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Identification of a Naturally Occurring Inhibitor of the Conversion of 1-Aminocyclopropane-1-Carboxylic Acid to Ethylene by Carnation Microsomes[1]

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ABSTRACT

During cell-free experiments with membranes isolated from carnation petals (*Dianthus caryophillus* L. cv White Sim), the conversion of 1-aminocyclopropane-1-carboxylic acid into ethylene was blocked by a factor derived from the cytosol. Subsequent characterization of the inhibitor revealed that its effect was concentration dependent, that it was water soluble, and that it could be removed from solution by dialysis and addition of polyvinylpolypyrrolidone. Activity profiles obtained after solvent partitioning over a range of pH values and after chromatography on silica gel, size exclusion gel, and ion exchange resins revealed that the inhibitor was a highly polar, low molecular weight species that was nonionic at low pH and anionic at pH values above 8. Use of selected solvent systems during paper and thin layer chromatography combined with specific spray reagents tentatively identified the compound as a hydroxycinnamic acid derivative. Base hydrolysis and subsequent comparison with known standards by high performance liquid chromatography, gas-liquid chromatography, and ultraviolet light spectroscopy established that the inhibitor was a conjugate with a ferulic acid moiety. Release of ferulic acid following treatment with β-glucosidase also indicated the presence of a glucose moiety, and unequivocal identification of the inhibitor as 1-*O*-feruloyl-β-D-glucose was confirmed by gas chromatography-mass spectroscopy and by ultraviolet light, ^{1}H-, and ^{13}C- nuclear magnetic resonance spectroscopy. Feruloylglucose constituted about 0.1% of the dry weight of stage III (preclimacteric) carnation petals, but concentrations fell sharply during stage IV (climacteric), when ethylene production peaks and the flowers senesce. In a reaction mixture containing microsome-bound ethylene forming enzyme system, 98% of all ethylene production was abolished in the presence of 50 μm concentrations of the inhibitor.

Current evidence suggests that the conversion of *S*-adenosylmethionine to ethylene via ACC[4] is the major pathway of ethylene biosynthesis in higher plants (1). The EFE, which mediates the conversion of ACC to ethylene, shows reduced activity when the cell membrane is disrupted (11), and treatments that alter membrane structure, including Triton X-100 (2–4) and exposure to temperature extremes, also inhibit ethylene formation (9, 19, 20). Microsomal membranes obtained from the petals of senescing carnation flowers have been shown to catalyze the conversion of ACC to ethylene (12), and, *in toto*, these observations indicate that the EFE is part of a membrane-bound complex. Nevertheless, isolation of purified EFE has not been achieved, and several lines of evidence suggest that the reactions observed with microsomes *in vitro* are not analogous to those occurring in vivo. Principal concerns result from the low affinity of the *in vitro* system for ACC (13) and its poor stereodiscrimination between isomers of 1-amino-2-ethylcyclopropane-1-carboxylic acid (18). However, Guy and Kende (7) observed that vacuoles isolated from protoplasts of pea do stereodiscriminate between the isomers of AEC and yet exhibit a low K_m for ACC.

During *in vitro* studies with the carnation microsomal system that converts ACC to ethylene, ethylene production was strongly inhibited by the WSF co-isolated from the petals (12). Whether the microsomes function in precisely the same way as intact EFE remains unclear, but the cytosolic inhibitor previously described (10, 12) and shown to block the ACC to ethylene step *in vitro* may also regulate the activity of EFE *in situ*. In the present study, therefore, we have used a range of chromatographic techniques to isolate and identify the inhibitor and have characterized its structure using UV spectroscopy, ^{1}H- and ^{13}C-NMR, and MS. The effects of the isolated inhibitor on ethylene biosynthesis were also tested, and the pattern of change in concentrations of the inhibitor during opening and senescence of carnation flowers was defined.

