Ethylene Formation in Sugar Beet Leaves

EVIDENCE FOR THE INVOLVEMENT OF 3-HYDROXYTYRAMINE AND PHENOLOXIDASE AFTER WOUNDING

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ABSTRACT

Ethylene production by sugar beet (Beta vulgaris L.) leaf discs is inhibited by white (or red, >610 nm) light or by wounding. In contrast, in wounded leaf discs, ethylene production is stimulated by light. The effect of light on wounded leaf discs has been studied by using an in vitro system which mimics the loss of compartmentation in the wounded leaf. Chlorophyll-free extracts from sugar beet leaves stimulate the production of the superoxide free radical ion (as a prerequisite for ethylene formation) by illuminated chloroplast lamellae. The substance from the crude leaf extracts which is active in stimulating the production of the superoxide free radical ion has been identified as 3-hydroxytyramine (dopamine). Exogenous dopamine between 5 μM and 100 μM stimulates ethylene formation by illuminated chloroplast lamellae from methional. It also stimulates the production of the superoxide free radical ion, the formation of which apparently involves both a lamellar phenoxydase and photosynthetic electron transport as a 1-electron donor, and is cyanide-sensitive.

MATERIALS AND METHODS

Sugar beet leaves (Beta vulgaris L.) from glasshouse cultures were gifts from the Kleinwanzlebener Saatzucht AG Einbeck (FRG). Leaf discs of approximately 2.3 cm in diameter were obtained by cutting leaves (about 10 cm long) with a cylindrical, sharpened iron tube. Wounding was performed by gently pressing the leaf discs with a plastic syringe piston on a plastic board, without disrupting the epidermal layer.

Crude extracts were prepared by homogenizing 50 g deveined leaves in 30 ml mm Tricine-NaOH buffer (pH 8) in a Sorvall Omni-Mixer at full speed for 1 min. The homogenate was pressed through a nylon net, and the green suspension was centrifuged for 15 min at 20,000g. The supernatant fraction contained less than 0.05 mg Chl/ml.

Superoxide dismutase was prepared by a modification of the procedure of Sawada et al. (29) as previously described (13). The activity units of superoxide dismutase were determined as described by McCord and Fridovich (23). Ethylene formation was monitored as previously described (10–12).

3-Hydroxytyramine was isolated from sugar beet leaves according to the procedure of Gardner et al. (16). The aqueous phase from sugar beet leaves (see ref. 16) was evaporated to dryness and dissolved in methanol containing 0.25% concentrated HCl. The filtered solution was loaded onto a cellulose column (prepared by stirring 15 g cellulose [Avicel, Merck-Darmstadt] in a 150-ml butane-2-formic acid-acetone-water mixture, 40:1:2.6, v/v). The column was washed with increasing amounts of the same solution. The fractions containing 3-hydroxytyramine were monitored by UV spectrophotometry (λmax = 279 nm) and pooled.

Methional was synthesized as described (11). Chloroplast lamellae were prepared from spinach (27) or from sugar beet leaves (6, 9). Hydroxylamine oxidation, a test system for the production of O2−, and the decarboxylation [1-14C]glyoxylate, a test for the production of H2O2, were measured as described earlier (7, 14). Phenoloxidase (α-diphenol-oxygen oxidoreductase) activity was measured manometrically by following the uptake of O2 in the presence of the indicated substrates in Warburg vessels at 15 C.

Trypsin treatment was performed essentially as previously described (30) in 20 mm tris-HCl (pH 7.6) buffer, 10 mm KCl, and 5 mm MgCl2. Twelve ml of a chloroplast suspension (200 μg Chl/ml) were preincubated at 25 C for 3 min and the digestion was started by the addition of 0.12 ml trypsin solution (1 mg/ml). At successive intervals, 1-ml aliquots were removed and added to 1 ml of a solution containing trypsin inhibitor (400 μg/ml) and BSA (10 mg/ml) and immediately cooled to 0 to 4 C. Photosynthetic DCPIP reduction was measured spectrophotome-

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2 Abbreviation: DCPIP: dichlorophenol indophenol.

