine (DOPA), (3,4-dihydroxyphenyl)ethylamine (DOPAmine), epinephrine, and 4-methylcatechol (each at 500 μM) was also measured on the same day. Protein determination was done according to Miller's (1959) method.

Instrumental Methods. Infrared (IR) spectra of the purified compound B before acetylation in KBr pellets and of that after acetylation as a thin film between sodium chloride windows were recorded on a Shimadzu IR 400 spectrophotometer. Proton magnetic resonance (^1H NMR) spectra of the acetylated compound B were recorded on a Varian $A_{60}A$ PMR spectrometer using $CDCl_3$ as the solvent and tetramethylsilane as an internal standard. Acetylated compound B was analyzed by mass spectrometry (MS) using the direct probe inlet to a VG Micromass 7070F operated at an ion source temperature of 200 °C and electron energy of 70 eV. The elemental composition of acetylated compound B was determined on a Carlo Erba Model 1108 elemental analyzer.

RESULTS AND DISCUSSION

Data on total phenolics and phenolic acids of five varieties of green pepper and their response toward pepper PPO are presented in Table I. A great variation in the total phenolic content as well as in the response of phenolics toward PPO among the five varieties of green pepper was noted (Table I). However, no correlation was observed between the total phenolic content and pepper PPO activity with respect to the extent of blackening of green pepper berries. Extent of blackening was, however, judged only by visual assessment of the dried berries. Coseteng and Lee (1987) also did not find a definite relationship between degree of browning among apple cultivars and apple PPO activity in terms of polyphenol concentrations. Phenolic acid content of five varieties of green pepper showed much less variation and the values ranged from 14.5 to 20 mg/100 g of green berries.

Table I. Phenolics of Five Varieties of Green Pepper Berries and Their Response toward Pepper Polyphenol Oxidase (PPO)

variety	total phenolics, mg/100 g fresh weight	total phenolic acids, mg/100 g fresh weight	sp act. of pepper PPO,[a] O_2 consumed min^{-1} (mg of protein)$^{-1}$
Panniyur	743.6	16.2	1786
Balankotta	1056.0	19.1	286
Chetan	955.1	20.8	2119
Uttharinkotta	1132.4	18.4	369
Karimunda	1219.4	14.5	1095

[a] The *n*-butanol extracts, after removal of the solvent, were tested as substrates toward pepper PPO as described under Materials and Methods.

Specific activity of partially purified pepper PPO toward DOPA, DOPAmine, epinephrine, and 4-methylcatechol as standard substrates was found to be 65, 1951, 690, and 334 μmol of O_2 consumed min^{-1} (mg of protein)$^{-1}$, respectively. The enzyme displayed only a weak activity toward DOPA but gave 30-fold higher activity toward DOPAmine, suggesting that the presence of a carboxyl group in a compound makes it a poor substrate. This contention is further supported by the fact that phenolic acids were found to be very poor substrates for pepper PPO (Variyar et al., 1988). In contrast, considerable activity of pepper PPO toward epinephrine, DOPAmine, and compound B suggested the requirement of a basic nitrogen in the substrate molecule to enhance the activity of the enzyme.

Pepper PPO was found to be inactive toward aqueous extracts of sun-dried black pepper obtained from each of the five varieties. This indicated the complete loss of enzyme-active phenolic compounds during the drying process. The total phenolic content in all five varieties of black pepper was also found to be decreased to approximately one-third the value of the respective green pepper. Thus, it appears that both phenolase-active and -inactive phenolics are present in green pepper berries and the blackening of green berries during the drying process is essentially associated with the enzymatic oxidation of active phenolics. However, the degree of blackening of dried pepper corns appears to be dependent on the availability, concentration, and nature of active phenolics present in green pepper. It was reported earlier that the phenolics in black pepper are a mixture of phenolic acid glycosides as well as flavanol glycosides, which on hydrolysis gave rise to nine phenolic acids along with significant quantities of quercetin and kaemferol (Schulz and Herrmann, 1980a; Vosgen et al., 1980). The nature of phenolic acids identified in all five varieties of pepper in the present study is similar to that reported earlier (Schulz and Herrmann, 1980a). However, we could not detect even trace amounts of flavanol in any of the varieties, either in fresh green or dried form.