MATERIALS AND METHODS

Plant Material

Carnation flowers (*Dianthus caryophillus* L. cv White Sim) were purchased at the tight-bud stage from a commercial source and placed individually in glass tubes of deionized water on a laboratory bench under 11.3 $W \cdot m^{-2}$ of continuous cool white fluorescent light. Senescing flowers were harvested at the stage of maximum ethylene production when the petals showed signs of in-rolling and a loss of turgidity (6). Five developmental stages were distinguished to facilitate visual selection of flowers: stage I, young flower only partly open; stage II, open flower, no ethylene production; stage III, mature flower, petals fully open but showing no in-rolling, small

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[4] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; BAW, *n*-butanol/acetic acid/water; CME, crude methanolic extract; EFE, ethylene forming enzyme; PC, paper chromatography; WSF, water soluble fraction; EPPS, *N*-2-hydroxyethyl-piperazine propane sulfonic acid; TMS, trimethylsilyl.

amounts of ethylene produced (preclimacteric); stage IV, senescing flowers showing petal in-rolling and maximum ethylene production (climacteric); stage V, petal tips brown, ethylene production very low, flowers moribund.

Microsomal and Inhibitor Fractions

Microsomal membranes were isolated in 10 mM EPPS buffer (pH 8.5) from 50 g of petals of stage IV flowers using minor modifications of a method previously described (12). The homogenate was spun at 131,000*g* for 70 min and the supernatant (WSF), containing the inhibitor, was freeze-dried and 250 mg of the yellowish powder subsequently extracted for 4 h with 80% methanol at 4°C. Solid material was removed in a clinical centrifuge held at top speed for 10 min and the extraction procedure repeated until the pellet was white and the supernatant clear. The combined methanolic extracts were evaporated under vacuum at 35°C, and the remaining aqueous volume, representing the CME, was made to 10 mL, adjusted to pH 2.0 with 0.4 M HCl, and partitioned four times against ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness and the residue redissolved in 300 μL of absolute methanol and labeled ethyl acetate fraction.

The microsomal pellet used for bioassay was resuspended in 2 mM EPPS buffer (pH 8.5) and dialyzed overnight at 4°C against 4 L of the same buffer. The dialyzed membrane was recentrifuged at 131,000*g* for 1 h and stored at −15°C.

Microsomal Bioassay

The reaction mixture was composed of 100 μL of 10 mM ACC as substrate, 100 μL of membrane preparation (1 mg protein mL^{-1}) and, in the appropriate tests, 100 μL of inhibitor (WSF or as specified), all brought to 1 mL with 68 mM EPPS buffer at pH 8.5. The 6-mL test tubes were closed with rubber septa and incubated at 33°C for 2 to 3 h before headspace analysis for ethylene. One-mL samples were injected into a Perkin-Elmer 900 gas chromatograph equipped with a flame-ionization detector and a 60 × 0.32 cm glass column packed with 60 to 100 mesh Al_2O_3 and run isothermally at an oven temperature of 60°C, using nitrogen as the carrier gas at a flow rate of 30 $mL \cdot min^{-1}$.

Silica Gel Chromatography

CME equivalent to 50 mg fresh weight of petals was dried on 5 mm discs of filter paper (Whatman GF/D), loaded on silica gel (5 g, 60–80 mesh, Nutritional Biochemicals) in a 1 × 30 cm glass column, eluted with solvents of increasing polarity and inhibitory activity monitored with the carnation microsome bioassay.

Gel Permeation Chromatography

A similar amount of WSF was loaded on a Sephadex G-10 column (5 g, 1 × 30 cm, glass) and eluted continuously with 2 mM EPPS buffer (pH 8.6), at 2.0 $mL \cdot min^{-1}$, and 1 mL fractions monitored for inhibition of ethylene as described above.

Ion Exchange Chromatography

CME was adjusted to pH 2.5 and a sample applied to the head of a 1 × 10 cm glass column packed with Dowex-50, H^+ form. After elution with water acidified to pH 3.0 with 0.1 M acetic acid, residue from the dried eluate was dissolved in water, adjusted to pH 9.0 with 2 M NH_4OH, and applied to a column of Dowex-1, OH^- form, and washed with five bed-volumes of water adjusted to pH 9.0 with 2 M NH_4OH. Eluates from both columns were tested for inhibitor activity.

Paper Chromatography and TLC

Ethyl acetate fraction was strip-loaded on 15 × 57 cm sheets of Whatman No. 3 paper and developed in 15% (v/v) acetic acid/water by descending chromatography. Air-dried replicate chromatograms were spray-tested for color reactions with ferric chloride-potassium ferricyanide for phenol functional groups, ammoniacal silver nitrate for reducing compounds, diazotized *p*-nitroaniline for cinnamic acids, and $AlCl_3$ for flavonoids. Fluorescence under UV light after exposure to NH_3 vapor served as a rapid test for phenolic compounds.