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metrical at 590 nm in a Zeiss spectrophotometer fitted for side illumination. Reaction mixtures contained, in 3 ml: chloroplasts equivalent to 10 μg Chl/ml, 20 μM DCPIP, 5 mM NH₄Cl, 20 mM tris-HCl (pH 7.6), 10 mM KCl, and 5 mM MgCl₂. The reactions were measured at room temperature and an extinction coefficient for DCPIP of 19.8 mm⁻¹ cm⁻¹ was assumed.

[1-¹⁴C]Sodium glyoxylate was purchased from Amersham-Buchler; trypsin and trypsin inhibitor were from Boehringer Mannheim.

RESULTS

Two basic mechanisms have been attributed to the degradation of Chl and other significant signs of senescence of green plant tissue: (a) chemical destruction of Chl and membrane lipids by peroxides and/or oxygen radicals (32, 33); (b) induction and/or activation of degradative enzymes by ethylene (1). In both mechanisms, an oxygen-activating system is apparently the basic requirement for the production of the active inducing principle, e.g. peroxides, oxygen radicals, and ethylene. We have investigated the induction of endogenous systems of oxygen reduction and ethylene formation, which could be responsible for the observed effect (senescence and/or resistance) under physiological conditions.

Ethylene Formation in Sugar Beet Leaf Discs. Discs of about 200 mg of young sugar beet leaves, fresh weight, floating for 2 hr on the surface of water (20 C) in closed vessels, evolve about 15 to 30 nmol ethylene/g fresh weight-hr. This rate is reduced to approximately 5 nmol when the leaf discs are illuminated (20,000 lux). Wounding the leaf tissue leads to an inhibition of ethylene production in the dark (3 nmol ethylene/g hr⁻¹), however, illumination of wounded discs results in a stimulation of ethylene production (10 nmol ethylene/g hr⁻¹). Although the absolute rates of ethylene production were quite different in the individual experiments, the same trends have been observed in 11 separate experiments with discs from young sugar beet leaves.

Influence of Leaf Extracts on Oxygen Activation by Isolated Chloroplast Lamellae from Sugar Beet and Spinach Leaves. In order to study the mechanism of light-stimulated ethylene formation in the wounded leaf, in vitro experiments were performed. The following results describe conditions and cofactors of a light-dependent oxygen-activating system from sugar beet leaves, which is also active in ethylene production from methionine, a model substrate.

Experimental conditions were chosen in order to try to mimic the situation in vivo in a wounded leaf tissue. Isolated chloroplast lamellae were incubated in the light with Chl-free extracts after centrifugation of leaf homogenates. The incubation mixture contained 0.3 mM hydroxylamine, which, in the presence of the superoxide free radical ion, is converted to nitrite (14). This system is capable of testing for monovalent oxygen reduction according to the equation:

\[ \text{NH}_4\text{OH} + 2\text{O}_2^- + \text{H}^+ \rightarrow \text{NO}_2^- + \text{H}_2\text{O}_2 + \text{H}_2\text{O} \]  

Figure 1a shows that Chl-free extracts from sugar beet leaves stimulate the formation of nitrite from hydroxylamine in contrast to extracts from spinach which exhibit an inhibition. In order to characterize the nature of the active compound in the sugar beet leaf extract further, the decarboxylation of [1-¹⁴C]glyoxylate was measured. This system tests for the production of H₂O₂ (7), according to the equation:

\[ \text{H}_2\text{O}_2 + \text{R-COO}^- + \text{COOH} \rightarrow \text{RCOOH} + \text{¹⁴CO}_2 + \text{H}_2\text{O} \]  

Figure 1b shows that heating the Chl-free sugar beet leaf extract (5 min at 80 °C) increases the decarboxylating activity. This activity is completely inhibited by 30 mM ascorbate. The inhibitory effect of ascorbate on this reaction suggests that the active compound might be a substance similar to the oxygen-reducing factor previously isolated from spinach or sugar beet chloroplast lamellae, after heat treatment (9). In contrast to the oxygen-reducing factor, the active compound from sugar beet leaf extracts seems to be producing both O₂⁻ and H₂O₂.

