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The Effect of Indole-3-Acetic Acid on Ethylene Formation in Wheat Seedlings

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Abstract. Isoperoxidase B 1 isolated from winter wheat (*Triticum aestivum* L., cv. Jubilar) seedlings was shown to catalyze ethylene formation from α-keto, γ-methylmercaptobutyric acid (KMBA). In the presence of Mn^{2+}, indole-3-acetic acid (IAA), and *p*-coumaric acid, the kinetics by isoperoxidase B 1 catalyzed conversion of KMBA into ethylene and other products was similar to that of IAA oxidation. The reaction rate was therefore controlled by IAA through its electron-donating properties.

Exogenous IAA induced ethylene formation in the segments of etiolated wheat coleoptiles. IAA-induced ethylene production was enhanced by L-methionine and mitomycin C. Aminoethoxy-analogue of rhizobitoxine, ferulic acid, sodium benzoate, cycloheximide and actinomycin D exhibited significant inhibitory effects. These data indicate that the overall reaction mechanism in coleoptile segments involves RNA and protein synthesis.

The site of IAA action is not specific; 2,4-dichlorophenoxyacetic, α-naphthylacetic and indole-3-butyric acids, respectively, possessed comparable inductive effect as IAA. Indole-3-propionic acid, indole, L-tryptophan and glucobrassicin had only low inductive efficiency, and moreover indole and L-tryptophan slowed down IAA-induced ethylene formation,

Although the effect of IAA on ethylene formation is well known and has been described in several plant organs, *e.g.* bean hypocotyls (SAKAI and IMASEKI 1971), pea epicotyls (KANG *et al.* 1971), pea roots (STEEN and CHADWICK 1973) and sorghum mesocotyls (FRANKLIN and MORGAN 1978), its mode of action is still not quite clear. In most of the above tissues, the inductive effect of IAA on ethylene biosynthesis has been found. Comparable IAA effect was not found in ageing tissues, *e.g.* in apples (LIEBERMAN and KUNISHI 1975).

L-methionine is the precursor of ethylene in plant cells and two enzymes are believed to catalyze the overall reaction: transaminase and peroxidase (YANG 1975). From our previous results (MACHÁČKOVÁ *et al.* 1975, ZMRHAL and MACHÁČKOVÁ 1978) we know that IAA serves as an electron donor in some oxidase reactions catalyzed by wheat isoperoxidase B 1. The purpose of the present study was:

1. to determine whether isoperoxidase B 1 is able to catalyze the production of ethylene *in vitro*;
2. to test the influence of IAA and related compounds on ethylene formation *in vivo*.

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MATERIAL AND METHODS

Isolation and Characterization of Isoperoxidase B 1

The following steps were involved in the isolation procedure: homogenization of the upper parts of wheat seedlings, centrifugation, ammonium sulphate precipitation, column chromatography on Sephadex G 25, Sephadex G 100 and DEAE-cellulose, followed by freeze-drying of isoenzyme B 1 fraction. Pure isoperoxidase B 1 was chemically characterized (amino acids, sugars).

Details of the isolation procedure as well as those of chemical composition of the isoenzyme molecule were published elsewhere (Zmrhal and Macháčková 1978).

Preparation of KMBA

α-keto, γ-methylmercaptobutyric acid (KMBA) was used as substrate for isoperoxidase B 1.

The mixture of 0.5 M L-met, 0.05 M KCl, catalase (1 μg ml^{-1}) and L-amino acid oxidase (100 μg ml^{-1}) in 0.1 M Tris—HCl buffer pH 7.8 was incubated for 1 h at 37 °C. Reaction was terminated by the addition of solid m-phosphoric acid to provide pH $\sim$ 3. The product was extracted three times with diethyl ether, ether evaporated in vacuum (Mapson *et al.* 1969) and the product identified by the melting point of its 2,4-dinitrophenylhydrazone (149 °C).