To isolate the inhibitor, a dried chromatogram was cut into 10 equal transverse strips and eluates (80% methanol) tested in the microsome bioassay. Eluate from the most inhibitory strip of a replicate chromatogram was rechromatographed in 4:1:5 BAW (v/v/v) and replicate dried chromatograms sprayed with color reagents and bioassayed as described above. The residue from the most inhibitory strip of a third replicate chromatogram was dissolved in 300 μL of absolute methanol prior to analytical HPLC.

Samples were also separated on TLC plates (Merck, 0.25 mm, 60F-254) and the chromatograms evaluated under UV light at 366 and 254 nm for bright blue fluorescence and dark quenching, respectively.

HPLC

Separation was achieved with a Spectra-Physics 8100 liquid chromatograph on a Supelco 4.6 × 150 mm, C_{18}, 5-μm, reverse-phase column held at 40°C, using a Brownlee C_{18}, Spheri-5, guard column and an SP 8400 UV detector set at 320 nm. Methanolic solutions of reference compounds and inhibitor extract, previously purified by two separations on paper, were injected without derivatization via a Rheodyne automatic injector valve equipped with a 20-μL loop. A binary solvent gradient was formed with methanol and water using acetic acid to suppress ionization of acid groups. Peak areas were determined with an SP 4270 integrator and the peaks tentatively identified by retention times and co-elution of sample peaks with known standards.

GLC

Dried samples were dissolved in 50 μL of pyridine and silylated with 200 μL of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (Supelco) in 1-mL Reacti-Vials. Mixtures were capped, stored in the dark at room temperature for 1 h, and then injected into a Hewlett-Packard 5710A gas chromatograph equipped with a flame-ionization detector and a fused-silica DB-5 capillary column (15 m × 0.32 mm i.d., J. W. Scientific). Helium was used as the carrier gas at a flow rate

of 1.1 mL·min^{-1} and the oven temperature programmed from an initial 2 min hold at 150°C to 320°C at 4° min^{-1}. Injector and detector temperatures were set at 250° and 300°C, respectively.

UV-VIS Spectroscopy

Commercial standards and purified samples from the HPLC were dissolved in methanol in 1-mL cuvettes and absorption spectra run from 200 to 500 nm in a Perkin-Elmer Lambda UV-VIS Spectrophotometer. Spectral shifts were also compared after adding an excess of NaOH or NaOAc to the cuvettes.

Alkaline and Enzyme Hydrolysis

Three sample volumes of HPLC-purified inhibitor were combined, dried, and one drop of methanol plus 1.5 mL 2M NaOH were added to hydrolyze the residue. The vial was flushed with nitrogen, capped, and left at room temperature for 4 h. For enzyme hydrolysis, additional samples were dissolved in 0.25 mL of citrate-phosphate buffer (pH 5.0), and 10 mg of β-glucosidase from sweet almonds (Boehringer Mannheim) were added and the mixture incubated at 37°C for 30 min. Hydrolysates were acidified to pH 2.0, the aglycone partitioned into ethyl acetate, washed 3 × with distilled water, evaporated to dryness, and identity of the aglycone verified by GLC and GC-MS.

Sugar Analysis

After partition against ethyl acetate, the aqueous fraction of the alkaline hydrolysate was passed through a 1 × 10 cm ion-exchange column packed with a 1:1 mixture of Dowex 50, H^+ and Dowex 1, OH^-. The sugar moiety was eluted with water, chromatographed on paper in 4:1:5 BAW (v/v/v), and tentatively identified using a 1% (v/v) solution of aniline hydrogen phthalate and authentic sugar standards. For co-chromatography during GLC, TMS derivatives were formed by addition TRI-SIL "TBT" (Pierce) to samples in screw-capped vials held at 60°C for 30 min before injection of 1-μL samples into a gas chromatograph as described above.

GC-MS

Purified samples were analyzed in the electron-ionization mode using an HP 5987A MS coupled to an HP 5890A gas chromatograph and an ionization temperature of 200°C, an emission current of 300 μA, an ionization current of 70 eV, and chromatography conditions as noted above.