Identification of the Compound in Sugar Beet Leaf Extracts That, Together with Illuminated Chloroplast Lamellae, Stimulates Oxygen Reduction. The results presented in Figure 1 made it very likely that a low molecular compound was responsible for the stimulation of monovalent oxygen reduction in the light. The crude extract was further fractionated. After heating, the extract was evaporated to dryness and further dried over P₂O₅ for 30 hr. The dry material was then extracted for 24 hr in the Soxhlet with methanol and a brown solution was obtained. This solution was evaporated to dryness, dissolved in a small volume of H₂O₂, and the pH was adjusted to 6 with acetic acid. The resulting brown solution was then loaded onto a polyamide column (Macherey-Nagel, polyamide SC 6 – AC). The column was eluted with water until the effluent was almost colorless. This effluent was designated as “H₂O₂-sol. extract”. After extraction with water, the polyamide column was eluted with methanol and the yellow fractions obtained were evaporated to dryness. All fractions were tested for the stimulation of glyoxylate decarboxylation. The main component of the methanol extracts from the column shows no activity and was identified as vitexin, according to Mabry et al. (21). The water-soluble extract, however, stimulates both the decarboxylation of glyoxylate (data not shown) and hydroxylamine oxidation by illuminated chloroplasts (Fig.
About 10 A units (1 unit of absorption representing an amount of H$_2$O-sol extract with an extinction of 1 at 260 nm in 1 ml) maximally stimulated nitrite formation from hydroxylamine; higher concentrations inhibited hydroxylamine oxidation. In the presence of 1 mM KCN, an inhibition by H$_2$O-sol extract of hydroxylamine oxidation was observed. Figure 2 also shows that dopamine has a similar effect on nitrite formation by illuminated chloroplasts both in terms of the inhibition seen at higher concentrations of dopamine, and the inhibition of nitrite formation in the presence of KCN.

The water-soluble extract was further purified according to the procedure described by Gardner et al. (16). Figure 3 compares the UV spectrum of the isolated product, capable of stimulating hydroxylamine oxidation, with dopamine. The spectra of both are essentially identical and have an absorption maximum at 279 nm (in H$_2$O).

The active substance and dopamine show identical Rf values on paper chromatograms (butanol-acetic acid-water, 4:1:5, v/v/v) and on paper electrophoresis. Both substances react with the formation of the identical red color upon addition of diazotized sulfanilic acid, and the UV spots of both compounds on paper chromatograms turn brown after a long exposure to air. Thus, the active component from sugar beet leaves is concluded to be identical to dopamine.

The product of the photochemical reaction catalyzed by dopamine is the superoxide-free radical ion. This is seen from the complete inhibition of nitrite formation from hydroxylamine by 20 units of superoxide dismutase (14, 23) (data not shown).

The same concentration dependencies exhibited by dopamine on hydroxylamine oxidation by illuminated chloroplasts are also seen for ethylene production from methionyl (or 2-keto-4-mercaptoacetamide) (Fig. 4).

**Mechanism of Production of O$_2^-$ by Isolated Chloroplasts in Presence of Dopamine.** Figure 5 shows the dependence of O$_2^-$ and H$_2$O$_2$ formation by isolated chloroplasts on the dopamine concentration. Nitrite formation is stimulated at low dopamine concentrations (about 10 $\mu$M) and inhibited at concentrations higher than 30 $\mu$M. In contrast, the decarboxylation of glyoxylate shows typical saturation-type kinetics with an apparent V$_{max}$ occurring at about 30 $\mu$M. The different kinetic for the production of O$_2^-$ and H$_2$O$_2$ suggests that they have different reaction mechanisms.

Different kinetic data for the formation of O$_2^-$ and H$_2$O$_2$ are again observed when caffeic acid and nitrite are added to the reaction system (data not shown). The formation of O$_2^-$ is not influenced at low concentrations up to 10 $\mu$M, but, as in the case of dopamine, is inhibited at concentrations higher than 30 $\mu$M. H$_2$O$_2$ formation, however, is stimulated by caffeic acid up to 60 $\mu$M without reaching saturation. Apparently, caffeic acid, in contrast to dopamine, only catalyzes the production of H$_2$O$_2$ by illuminated spinach chloroplasts.

