AN INHIBITOR OF INDOLEACETIC ACID OXIDASE FROM PEA TIPS [1]

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Reduction of IAA oxidase activity in etiolated pea buds by exposure to light was first reported by Tang and Bonner (9). Hillman and Galston (4) have shown that the IAA oxidase activity of the buds of etiolated pea plants is inhibited by red light (640 mμ) and that the effect is reversed by near infrared (730 mμ) light. They presented evidence that the inhibition is due to increased concentration of a dialyzable inhibitor. Our investigation of the structure of this IAA oxidase inhibitor has revealed that it is a derivative of kaempferol. Although kaempferol and related flavanoids have been found previously in many plant species the role that these compounds play in the biochemistry of the plant has remained obscure. The finding here that a derivative of kaempferol is an IAA oxidase inhibitor suggests that flavanoids may exert an effect on plant growth through their action on the IAA oxidase system.

EXPERIMENTAL

IAA OXIDASE ENZYME: Black Valentine bean roots were used as the source of the IAA oxidase (IAAO) since they proved to be essentially free from cofactors and inhibitors of the enzyme. The enzyme was prepared from 8 day old Black Valentine bean plants grown under artificial light at 2,000 ft-c, daylength 16 hours and day and night temperatures of 23 C and 18 C, respectively. The roots were rinsed with tap water and blotted with paper towels, then cut into 1-inch lengths and dropped immediately into dry ice. Frozen root sections, 25 g, were pulverized in a chilled mortar; the resulting fine powder was mixed with 150 ml of 0.02 M KH_2PO_4 buffer, pH 6.1, and stirred for 20 minutes. Centrifugation at 20,000 $\times g$ for 10 minutes yielded a clear supernatant which when diluted to 250 ml with buffer yielded enzyme stock solution (E) which could be stored frozen for months with little loss of activity.

SOLUTIONS: A stock solution (S) containing the substrate IAA at 1×10^{-3} M and Mn^{++} at 5×10^{-4} M was prepared by dissolving 175 mg of IAA and 98.95 mg of $MnCl_2 \cdot H_2O$ in a liter of 0.01 M KH_2PO_4 buffer, pH 6.1. A 1×10^{-3} M stock solution of 2,4-dichlorophenol (DCP), for use as cofactor in the standard assay, was prepared by dissolving 163 mg of the compound in one liter of buffer.

ASSAY: In the standard assay for inhibitor activity, the assay cups, 50 ml beakers, contained 1 ml of E, 2 ml of S, 0.5 ml of DCP, 0 to 5 ml inhibitor solution, and sufficient 0.01 M buffer to bring the total volume to 10 ml. Samples were incubated in a Dubnoff metabolic shaker at 26 C. Residual IAA was determined with Salkowski ferric chloride reagent by the method of Tang and Bonner (9).

GROWTH OF PEAS: Peas (*Pisum sativum* L. var. Alaska), obtained from W. Atlee Burpee Co., Philadelphia, Pa., were grown in vermiculite in darkness at 23 to 25 C and 50 % relative humidity. At 7 or 8 days the plants were exposed for 20 minutes to 800 kiloergs of red light from a source similar to that of Nitsch and Nitsch (6). The terminal buds were harvested 18 to 20 hours later and dropped into a beaker cooled in dry ice. The frozen tips usually were extracted immediately after harvest but occasionally were stored frozen for a week or so.

PRELIMINARY WORK: An initial experiment was performed to confirm the red light effect on IAA oxidase (IAAO) of etiolated pea buds. Homogenates of buds from dark control and red light-treated etiolated pea plants were prepared in a fashion similar to the bean root enzyme preparation except that the pea bud homogenate stock solutions represented 500 mg tissue per milliliter. With DCP as cofactor at 5×10^{-5} M, and varying enzyme concentration, the presence of inhibitor in the red light bud homogenate was very apparent. On the other hand, homogenate of dark control buds with no added cofactor, showed increasing IAA destruction with increasing enzyme concentration.

Both homogenates were dialyzed against an equal volume of water at 4 C for 72 hours. A 10 μl aliquot of each dialysate was chromatographed on paper. Each dialysate showed the presence of substances which gave a brown color with diazotized sulfanilic acid (1) and a bright yellow aluminum lake. In 20 % KCl as solvent, the R_f of the major substance in dark control dialysate was 0.28 while that of red light dialysate was 0.12. This value (0.12) for dialysate of red light treated buds compares to the R_f 0.14 obtained later for the purified inhibitor.