NMR Spectroscopy

Spectra were obtained with a Bruker AM 250 NMR spectrometer and peak assignments established on the basis of data in reference literature and indices and by direct comparison with standards for each component of the inhibitor. To obtain the large sample sizes required for NMR analysis, eluates from the second purification run on paper were applied to Whatman preparative TLC plates and developed in ethyl acetate/isopropanol/water (65:24:11, v/v/v). Homogeneity of the eluted inhibitor was confirmed during HPLC and the eluate subsequently lyophilized for 12 h before dissolving the residue in hexadeuterodimethyl sulfoxide for measurement of ^{1}H- and ^{13}C-NMR at frequencies of 250 and 62.5 MHz, respectively.

RESULTS

An amount of crude cytosolic extract equivalent to 0.2 g fresh weight of the petals inhibited the *in vitro*, microsomal conversion of ACC to ethylene by 80% (Table I). When the extract was partitioned against ethyl acetate over a range of pH values, maximum inhibitory activity in the organic phase was detected at pH 2.0 with none at pH 10.0. Large amounts

Table I. *Effect of Dilution of the Water Soluble Fraction from 50 g of Carnation Petals on the Ethylene Converting Activity of Isolated Microsomes*

Supernatant obtained at 131,000*g* was concentrated to 2 mg protein per mL and then diluted with 10 mM EPPS buffer (pH 8.5). The reaction mixture contained 0.2 mL of 20 mM ACC; 100 μg membrane suspension and 0.2 mL of diluted inhibitor in amounts described in the table.

Equivalent Weight of Fresh Petals	Ethylene	Inhibition
g	*nL·100 μg^{-1} protein·2 h^{-1}*	%
0.0	6.18 ± 0.38	
0.002	6.16 ± 0.18	0.3
0.02	5.51 ± 0.82	10.8
0.2	1.22 ± 0.11	80.3
2.0	0.129 ± 0.013	97.9
20.0	0.036 ± 0.005	99.5

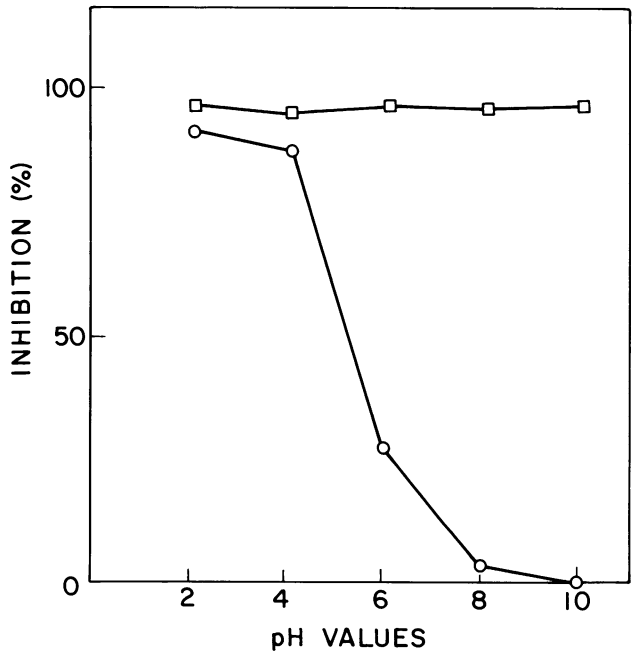


Figure 1. The effect of pH on the solvent partitioning properties of the inhibitor. Samples of crude cytosolic extract diluted 10-fold were adjusted to pH values of 2, 4, 6, 8, and 10 and partitioned against ethyl acetate. Dried ethyl acetate and aqueous fractions were each solubilized in 5 ml of buffer and bioassayed. Ethylene inhibitory activity from organic phase, O——O; from aqueous phase, □——□.

Table II. *Inhibitor Activity Profile during Ion Exchange Chromatography*

Acidified CME (0.25 mL) was applied to Dowex-50 (H^+ form), eluted with water at pH 3, the eluate dried, redissolved in water, and the pH adjusted to 9 and applied to Dowex-1 (OH^- form). Eluates from both columns were dried and assayed. The reaction mixture contained 4 mM ACC and 100 μg protein at final concentration.