Ethylene Formation from KMBA

Ethylene formation was assayed in stoppered 10 ml vials containing air at 10^5 Pa pressure and 3 ml of the following reaction mixture: 0.2 mM IAA, 1 mM Mn^{2+} ($MnCl_2$), 0.05 mM HCA, 50 μg KMBA and 50 μg isoperoxidase B 1 in 0.05 M potassium phosphate buffer pH 5.6. The reaction was initiated by the addition of enzyme to the reaction mixture. The incubation period was 30 min at 30 °C on a Dubnoff incubator. The reaction was terminated by adding TCA to the concentration of 6%. Samples of the gas were withdrawn using 1 ml disposable plastic syringes (Becton Dickinson Co. Ltd.). Ethylene was determined by gas chromatography of the samples on Perkin-Elmer F 30 chromatograph using Porapak N column and nitrogen as the carrier.

Ethylene Formation in Wheat Coleoptile Segments

Surface sterilized (70% ethanol, 5 min) and imbibed (6 h in redistilled water) seeds of winter wheat (cv. Jubilar) were placed on the wet filter paper discs in Petri dishes and allowed to germinate 4 days in the darkness at 25 °C. Coleoptiles 30—35 mm in length were selected and segments 10 mm in length were cut of them starting 3 mm below the apex. The first leaves

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Abbreviations used: IAA = indole-3-acetic acid; KMBA = α-keto, γ-methylmercaptobutyric acid; HCA = 4-hydroxycinnamic(p-coumaric) acid; FA = 3-methoxy, 4-hydroxycinnamic (ferulic) acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = α-naphthylacetic acid; IBA = indole-3-butyric acid.

were carefully removed (Anderson *et al.* 1975) and the segments were preincubated 2 h in redistilled water. Then 15—20 segments were transferred into 10 ml glass vials containing 2 ml of 0.05 M potassium phosphate buffer pH 6.0 with the substances under the test (see Tables 1 and 2). The vials were stoppered immediately and incubated at 20 °C. At desired time intervals, the samples of gas phase were withdrawn and the same volume of air was injected back into the vials to maintain original pressure. Ethylene content in the gas samples was determined as described above.

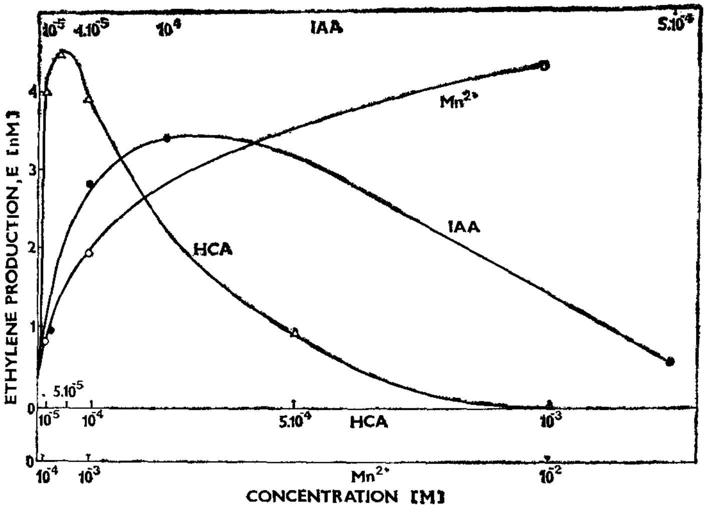

Fig. 1. The dependence of ethylene production from KMBA on IAA (A), HCA (B) and Mn^{2+} (C) concentrations. The reaction mixture of total volume 3 ml contained — A: 50 μM HCA, 1 mM Mn^{2+}, 50 μM KMBA and 50 μg isozyme B 1 in 0.05 M potassium phosphate buffer pH 5.6. — B: 0.4 mM IAA, 1 mM Mn^{2+}, 50 μM KMBA and 50 μg isozyme B 1 in 0.05 M potassium phosphate buffer pH 5.6. — C: 0.4 mM IAA, 50 μM HCA, 50 μM KMBA and 50 μg isozyme B 1 in 0.05 M potassium phosphate buffer pH 5.6. Incubation proceeded in all cases 30 min at 30 °C.