Pepper phenolase enzyme showed highest activity toward unhydrolyzed *n*-butanol fractions of pepper samples and only negligible activity toward diethyl ether and ethyl acetate fractions. The *n*-butanol fraction on TLC resolved into four distinct spots having R_f values at 0.66 (A), 0.44 (B), 0.39 (C), and 0.24 (D). Panniyur and Uttharinkotta varieties gave qualitatively similar separation with spot

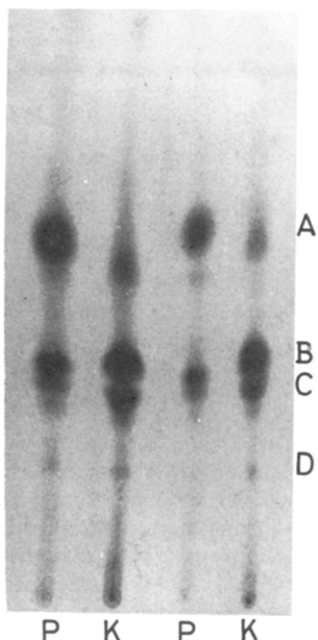
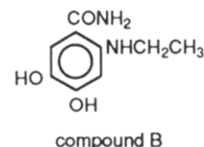


Figure 1. TLC chromatogram of green pepper extracts of Panniyur (P) and Karimunda (K) varieties spotted at two different concentrations.

A predominating, whereas the other three varieties gave similar qualitative separation with spot B as the predominant compound. Figure 1 shows a representative chromatogram of *n*-butanol-soluble components of two commercial varieties of green pepper, namely Panniyur and Karimunda. After acid hydrolysis of the butanol extract, the spots at $R_f = 0.66$ and 0.39 were absent and a few new spots appeared at R_f values above 0.9 along with a spot at $R_f = 0.44$. When this spot at $R_f = 0.44$ was scraped and rechromatographed on a TLC plate along with isolated compound B from unhydrolyzed *n*-butanol extracts, both gave a single spot corresponding to a R_f of 0.44. This clearly indicated that the spot at $R_f = 0.44$ in the hydrolysates was indeed compound B. The compound A was earlier identified as (3,4-dihydroxyphenyl)ethanol glucoside (Variyar et al., 1988). It was observed consistently that compound B undergoes discoloration on a preparative silica gel TLC plate when the developing solvent system was completely removed from the plate. The presence of a small amount of formic acid in silica gel, however, helps the recovery of compound B without its discoloration.

The IR spectra of compound B in KBr pellets showed broad λ_{max} at 3500, 3400, 1650, 1640, and 1400 cm^{-1}, indicating the presence of a hydroxyl, an amine, and a primary amide group in the molecule. Spectral characteristics of compound B after acetylation and subsequent purification are as follows: IR (film) 3440 and 3240 (amide/amine), 1770 ($OCOCH_3$), 1650 and 1640 ($CONH_2$), 1430 ($CONH_2$) and 1510 and 760 cm^{-1} (aromatic). Its EIMS, however, did not show a molecular ion peak but gave predominant peaks at m/z as follows: 306 (M - NH_2), 291, 248, 220, 205 (base), 162, 151, and 135. Its ^1H NMR ($CDCl_3$) showed δ 1.5 (t, $J = 7$ Hz, 3 H, CH_2CH_3), 2.18 (s, 3 H, $NCOCH_3$), 2.27 (s, 6 H, 2 × $OCOCH_3$), 2.9 (br q, 2 H, CH_2CH_3), and 7.0 (s, 2 H aromatic proton). The elemental composition of acetylated compound B is as follows: Anal. Found (calcd) for $C_{15}H_{18}O_6N_2$: C, 55.7 (55.9); H, 5.4 (5.6); O, 30.6 (29.8); N, 8.3 (8.7).