Treatment	Ethylene	Inhibition
	$nL \cdot 100\ \mu g^{-1}\ protein \cdot h^{-1}$	%
Control	2.66 ± 0.05	
Acidified CME	0.21 ± 0.02	92.1
Water eluate, pH 3, from Dowex-50	0.36 ± 0.03	86.5
Water eluate, pH 9, from Dowex-1	3.19 ± 0.11	0
1 M formic acid eluate from Dowex-1	0.31 ± 0.07	88.4

of the inhibitor were also detected in all aqueous phases (Fig. 1), and when *n*-hexane was substituted for ethyl acetate, no inhibitory activity was detected in the organic phase at any pH. Both ethyl acetate and *n*-hexane failed to elute the acidified inhibitor from a silica gel column, but strong inhibition was obtained with a more polar eluant of 60% MeOH in ethyl acetate. During gel permeation chromatography, all activity in the CME emerged in one peak in the early stages of elution but well behind the void volume, indicating a M_r somewhat less than 700 D. Dialysis of the crude WSF after boiling supported that conclusion and also demonstrated that the inhibitor was not heat labile or proteinaceous. In total, these observations and the retention of the inhibitor on a Dowex-1 anion exchange column at pH 9.0 (Table II) indicated that the inhibitor was acidic, amphipathic, and that it had a relatively low M_r.

The addition of PVPP to a fresh petal homogenate sharply reduced inhibitor activity, and treatment of the methanol eluate from the PVPP pellet or of the active fraction from the silica gel column with ferric chloride-potassium ferricyanide, produced an intense blue color, characteristic of phenolics. When the inhibitor extract was chromatographed on paper or on thin layer plates, the most inhibitory section on each chromatogram fluoresced strongly under UV light and gave intensely positive color reactions in the four reagent tests for phenolics. The pink bands obtained after spraying with diazotized *p*-nitroaniline were also strongly indicative of a cinnamic acid derivative, and several chromatographic characteristics suggested a strong similarity to chlorogenic acid (5-caffeoylquinic acid). However, chlorogenic acid was retained at the starting point during TLC in relatively neutral solvents (MC and EIW, Table III), whereas the HPLC purified inhibitor was more mobile under those conditions, indicating that it was not charged at neutral pH, *i.e.* that it did not have a free carboxyl group. In addition, R_F values of the aglycone, obtained after alkaline hydrolysis, were consistent with those recorded for authentic ferulic acid chromatographed in five different solvent systems (Table III), and UV spectra for ferulic and the aglycone were nearly identical. Although the intact inhibitor and chlorogenic acid had similar UV spectra, marked differences were observed after induction of spectral shifts following addition of NaOH and NaOAc (Table IV). Their lack of identity was also confirmed during HPLC (Fig. 2).

Hydrolysis of the inhibitor with β-glucosidase was indicative of a ferulic acid-glucose conjugate, and the presence of β-glucose was confirmed by color tests during PC and co-chromatography during GLC. Enzymatic hydrolysis of the inhibitor following methylation with diazomethane produced a compound that co-chromatographed with 3,4-dimethoxycinnamic acid and not with the methyl ester of the ferulic acid moiety. This indicated the presence of a ferulic acid derivative with a β-glucose substituent conjugated at the carboxyl end and not at the phenolic hydroxyl via an ether linkage.

Although the molecular ion of the silylated inhibitor was not observed during electron impact GC/MS, an abundant fragment with a mass of 249, characteristic of the ferulic moiety, was observed in the spectrum. Moreover, at least 10 ions readily identified as fragments derived from the putative glucose moiety of the inhibitor were recorded on the mass spectrum. Among these peaks there were four of the five masses that characterize glucose (5), including the base peak at m/e 147. All major ions in the spectrum of the purified aglycone were identical to those from authentic ferulic acid.

An estimate of the M_r of the inhibitor was obtained with chemical ionization MS. A M_r minus 15 signal recorded at m/e 701, probably representing the loss of a methyl group

Table III. *Comparison of R_F Values of the Inhibitor, Its Aglycone and Phenolic Standards during PC and Silica Gel TLC Using Five Different Solvent Systems*

Compound	R_F values					
	TLC				PC	
	BAW[a]	MC[b]	CA[c]	EIW[d]	BAW	15% HoAc[e]
Inhibitor	0.56	0.30	0.00	0.62	0.62	0.81
Aglycone	0.80	0.52	0.63	0.77	0.83	0.52
Reference compounds:						
Chlorogenic acid	0.33	0.00	0.03	0.07	0.61	0.81
Caffeic acid	0.72	0.35	0.21	0.82	0.79	0.56
Ferulic acid	0.82	0.53	0.63	0.75	0.84	0.52

[a] BAW = *n*-butanol/acetic acid/water (4:1:5, v/v/v). [b] MC = 30% methanol/70% chloroform. [c] CA = 95% chloroform/5% acetic acid. [d] EIW = ethyl acetate/isopropanol/water (65:24:11, v/v/v). [e] HoAc = acetic acid.