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ISOLATION OF INHIBITOR: In a preliminary experiment, 200 mg of frozen red light-treated buds were pulverized in a chilled mortar and the resulting powder stirred in 25 ml of ethanol for 20 minutes, then filtered. The filtrate was evaporated to dryness and the residue was extracted with 1 ml of methanol. Removal of the methanol left a residue which was taken up in 5 ml of 0.01 M phosphate buffer, pH 6.1, and tested in the IAAO assay. At a level representing 80 mg of tissue per 10 ml reaction mixture, this solution produced 100 % inhibition of IAA destruction during 60 minutes incubation.

From a similar extraction a 5 μl aliquot of methanol extract was spotted on Whatman No. 1 paper and chromatographed in butanol: 27 % acetic acid (1:1). Spraying the chromatogram with diazotized sulfanilic acid (DSA) reagent (1), then 10 % sodium carbonate, revealed major amounts of two phenolic compounds with R_f's of 0.22 and 0.58. These two areas from a similar chromatogram were eluted with 0.01 M phosphate buffer. IAAO assay of these eluates indicated that the R_f 0.58 fraction was an IAAO inhibitor. On other chromatograms the R_f 0.58 spot appeared dull brown under UV light alone but fluoresced yellow-green under UV light in the presence of ammonia vapors. This spot also gave an olive color with ferric chloride and formed a yellow lake with aluminum chloride, which fluoresced yellow green under UV light. The R_f in ethyl acetate was 0.02; in 15 % acetic acid 0.68 and in 20 % KCl 0.14. These combined properties suggested that this inhibitor might be a flavonol-3-glycoside. However, flavonol glycosides normally have a UV absorption maximum in the 340 to 360 mμ region and this material exhibited maxima only at 317 and 268 mμ.

To obtain sufficient material for further study, 95.5 g of frozen red light-treated terminal buds were ground in a chilled mortar and the resulting powder was stirred 1 hour in 500 ml ethanol, then filtered. Evaporation of the filtrate yielded 4.4 g of oily yellow residue. This was chromatographed on a 1.5 × 45 cm Whatman ashless cellulose powder column using propanol: acetic acid: water (20:1:10). The eluate was cut into several 10 to 15 ml fractions and these were examined by paper chromatography. Fractions containing the inhibitor were pooled, concentrated, and chromatographed a second time on cellulose. The resulting inhibitor-containing fractions were pooled and taken to dryness yielding 1.23 g gummy yellow residue.

This residue was further purified on an LKB pressurized paper chromatographic column, size 4, using isopropanol: water (4:1). The eluate was collected in 14 to 15 ml fractions by use of an automatic fraction collector set for a 15-minute collection period. Optical density measurements showed concentration of the 317 mμ absorbing material in tubes 65 to 88. The contents of these tubes were combined and the solvent evaporated yielding 254 mg of tan residue. On paper chromatograms this residue yielded a single well-defined light yellow spot at R_f 0.69 in butanol: acetic acid: water (4:1:5). In water saturated with butanol, the yellow spot appeared at R_f 0.59 but an additional spot which fluoresced blue in the presence of ammonia and UV light appeared at R_f 0.38.

The 254 mg of tan residue was further chromatographed on the LKB pressurized paper chromatographic column in water saturated with butanol. A sharp peak of the 317 mμ-absorbing component occurred in fractions 51 to 59. These were pooled and evaporated to dryness in vacuo, using a Rinco evaporator, yielding 78 mg of glassy tan solid residue. This material appeared chromatographically pure in several solvent systems. Analysis showed 52.42 %