The presence of a singlet of 6 H at δ 2.27 and a singlet of 3 H at δ 2.18 in the ^1H NMR spectrum indicates three acetyl groups, two of which are of similar nature but distinct from the third. The presence of three acetyl group is also supported by their loss of mass 43 each giving rise to peaks at m/z 248, 205, and 162 in the MS spectrum. A strong absorption on Dowex-50 column together with IR data of unacetylated and acetylated compound B indicates the presence of an amino group in the molecule. Further, the absence of an IR band at 1550 cm^{-1} in the IR spectrum of the acetylated compound B corresponding to N–H stretching of a secondary amide rules out the presence of a primary aromatic amino group in the original molecule. Thus, a singlet of 6 H at δ 2.27 in the NMR spectrum of acetylated compound B arises from the acetylation of two phenolic hydroxyl groups, and a singlet of 3 H at δ 2.18 must be an *N*-acetyl group arising from a secondary aromatic amine in the original compound B. A triplet of 3 H at δ 1.5 indicates the presence of a methyl group, while a broad quartet at δ 2.7 indicates a methylene group in the side chain of the molecule. Thus, compound B appears to possess an alkyl (CH_2CH_3) substituted secondary aromatic amino group. The presence of a characteristic doublet at 1640 and 1650 cm^{-1} due to a >C=O stretch together with a C—N stretch band at 1425–1430 cm^{-1} in the IR spectra suggests the presence of a primary aromatic amide group in the molecule, which is also supported by a doublet at 3440 and 3240 cm^{-1} in the IR spectra and also by a strong M - NH_2 fragment in the MS spectra of the acetylated product. Hence, the presence of two phenolic hydroxyl groups, a secondary alkyl-substituted amino group, and an aromatic amide group in compound B could be deduced. Since compound B was shown to be an efficient substrate for pepper PPO, the two hydroxyl groups in compound B must be ortho to each other (Mayer and Harel, 1970). Thus, the structure of compound B could be assigned as 3,4-dihydroxy-6-(*N*-ethylamino)benzamide. The occurrence of this compound



in green pepper is reported for the first time. Besides its prominent role in the blackening of green pepper berries, this compound is likely to have other physiological roles since related to phenolic amides (Yoshihara et al., 1978; Nakatani et al., 1980) and phenolic amines (Callingham and Barrand, 1979) have been reported to have various pharmacological properties.

LITERATURE CITED

Callingham, B. A.; Barrand, M. A. The catechol amines: adrenaline; noradrenaline, dopamine. In *Hormones in Blood*, 3rd ed.; Horace, G. C., Thomas, J. V., Eds.; Academic: New york, 1979; pp 143–207.

Coseteng, M. Y.; Lee, C. Y. Changes in Apple Polyphenoloxidase and Polyphenol Concentrations in Relation to Degree of Browning. *J. Food Sci.* **1987**, *52*, 985–989.

Mayer, A. M.; Harel, E. Polyphenol Oxidases in Plants. *Phytochemistry* **1979**, *18*, 193–215.

Miller, G. L. Protein Determination of Large Number of Samples. *Anal. Chem.* **1959**, *31*, 964.

Nakatani, N.; Inatani, R.; Fuwa, H. Structure and Synthesis of two Phenolic Amides from Piper nigrum L. *Agric. Biol. Chem.* **1980**, *44*, 2831–2836.

Purseglove, J. W.; Brown, E. G.; Green, C. L.; Robbins, S. R. J. Spices; Longman: New York, 1981; Vol. 1, pp 10–99.

Regional Research Laboratory, Trivandrum, India. Value Added Products from Spice and Spice Products, Project No. RLT/83/44, 1986, pp 1–61.

Schulz, J. M.; Herrmann, K. Occurrence of Hydroxybenzoic acids and Hydroxycinnamic Acids in Spices IV. Phenolics of Spices. *Z. Lebensm. Unters. Forsch.* **1980a**, *71*, 193–199.

Schulz, J. M.; Herrmann, K. Analysis of Hydroxybenzoic Acid and Hydroxycinnamic Acids in Plant Material I. Sample Preparation and Thin-layer chromatography. *J. Chromatogr.* **1980b**, *195*, 85–94.

Swain, T.; Hillis, W. E. Phenolic Constituents of Prunus Domestica I. Quantitative analysis of Phenolic Constituents. *J. Sci. Food Agric.* **1959**, *10*, 63.

Variyar, P. S.; Pendharkar, M. B.; Banerjee, A.; Bandyopadhyay, C. Blackening in Green Pepper Berries. *Phytochemistry* **1988**, *27*, 715–717.

Vosger, B.; Kiok, G.; Herrmann, K. Flavonol Glycosides of Pepper (Piper nigrum L.), Clove (Syzgium aromaticum L., Merr. et Perry) and Allspice (Pimenta dioca L. Merr.), 3. Phenolics of Spices. *Z. Lebensm. Unters. Forsch.* **1980**, *170*, 204–207.

Yoshihara, T.; Takamatsu, S.; Sakamura, S. Three New Phenolic Amides from the Roots of Eggplant (Solanum melongena L.). *Agric. Biol. Chem.* **1978**, *42*, 623–627.

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