Table IV. *UV Absorption Spectra and Spectral Shifts of the Inhibitor, Its Aglycone and Commercial Standards of Chlorogenic and Ferulic Acids*

Compound	Absorption Maxima (nm)		
	MeOH	MeOH + NaOH	MeOH + NaOAc
Inhibitor	326,290	378,296sh	380,326
Chlorogenic acid	326,300sh[a]	382,260sh	370sh,312
Aglycone	319,294	344,300	308,285
Ferulic acid	317,291sh	344,300	309,286

[a] sh: shoulder.

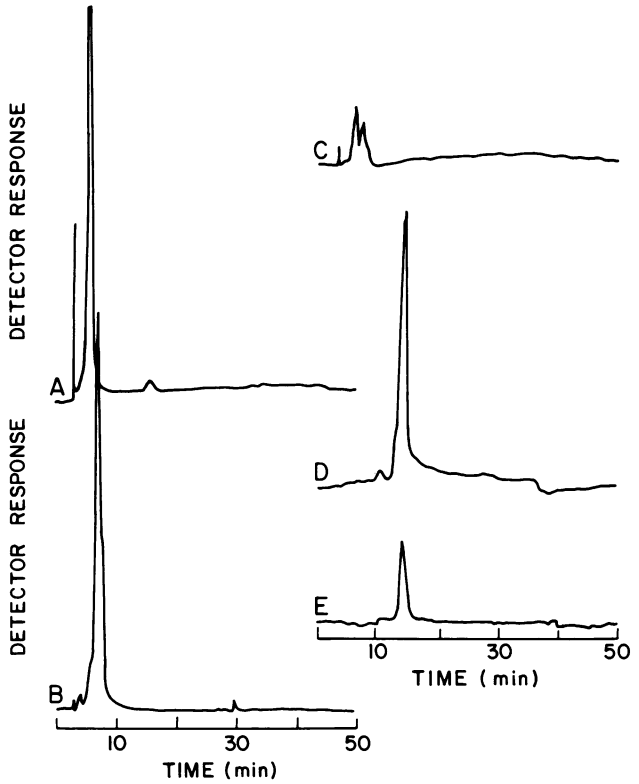
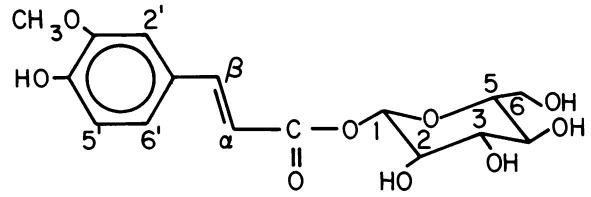


Figure 2. HPLC analyses of the partially purified inhibitor and its aglycone with detection at 320 nm. Gradient elution (solvent A, 5% acetic acid in methanol; solvent B, 5% acetic acid in water): starting from 10% A and 90% B (isocratic) for 5 min to 50% A (linear gradient) in 20 min, a 5 min hold and then to 100% A (linear gradient) over 20 min. A, Freshly prepared chlorogenic acid; B, partially purified inhibitor; C, coinjection of chlorogenic acid and inhibitor; D, aglycone of inhibitor; and E, ferulic acid standard.

from a TMS residue (16), provided additional evidence that the inhibitor was correctly identified as the glucosyl derivative of ferulic acid (Fig. 3).

A ^{1}H-NMR spectrum showed the presence of three aromatic protons (2'H, 7.28 ppm singlet; 6'H, 7.11 ppm, doublet; 5'H, 6.81 ppm doublet) with values characteristic of those in ferulic acid and a J value of 15.8 Hz, indicative of the *trans* isomer of ferulic acid (17). The large coupling constant of J = 8.0 at positions 5' and 6' established that substituents on the aromatic ring were not present at those positions. Peaks that appeared between 3.00 and 5.50 ppm resembled the spectrum of standard β-glucose and not quinic acid. A prominent doublet with a coupling constant of 7.72 Hz resonating



1-O-FERULOYL-β-D-GLUCOSE

Figure 3. Proposed chemical structure of the inhibitor, 1-*O*-feruloyl-β-D-glucose.

at 5.43 ppm was characteristic of a glucose anomeric proton with a β-configuration.