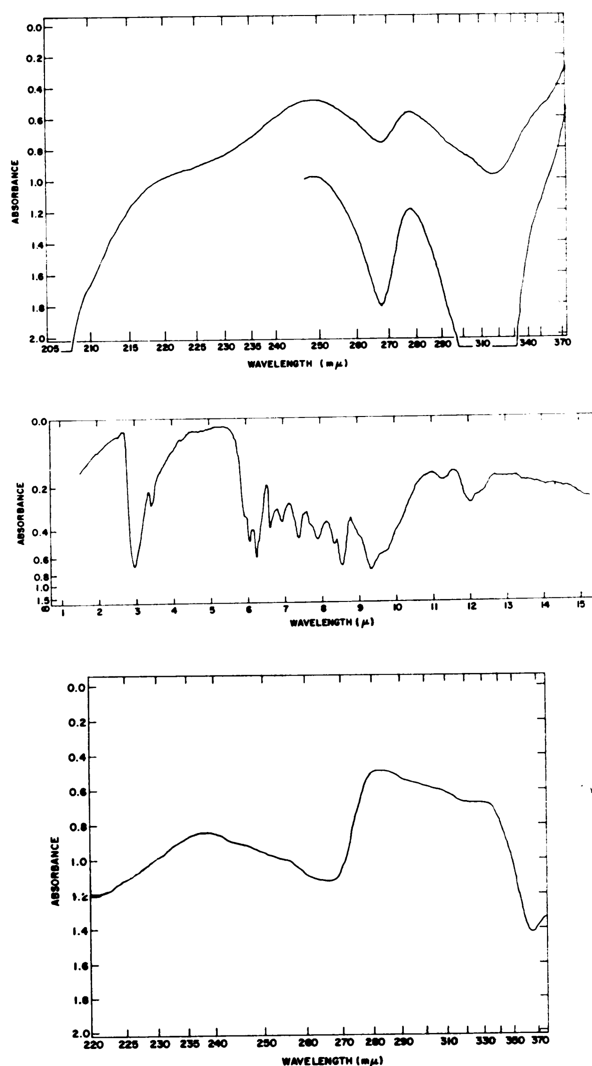

FIG. 1 (*top*). UV absorption spectrum of IAA oxidase inhibitor (25 ppm in ethanol).

FIG. 2 (*center*). IR absorption spectrum of IAA oxidase inhibitor (KBr pellet).

FIG. 3 (*bottom*). UV absorption spectrum of flavonol liberated by acid hydrolysis of inhibitor (15 ppm in ethanol).

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TABLE I
EFFECT OF FLAVANOIDS ON IAA OXIDASE

FLAVANOID	(ppm)	DCP (m/l)	% IAA DESTROYED AFTER 60 min
...	...	...	26
...	...	5×10^{-5}	56
Natural Inhibitor	400	"	2
"	200	"	62
Kaempferol	15	"	0
"	6	"	51
Kaempferide	300	"	6
"	150	"	28
Rhamnocitrin	1500	"	0
"	300	"	40

carbon, 5.12 % hydrogen, and no nitrogen. The UV absorption spectrum (fig 1) showed λ-max at 317 mμ ($E^{1\%}$ 1 cm = 3.88×10^2) and at 268 mμ ($E^{1\%}$ 1 cm = 3.04×10^2). The infrared absorption spectrum (fig 2) showed a band characteristic of OH at 2.94 μ, saturated CH absorption at 3.4 and 3.48 μ, possibly α,β- unsaturated carbonyl absorption at 5.9 μ, aromatic-CH = CH- in the 6 μ region, and a broad band at 9.3 μ, possibly due to a -CO- group. This substance at 400 ppm completely inhibited IAA destruction in the standard enzyme assay (table I).

HYDROLYSIS OF INHIBITOR: The purified inhibitor, 16.1 mg, and 6 ml 1 % H_2SO_4 were heated on a steam bath for 1.5 hours. During the heating a dark yellow colored flocculent precipitate separated from the solution. The suspension was chilled in ice and the precipitate collected by centrifugation. After washing with water and drying in vacuo 2.3 mg of solid were obtained, mp 228 to 262 C. Recrystallization from a water-ethanol solution gave a light yellow colored product which turned brown and softened at 255 C, then melted with decomposition at 268 to 272 C.