RESULTS

Ethylene Formation from KMBA

In the presence of Mn^{2+} ions, a phenolic cofactor and an electron donor isoperoxidase B 1 catalyzed the turnover of KMBA to ethylene and other products. HCA was the most effective phenolic cofactor. The dependence of ethylene formation on the concentrations of respective reactants is shown in Fig. 1.

IAA and HSO_3^- served as electron donors. HSO_3^- ions were three times more effective than IAA, but we found that these anions decomposed KMBA also nonenzymatically.

Exogenous H_2O_2 eliminated the necessity of Mn^{2+} and IAA. Addition of 10^{-5} M FA to the reaction mixture introduced a lag period (5—10 min) prior to the onset of ethylene formation. Using 10^{-4} M FA, no ethylene was detected for 60 min. H_2O_2 eliminated the FA action.

pH optimum of ethylene formation was 5.6.

Ethylene Formation in Wheat Coleoptile Segments

Coleoptile segments produced no ethylene when incubated in buffer, either with or without 10^{-3} M and 10^{-4} M L-met. But IAA was able to induce ethylene formation. The time-course of ethylene formation for various IAA concentrations is given in Fig. 2. pH optimum for IAA-induced ethylene formation was 5.5—6.0.

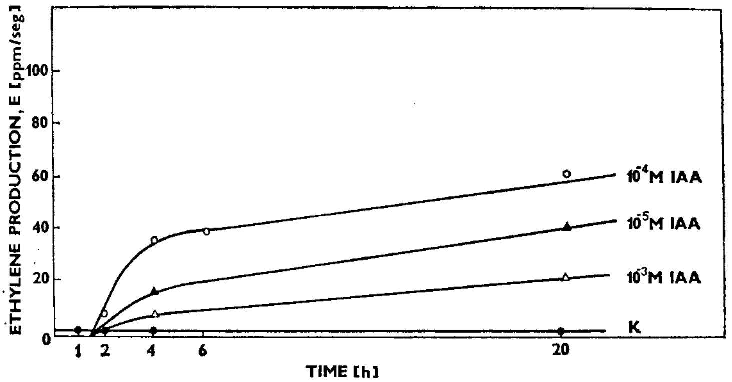

Fig. 2. Time-course of ethylene production by wheat coleoptile segments in the presence of various IAA concentrations. Incubation proceeded in 2 ml of IAA solutions in 0.05 M potassium phosphate buffer pH 6.0 for 20 h at 30 °C.

The influence of tested compounds on IAA-induced ethylene formation is summarized in Table 1 and their ability to induce ethylene formation in Table 2.

The synergic action of mitomycin C and IAA was totally inhibited by cycloheximide (10 μg ml^{-1}) and rhizobitoxine analogue (5 μg ml^{-1}). Also 2,4-D, NAA and IBA-induced ethylene formation was inhibited by the mentioned compounds.

Because KMBA had no influence on IAA-induced ethylene formation and also had no inductive effect, we think that it was metabolized in another way than *in vitro*. On gas chromatogram we found two peaks different from ethylene (not identified) and the incubation mixture turned red during incubation with KMBA.

Permanent IAA presence was not necessary for induction of ethylene formation. Incubation of segments in IAA solution for 15 min was sufficient to induce ethylene formation. The amount of ethylene formed was proportional to the duration of incubation in IAA solution till 2 h. After 2 h preincubation of segments in IAA solution the ethylene production was the same as in the case of continual IAA presence.

The attempts to demonstrate ethylene formation in wheat coleoptile homogenates did not meet with success despite the conditions resembling those used for intact segments. Further details, therefore, are not given here.