J-Modulated ^{13}C-NMR analysis provided final confirmation of structure. The procedure takes into account the associated proton environment of each carbon, and the spectrum obtained facilitates assignment and confirmation of carbon peaks. The data on chemical shifts and peak to peak orientation of every carbon in the inhibitor, in ferulic acid, and in β-glucose and quinic acid are shown in Table V. The first nine peaks plus the methoxy peak of the inhibitor obviously derive from the carbons in ferulic acid, while the last six peaks derive from glucose, thus confirming the identity of the inhibitor as 1-*O*-feruloyl-β-D-glucose.

The inhibitor was relatively abundant in young carnation flowers and accumulated to maximum levels just prior to the climacteric-like rise in ethylene that begins in stage III of the maturation and senescence process (Fig. 4). It constituted about 0.1% of petal dry weight (2676 nmol·g^{-1} dry weight) at stage III but then declined rapidly through stage V to levels less than one-quarter of the highest recorded values. Concentrations of free ferulic acid did not change over the same period.

During the in vitro enzyme assay, ethylene formation was essentially completely suppressed when the microsomes were incubated with ACC in the presence of 50 μM solutions of the inhibitor or its aglycone (Table VI). As well, Itzhaki *et al.* (10) have reported that a partially purified preparation of what is presumably the same inhibitor from carnation petal cytosol blocks the conversion of ACC to ethylene by isolated microsomes and in intact petals.

DISCUSSION

Several properties distinguish the inhibitor of ethylene synthesis that we describe from the high M_r proteinaceous inhibitor previously isolated from mung bean by Sakai and Imaseki (15). The carnation inhibitor was found in high concentrations in the cytosol fraction of petals and was readily removed from aqueous extracts by dialysis, PVPP slurry, and anion exchange at moderately high pH. Furthermore, boiling did not affect inhibitor activity, indicating that it was heat-stable. Gel filtration demonstrated that the inhibitor had a M_r of less than 700 D, and several color reactions plus its behavior at high pH suggested that it was a phenolic derivative.

Following alkaline hydrolysis, the components of the inhibitor were clearly identified as ferulic acid and β-glucose. Four pieces of evidence confirmed that the glucosyl moiety was

Table V. *^{13}C-NMR Spectroscopy of Ferulic and Quinic Acids, β-D-Glucose and the Inhibitor*

Carbon Position	Chemical Shift (ppm) and Sign of *J* Modulation (+,−)[a]			
	Inhibitor	Ferulic acid	β-Glucose	Quinic acid
COOH	165.5 (+)	167.9 (+)		175.6 (+)
C-4′	148.2 (+)	149.0 (+)		
C-3′	147.2 (+)	147.9 (+)		
C-α	146.5 (−)	144.4 (−)		
C-1′	124.7 (+)	125.7 (+)		
C-6′	123.5 (−)	122.8 (−)		
C-2′	113.8 (−)	115.6 (−)		
C-5′	112.8 (−)	115.5 (−)		
C-β	111.3 (−)	111.2 (−)		
C-1	94.3 (−)		96.7 (−)	74.6 (+)
C-3	77.9 (−)		76.7 (−)	74.5 (−)
C-5	76.5 (−)		76.5 (−)	66.7 (−)
C-2	72.6 (−)		74.9 (−)	40.5 (+)
C-4	69.6 (−)		70.4 (−)	69.1 (−)
C-6	60.7 (+)		61.6 (+)	37.5 (+)
OCH_3	55.7 (−)	55.7 (−)		

[a] Carbon atoms associated with odd numbers of protons give rise to negative peaks; whereas those associated with zero or even numbers of protons give rise to positive peaks.