When preparations of spinach or sugar beet leaf chloroplast lamellae are tested for phenoloxidase (o-diphenol-oxygen oxidoreductase) with either caffeic acid or dopamine as substrates, only dopamine significantly stimulates oxygen uptake in the
but only view that dase hydroxylamine from sin treatment (30).

These results, together with the fact that KCN, a phenoloxidase inhibitor, reverses the dopamine stimulation of hydroxylamine oxidation by illuminated chloroplast lamellae, support the view that phenoloxidase is involved in the production of O$_2^-$, but only if the substrate is present in a certain concentration range (about 10 μM).

**Trypsin Activation of Lamellar Bound Phenoloxidase.** Trypsin treatment of chloroplast lamellae results in an inhibition of photosynthetic electron transport measured as DCPIP photoreduction (30). After about a 3-min treatment (trypsin to Chl ratio, 0.05, w/w), approximately 50% of the electron transport activity is lost. The phenoloxidase activity is, however, increased about 3-fold. No further increase of phenoloxidase activity is observed after longer times of treatment, although the photosynthetic activity decreases to almost zero after 8 min.

The effect of trypsin treatment on the rates of hydroxylamine photooxidation in the presence of either methylviologen or dopamine is compared in Table 1. In a manner similar to trypsin-induced inhibition of DCPIP photoreduction, methylviologen-catalyzed hydroxylamine oxidation is reduced by approximately 50% after a 3-min trypsin treatment. Dopamine-catalyzed hydroxylamine photooxidation is, however, stimulated after trypsin treatment.

**DISCUSSION**

The function of o-diphenols as regulating agents in ethylene biosynthesis has been suggested by several workers and, in most cases, an inhibition of ethylene synthesis by o-diphenols has been observed (1, 19, 20, 24, 34). Fuchs (15), however, has reported that catechol stimulates ethylene formation in green citrus peel plugs in a reaction which seems to be sensitive to the o-diphenol oxidase inhibitor, 2,3-naphthalene diol (22). He concluded that a phenoloxidase, together with an o-diphenol, might be involved in ethylene biosynthesis. Our findings share common features with Fuchs' observations. Isolated chloroplasts show an enhanced production of O$_2^-$ upon addition of the o-diphenol, dopamine, in a reaction which is cyanide-sensitive. The cyanide sensitivity strongly suggests that a phenoloxidase, together with dopamine, is responsible for the activation of oxygen. Furthermore, activated oxygen appears to be one of the prerequisites for ethylene formation. Our system then, might be responsible for the production of the oxygen species involved in the cleavage of an appropriate ethylene precursor.

This in vitro system, comprising illuminated chloroplast lamellae and dopamine, is a model which might closely mimic the actual situation in sugar beet leaf cells after wounding without disruption of the epidermis. The wounded cells must contain disrupted chloroplasts as well as all of the substances released from the different compartments of the leaf cells. We assume that dopamine comes into contact with chloroplasts only after wounding, resulting in a new physiological reaction; instead of NADP, oxygen would be the only electron acceptor under these altered conditions.

Isolated chloroplast lamellae can apparently react with o-diphenols three different ways: (a) lamellar bound phenoloxidase oxidizes appropriate o-diphenols in a dark reaction, yielding brown melamine-like compounds and water (3, 31); (b) light-dependent reactions not involving lamellar bound phenoloxidase which use catalytic amounts of o-diphenols and yield H$_2$O$_2$; the o-diphenols need not be substrates for lamellar phenoloxidase; (c) light-dependent reactions involving lamellar bound phenoloxidase, which use catalytic amounts of o-diphenols, substrates for lamellar phenoloxidase according to reaction type a, yielding the superoxide free radical ion.