DISCUSSION

In our previous study (Zmrhal and Macháčková 1978) we reported that purified wheat isoperoxidase B 1 catalyzes oxidation of a number of phenolic compounds and also of IAA. In all oxidase reactions studied, the requirements

TABLE 1

The effect of tested compounds on IAA—induced ethylene formation in wheat coleoptile segments. IAA concentration used was 0.1 mM and its action taken as 100 %. Tests were performed in 0.05 M potassium phosphate buffer pH 6.0

Compound	Functional characteristic	Concentration	Effect (in % of IAA action)	
			after 4 h of incubation	after 20 h of incubation
L-methionine	Substrate for ethylene formation	0.1 mM	140	140
Aminoethoxy-analogue of rhizobitoxine	Inhibitor of L-methionine transamination	5 $\mu g\ ml^{-1}$	0	0
		10 $\mu g\ ml^{-1}$	0	0
		50 $\mu g\ ml^{-1}$	0	0
Sodium benzoate	Free radical trap	1 mM	60	83
Ferulic acid	*o*-diphenol	1 mM	36	60
Cycloheximide	Proteosynthesis inhibitor	1 $\mu g\ ml^{-1}$	75	75
		5 $\mu g\ ml^{-1}$	60	60
		10 $\mu g\ ml^{-1}$	0	0
Mitomycin C	DNA replication inhibitor	5 $\mu g\ ml^{-1}$	180	180
Actinomycin D	DNA-dependent RNA formation inhibitor	2.5 $\mu g\ ml^{-1}$	78	88
		10 $\mu g\ ml^{-1}$	50	56
α-amanitin	RNA-polymerase II inhibitor	1 $\mu g\ ml^{-1}$	100	80
		2 $\mu g\ ml^{-1}$	100	70
		5 $\mu g\ ml^{-1}$	100	70
Kinetin	Plant hormone	1 μM	110	110
		10 μM	110	110
Gibberellic acid GA_3	Plant hormone	50 μM	100	100
Indole	Basic nucleus of IAA	0.1 mM	52	57
L-tryptophan	IAA precursor	0.1 mM	22	28
Sucrose	Supply of energy	2 %	100	100
ATP	Supply of energy	0.1 mM	100	100
		1 mM	100	100

for isozyme B 1 activity were Mn^{2+} ions, a phenolic cofactor and an electron donor. Results of the present work indicate that *in vitro* isoperoxidase B 1 is also able to catalyze formation of ethylene from KMBA, which is the transamination product of L-methionine. Enzymic conversion of KMBA into ethylene (and other products) was demonstrated in the reaction mixture containing Mn^{2+} ions, IAA and HCA. The necessity of the - two former compounds could be eliminated by exogenous H_2O_2. This sugd gests that Mn^{2+} and IAA are involved in isoperoxidase B 1 — catalyze

TABLE 2

The ability of tested compounds to induce ethylene formation in wheat coleoptile segments. The effect of 0.1 mM IAA is taken as 100 %. If not specified, 0.05 M potassium phosphate buffer pH 6.0 was used

Compound	Concentration	Effect (in % of IAA action)	
		after 4 h of incubation	after 20 h of incubation
Mitomycin C	5 μg ml^{-1}	0	0
2,4-dichlorophenoxyacetic acid	0.1 mM	100	100
α-naphthylacetic acid	0.1 mM	100	100
Indole-3-butyric acid	0.1 mM	100	100
Indole-3-propionic acid	0.1 mM	10	10
Indole	0.1 mM	< 5	< 5
L-tryptophan	0.1 mM	< 5	< 5
Indole-3-acetyl-L-asparagic acid	0.1 mM	50	36
Acetate buffer, pH 4.0	0.05 M	0	0
Glucobrassicin*	1 mM	90	90

* The sample of glucobrassicin was the kind gift of Dr. M. Kutáček, DrSc. from the Institute of Experimental Botany of the Czechoslovak Academy of Sciences.