TABLE II
COMPARISON OF R_f VALUES OF INHIBITOR HYDROLYSIS PRODUCT WITH THOSE OF KAEMPFEROL

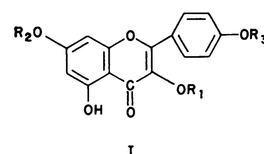
	FLAVONOL FROM PEA TIPS	KAEMPFEROL
Propanol : NH_4OH (7 : 3)	0.11	0.12
m-Cresol : acetic acid : H_2O (50 : 2 : 48)	0.58	0.55
Phenol : H_2O (80 : 20)	0.70	0.67
(88 : 12)	0.77	0.77
Butanol : acetic acid : H_2O (4 : 1 : 5)	0.90	0.91
15 % Acetic acid	0.07	0.06
2,6-Lutidine	0.97	0.97
Petroleum ether : benzene : ethyl acetate (45 : 45 : 20)	0.78	0.81

The UV absorption spectrum of this material (fig 3) was similar to that reported for kaempferol (7), λmax at 367 mμ ($\epsilon = 2.6 \times 10^4$) and 266 mμ ($\epsilon = 2.11 \times 10^4$). In table II are compared the R_f values of the isolated material in nine different solvent systems to those of authentic kaempferol (from Mann Research Laboratories, N. Y.).

In another run the acid hydrolysate was extracted with ethyl acetate before further workup. The ethyl acetate in turn was extracted with 15 % sodium bicarbonate solution. The bicarbonate extract was acidified with concentrated HCl, extracted with ethyl acetate, then the ethyl acetate removed in vacuo. By UV absorption spectra, paper chromatography, and cofactorial behavior in the IAAO assay it was demonstrated that the residue contained *p*-hydroxycinnamic acid. The 317 mμ λ-max of the inhibitor (fig 1) is probably due in part to the strong absorption at 310 mμ by the *p*-hydroxycinnamoyl group.

Paper chromatographic analysis of the aqueous portion of the acid hydrolysate, after extraction with ethyl acetate, indicated the presence of a single sugar. A phenylosazone of this sugar was prepared (8) which, after three recrystallizations from ethanol, melted at 203 to 204 C. As the melting point of this material was not depressed by an authentic sample of the phenylosazone of glucose, it is concluded that the only sugar released on acid hydrolysis of the IAAO inhibitor is glucose.

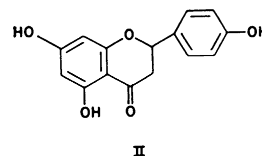
INHIBITORS: Inhibition of the IAAO system by the natural inhibitor, by kaempferol I ($R_1=R_2=R_3=H$), by kaempferide I ($R_1=R_2=H$; $R_3=CH_3$), and by rhamnocitrin I ($R_1=R_3=H$; $R_2=CH_3$) are compared in table I.



I

On a weight basis kaempferol is at least 30 times as effective as is the natural inhibitor. Kaempferol is also about 20 times as active as kaempferide and at least 100 times as active as rhamnocitrin.

FLAVANOID COFACTORS: The flavanone, naringenin (II), and flavone derivative, apigenin-7-glucoside (III), failed to inhibit IAAO, thus denying the possibility that inhibition of IAAO by flavanoids might be due to effects of meta hydroxyls at five and seven positions. Furthermore, II and III were moderately good cofactors for bean root IAAO.



II

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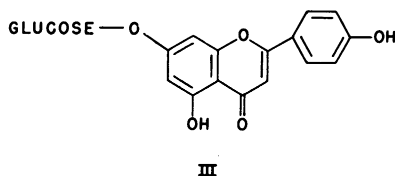


The relative effectivenss of DCP, naringenin, and apigenin-7-glucoside as cofactors for IAA destruction by bean root IAAO is shown in figure 4. The initial lag with apigenin-7-glucoside may mean that a small percentage of inhibitor was present, as the compound was not purified before use. Naringenin also failed to show any cofactor activity before two recrystallizations from aqueous ethanol.

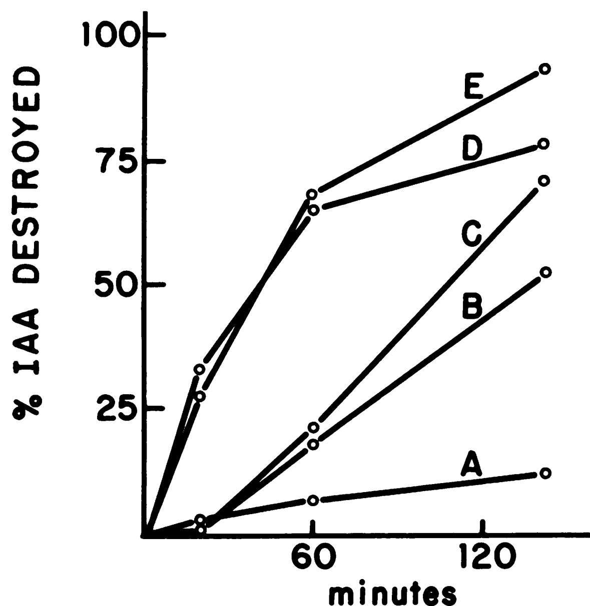
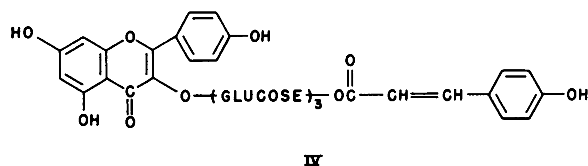