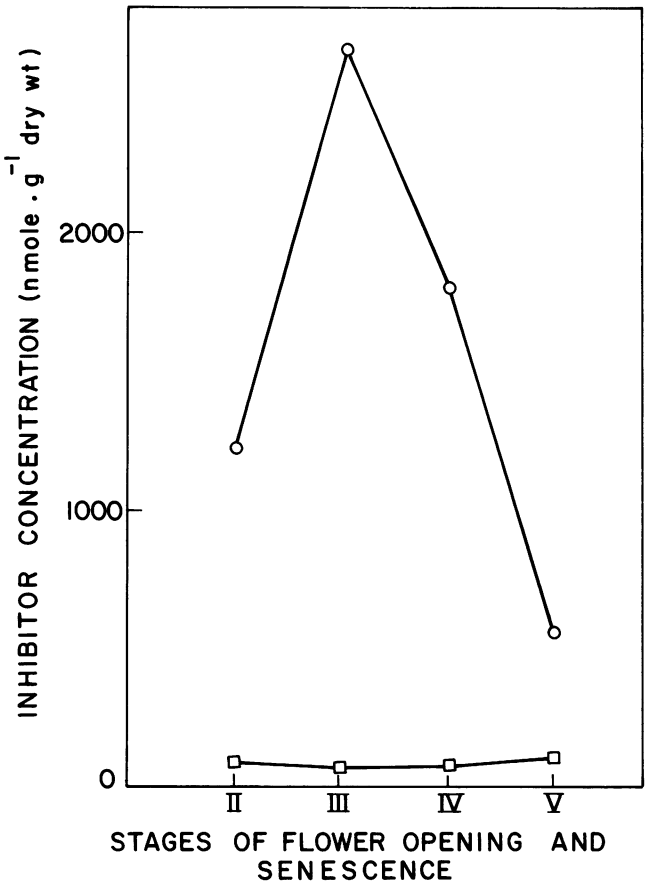


Figure 4. Levels of feruloylglucose and ferulic acid in carnation petals at different stages of senescence. Feruloylglucose, O——O; ferulic acid, □——□.

combined with the carboxyl group and not with the hydroxyl substituent on the ferulic acid moiety. First, the inhibitor showed an obvious alkaline-shift of UV-absorption, suggesting the presence of a free phenolic hydroxyl group. Second, the inhibitor was easily hydrolyzed under alkaline conditions.

Table VI. *Effect of Feruloylglucose and Ferulic Acid on the Conversion of ACC to Ethylene by Microsomes from Senescing Carnation Petals*

Reaction mixture (1 mL) contained 100 μg membrane protein and 1 mM ACC.

Treatment	Activity (Ethylene Production)	
	Ethylene	Inhibition
	nL · 100 μg^{-1} protein · h^{-1}	%
Control	0.962 ± 0.03	
Inhibitor concentration (μM):		
100	0.0	100
50	0.014 ± 0.005	98
10	0.518 ± 0.019	46.1
Ferulic acid (50 μM)	0.0	100

Third, the spectral characteristics of the inhibitor closely resembled those of esters of cinnamic acid in an alkaline environment, *viz.* a bathochromic shift, whereas *o*-coumaryl glucoside, *i.e.* a phenolic glycoside, would yield a hypsochromic shift (8). Fourth, methylation of the inhibitor and subsequent hydrolysis produced the expected 3,4-dimethoxycinnamic acid.

Unequivocal identification of structure was confirmed by combined evidence from GC/MS and ^{1}H- and ^{13}C-NMR spectroscopy. GC/MS established that the aglycone of the inhibitor was ferulic acid and that its moiety was glucose. The large number of hydroxyl groups on glucose made the TMS derivatives of the intact inhibitor highly unstable in the ionization chamber, and the molecular ion was therefore not observed. The ferulic fragment obtained by cleavage of the ester linkage was readily detected, and subsequent analysis by chemical ionization MS revealed a prominent mass-minus-15 peak specific for TMS derivatives (16), thus confirming the M_r of the inhibitor.

Analysis by ^{1}H-NMR verified the presence of the following structural features: a methoxy group, an aromatic ring with

substituents at the 3′ and 4′ positions, a double bond in *trans* configuration, multiple peaks from glucose and a β-linkage at the anomeric carbon of the glucose.

Final identification of the intact molecule was obtained using ^{13}C-NMR with *J*-modulation. Positive assignment of every peak produced by the inhibitor was achieved by matching chemical shifts and orientations of all peaks with known standards. The peaks were shown to be additively derived from ferulic acid and β-glucose, and the structure thus positively identified as 1-*O*-feruloyl-β-D-glucose (Fig. 3).

High levels of the inhibitor were present in presenescent carnation petals with maximum concentrations observed during stage III of flower opening and senescence (Fig. 4). The sharp decrease in concentration that followed is a concomitant of the large peak in ethylene production that is also characteristic of stage IV flowers (14). We might reasonably speculate therefore that the mechanism that controls ethylene synthesis is modulated by endogenous levels of the inhibitor. That possibility is currently under investigation.

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