formation of enzyme — H_2O_2 intermediary complex, which is a prerequisite need for subsequent KMBA oxidation. IAA serves probably as an electron donor and reaction rate depends on its concentration. The kinetics of isoperoxidase B 1 — catalyzed ethylene formation from KMBA is very similar to that of IAA oxidation reported previously (ZMRHAL and MACHÁČKOVÁ 1978). IAA could be replaced by HSO_3^- ions, which were an even more effective electron donor than auxin. It is difficult, however, to interprete these results, as hydrosulfite ions decompose KMBA also non-enzymatically. Ferulic acid caused a lag period prior to ethylene formation. This reflects lower isoperoxidase B 1 affinity to KMBA than to preferentially oxidized FA.

We have extended these results to find if IAA could regulate ethylene formation *in vivo* by the same way as *in vitro*, *i.e.* through its electron donator function. Segments of etiolated wheat coleoptiles produced no measurable amounts of ethylene by themselves, but they were able to do it at the presence of IAA. IAA effect, however, was rather of an inductory nature and could not be simply explained through regulation of peroxidase reaction rate. Thus, even if our tentative assumption was correct, we would not have been able to confirm it because of the simultaneous strong inductory effect of IAA.

IAA-binding site is probably of low specifity; coleoptile segments produced comparable amounts of ethylene under the influence of both IAA and synthetic auxins 2,4-D, NAA and IBA, respectivelly. Also some other indole derivatives were able to induce ethylene formation, but their efficiency was very low. Moreover L-try and indole slowed down IAA-induced ethylene formation. Such interactions could be interpreted as a competition for binding site between related compounds. Similar effects have been reported for N,N-dimethyltryptophan (ANDERSON *et al.* 1975).

We are convinced that peroxidase participates in ethylene formation in wheat coleoptiles; the reasons for it are the following: Both FA (o-diphenol) and sodium benzoate (free radical trap) significantly inhibited IAA-induced ethylene formation. Moreover, the stimulatory effect of L-met and, especially, the strong inhibitory effect of rhizobitoxine analogue, which is the potent inhibitor of L-met transamination (OWENS *et al.* 1971) suggest that ethylene is produced *via* L-met———KMBA———C_2H_4 route also in wheat coleoptiles. The failure to demonstrate *in vivo* conversion of KMBA to ethylene might reflect either inability to transport this substrate or inability to convert it into high-energy metabolite (ethylene formation in apples starts with S-adenosylmethionine — YANG 1975).

From recent literature we have learned about IAA effects on protonic pump (MENTZE *et al.* 1977). Wheat coleoptile segments, however, produced no ethylene in as acidic a medium as that of pH 4.0. On the other hand, we observed clear and strong inhibitory effects of cycloheximide and actinomycin D. These results suggest implication of both proteosynthesis and nucleic acid metabolism in IAA-induced ethylene formation.

The inhibitory effect of α-amanitin was too low to allow us to decide if RNA polymerase II was also involved. Finally, the surprising synergic action of mitomycin C and IAA might, possibly, be explained by the presence of indolic nucleus in mitomycin C molecule.

Based on the recent work of MATTOO and LIEBERMAN (1977) there is a reason to believe that enzymatic system: transaminase and peroxidase, which is responsible for ethylene formation in plant cells, is bound to the pectic moiety of the primary cell wall. Our previous results agree with this finding. Wheat isoperoxidase B 1 is believed to be localized in the primary cell wall (ZMRHAL and MACHÁČKOVÁ 1978).

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Note in proof. During processing of this manuscript 1-aminocyclopropane-1-carboxylic acid (ACC) was identified as an intermediate of ethylene biosynthesis (ADAMS, D. O., YANG, S. F.: Plant Physiol. **63** : 285—294, 1979 and LÜRSSEN, K., NAUMANN, K., SCHRÖDER, R.: Z. Pflanzenphysiol. **92** : 285—294, 1979). This might be the cause of incapability of wheat coleoptile segments to metabolize the α-keto-γ-methylmercaptobutyric acid (KMBA) as described above.

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