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**Fig. 5.** (a) Comparison of dopamine-catalyzed hydroxylamine oxidation (as test for O$_2^-$) with $[1^4]$glyoxylate decarboxylation (as test for H$_2$O$_2$) by isolated spinach chloroplast lamellae. (b) Comparison of hydroxylamine oxidation with glyoxylate decarboxylation by isolated spinach chloroplast lamellae in the presence of caffeic acid. The test systems were as described for Figure 1. Test for H$_2$O$_2$ (O--O): decarboxylation of $[1^4]$sodium glyoxylate. Test for O$_2^-$ (●●●): nitrite formation from hydroxylamine.

**Fig. 6.** Comparison of dopamine and caffeic acid as substrates for light-dependent hydroxylamine oxidation and lamellar phenoloxidase of isolated chloroplast lamellae from sugar beet leaves. For reaction conditions, see Figure 1.

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Table 1: Effect of Trypsin Treatment on Hydroxylamine Oxidation by Spinach Chloroplast Lamellae in the Presence of Either Methylviologen or Dopamine as Cofactors, as Compared to Dichlorophenol Indophenol Photoreduction and Phenoloxidase Activity

<table>
<thead>
<tr>
<th>Assay</th>
<th>Activity of Chloroplast Lamellae</th>
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<tr>
<td></td>
<td>Control</td>
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<td></td>
<td>μeq/mg Chl./h %</td>
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<tr>
<td>Hydroxylamine oxidation</td>
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<tr>
<td>with 600 μM methylviologen</td>
<td>33</td>
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<tr>
<td>with 300 μM dopamine</td>
<td>6</td>
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<tr>
<td>DCPIP reduction</td>
<td>188</td>
</tr>
<tr>
<td>Polyphenoloxidase with 5 μmol dopamine as substrate</td>
<td>120</td>
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</tbody>
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Fig. 7. Proposed mechanism for the involvement of phenoloxidase and dopamine in the light-dependent production of superoxide free radical ion by chloroplast lamellae.

In the following, we present mechanisms for the reactions of types b and c.

**Reaction Type c: Involvement of Phenoloxidase in Light-dependent Superoxide Formation by Chloroplast Lamellae.** The conclusions concerning the mechanism of this reaction are based on the following observations: (a) KCN, an inhibitor of phenoloxidase, reverses the stimulation of hydroxylamine oxidation by dopamine; (b) only substrates of lamellar phenoloxidase are also good catalysts for the stimulation of hydroxylamine oxidation; (c) activation of latent lamellar phenoloxidase also stimulates hydroxylamine oxidation in the presence of dopamine, a cofactor of oxygen reduction, but inhibits hydroxylamine oxidation in the presence of methylviologen, also a cofactor of oxygen reduction. Methylviologen is not a substrate of lamellar phenoloxidase.

These three observations lead to the conclusions summarized in Figure 7. Phenoloxidase and a 1-electron donor (the reducing site of photosystem I, cf. refs. 2 and 13) are a reactivating unit which forms an autoxidizable semiquinone (see also refs. 25 and 28). The product of the autoxidation of the semiquinone is the superoxide free radical ion, which can also yield H₂O₂ by dismutation. At higher concentrations of dopamine, the superoxide free radical reacts with the excess free dopamine, yielding O₂⁻ which is trapped by another molecule of o-diphenol. The reaction is inhibited by superoxide dismutase (8).

The difference between reactions b and c is the involvement of phenoloxidase in reaction c, while reaction b is a chemical, autocatalytic process. Reaction c represents a system similar to mixed function oxygenases, as outlined in equations 3 to 5:

\[ 2 \text{o-diphenol} + 3 \text{O}_2 + 2e^- \rightarrow 2 \text{o-quinone} + 2 \text{O}_2^- + 2 \text{H}_2\text{O} \quad (3) \]

\[ 2 \text{o-quinone} + 4e^- + 4 \text{H}^- \rightarrow 2 \text{o-diphenol} \quad (4) \]

This reaction might be involved in light-dependent hydroxylations as described by Bartlett et al. (4) and by Halliwel1 (17).

**Literature Cited**