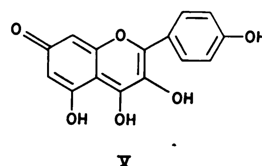
FIG. 4 (*lower right*). Effect of cofactors on IAA oxidase from bean roots. A = enzyme, 2×10^{-4} M IAA, 1×10^{-4} M manganous chloride. B = A + 1×10^{-4} M apigenin-7-glucoside. C = A + 2×10^{-5} M naringenin. D = A + 1×10^{-4} M naringenin. E = A + 5×10^{-5} M 2,4-dichlorophenol.

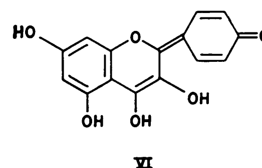
Discussion

An IAA oxidase inhibitor has been isolated from the terminal buds of etiolated pea plants exposed to red light, which on hydrolysis yields kaempferol, glucose, and *p*-hydroxycinnamic acid. Structure studies, which will be reported in detail later, have shown that the kaempferol : glucose : *p*-hydroxycinnamic acid ratio in the inhibitor is 1 : 3 : 1 and have indicated that the compound is structure IV.



Kaempferol, itself, is a considerably better IAA oxidase inhibitor than the kaempferol derivative isolated from the tips of pea plants. This is reasonable since the naturally occurring inhibitor appears conjugated with *p*-hydroxycinnamic acid, which is known to be an excellent IAA oxidase cofactor (3). Rhamnocitrin which has the 7-hydroxyl methylated, and kaempferide, which has the 4'-hydroxyl methylated, are much less active inhibitors than kaempferol. This suggests that kaempferol may be inhibiting the IAA oxidase system through its tautomeric structures, V and VI, which cannot exist, respectively, when the 7- and 4'-hydroxyls are methylated. Structures V and VI bear similarity to catechol and caffeic acid, excellent IAA oxidase inhibitors (3).





Naringenin, II, and apigenin-7-glucoside, III, are cofactors for the IAAO system. Since it has been established that naringenin, apigenin, and kaempferol exist in the terminal buds of at least one plant species (5), it is tempting to speculate that one way in which the red-far red photoreaction affects plant growth is through control of IAA oxidase cofactor-inhibitor balance. Thus activation of the red-far red pigment by red light may lead to the triggering of the oxidative enzyme systems which convert IAAO cofactors such as naringenin, apigenin, or related compounds to the kaempferol derivatives that serve as IAAO inhibitors. It must be recognized, of course, that operation of IAAO in vivo, is not yet proved, though work of Goldacre (2) with pea stem sections suggests it.

Summary

I. An inhibitor of IAA oxidase has been isolated from the buds of etiolated pea plants which were exposed to red light prior to harvest. This inhibitor is more abundant in red light treated buds than it is in completely dark grown controls. Structure studies indicate that the compound is a 3- (*p*-hydroxycin-

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namoyltriglucosyl) derivative of kaempferol. In a standard assay this flavonol derivative inhibits IAA oxidase 100 % at 400 ppm.

II. A new class of compounds, 3,7-dihydroxyflavones, inhibitory for IAA oxidase of bean roots and etiolated pea buds has been discovered. Kaempferol, the most active member of this class yet tested, shows 100 % inhibition at 5×10^{-6} M in the standard assay.

III. Two flavonoid cofactors for IAA oxidase, naringenin and apigenin-7-glucoside, have been found.

IV. It is proposed that one possible mechanism for the effects of red light on plant growth is through control of IAA oxidase cofactor-inhibitor balance.

Acknowledgments

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