Spontaneous Chemiluminescence of Soybean Embryonic Axes during Imbibition

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ABSTRACT
Isolated soybean (Glycine max L. var Hood) embryonic axes have a spontaneous chemiluminescence (about 150 counts per minute per embryo) that increases showing two phases, upon water imbibition. The first photoemission burst was measured between 0 and 7 hours of imbibition with a maximum of about 350 counts per minute per embryo after 2 hours. The second photoemission phase, between 7 and 30 hours, increased from about 220 to 520 counts per minute per embryo. Both chemiluminescence phases were inhibited by infused butylated hydroxyanisole while only the second phase was inhibited by infused salicylhydroxamic acid. On the basis of the sensitivity of the lipoxygenase reaction to both inhibitors (about 90%), the first burst is tentatively assigned to oxy-radicals mobilized upon water uptake by the embryonic axes, and the second phase is tentatively identified as due to lipoxygenase activity. The in vitro lipoxygenase activity of the embryonic axes was estimated by both the fraction of total oxygen uptake that was inhibited by butylated hydroxyanisole and by the fraction of photoemission that was inhibited by butylated hydroxyanisole and by salicylhydroxamic acid. Both approaches indicated marked increases (5-fold and 12-fold, respectively) of lipoxygenase activity between 2 and 30 hours of imbibition. The measured chemiluminescence per O₂ uptake ratio (the experimental quantum yield) for the lipoxygenase reaction (3.3 × 10⁻¹⁴ counts per O₂ molecule) was used to estimate the O₂ uptake due to lipoxygenase activity from the photoemission of the embryonic axes after 30 hours of imbibition. The value (0.54 microliters per minute per axis) was close to the butylated hydroxyanisole-sensitive O₂ uptake (1.2 microliters O₂ per minute per axis) of the same embryonic axes. Chemiluminescence may afford a noninvasive assay for lipoxygenase activity in intact plant tissues.

In recent years there has been a growing interest in the possible involvement of oxidative free radicals in some plant processes like fruit senescence (2, 9) wound healing (18), and seed aging (19). Low level chemiluminescence is currently regarded as a measurement of the steady state level of oxy-radicals and singlet oxygen formed during the lipoperoxidation process and seems to afford a useful noninvasive and nondestructive assay for these reactive species in intact tissues (5, 7, 10).

Soybean seeds show spontaneous low level chemiluminescence, which sharply increases upon imbibition (6, 8). Two processes, the nonenzymic autoxidation of unsaturated fatty acids and the lipoxygenase-catalyzed reaction (8), lead, through common intermediates (ROO) to light emission. Both processes have been identified on the basis of their Arrhenius activation energies (8). The peroxy-radicals from unsaturated fatty acids are intermediates of the autooxidation process whereas the lipoxygenase reaction yields peroxy-radicals in a side reaction (8, 11, 15). The predominantly red emission is consistent with 'O₂⁻ being one of the species responsible for photoemission (8, 10).

Considering the chemical reactivity of oxy-radicals and of 'O₂ (5, 7, 13) that could lead to membrane and nucleic acid damage, it is possible that an increased level of these reactive species during the early imbibition phase may have a deleterious role on embryo and seed performance during germination.

In this study we measured the spontaneous chemiluminescence of soybean embryonic axes during the first hours of imbibition and tested the sensitivity of the light emission to free radical traps and lipoxygenase inhibitors. Moreover, we have explored the possibility of estimating lipoxygenase activity in the intact tissues by chemiluminescence measurements.

MATERIALS AND METHODS
Embryonic axes were excised from freshly harvested soybean seeds (Glycine max L. var Hood) with a moisture content of 13 to 17% and showing over 95% germination at 26°C. The embryonic axes were incubated on filter paper covering wet cotton in Petri dishes at 26°C for a period of 30 h. The O₂ uptake of embryonic axes was measured polarographically with a Clark-type oxygen electrode at 26°C; the embryos were placed in 1.8 ml of 50 mM NaH₂PO₄—K₂H₂PO₄ buffer (pH 6.5), and O₂ uptake was recorded over the initial 5 to 10 min. Parallel controls were run in a conventional Warburg manometer yielding similar oxygen uptake rates.

Chemiluminescence of soybean embryonic axes was determined by using either: (a) a Johnson Foundation (Johnson Research Foundation, University of Pennsylvania, Philadelphia) photon counter (5) in which case the embryos were placed on filter paper covering wet cotton in a Petri dish (Fig. 1); or (b) a Packard Tri-Carb model 3320 liquid scintillation counter in the out-of-coincidence mode with the discriminator adjusted for low-level emission. In this latter case, the embryos were placed in the vials in contact with filter paper covering wet cotton as indicated in Figure 1. The vials were incubated at room temperature (26°C) and read for chemiluminescence for about 5 to 10 min.

Lipoxygenase activity was measured either as O₂ uptake or as chemiluminescence at 26°C in a reaction medium consisting of
Isolated axes infused with the lipoxygenase inhibitor SHAM exhibited only the first rise in photon emission and had completely inhibited the second rise of chemiluminescence. Isolated axes infused with the free radical scavenger BHA showed a markedly inhibited first rise of chemiluminescence (about 80% of the integrated chemiluminescence over the 0 to 7-h period) and an almost completely suppressed second rise of chemiluminescence. A fraction of the measured light emission, 150 to 180 cpm/embryo, about the photoemission of the dry embryos, was always present and was not affected by the inhibitors (Fig. 2).

**Relationship between Light Emission and In Vitro Lipoxygenase Activity.** Since the lipoxygenase reaction is one of the sources of the luminescence of soybean seeds (6, 8), it appeared feasible to measure light emission as an indication of the in vitro activity of the enzyme in the isolated embryonic axes. One of the necessary conditions to make such a procedure acceptable is to know the quantitative relationship between lipoxygenase activity and chemiluminescence. As seen in Figure 3A, the in vitro activity of the purified enzyme measured both by O₂ uptake and by chemiluminescence kept a linear relationship with enzyme concentration. Over the assayed range, the ratio of photons measured to O₂ uptake (i.e. our experimental quantum yield of the reaction) had the constant value of 3.3 × 10⁻¹⁴ counts/O₂ molecule. The Kᵣ values for linoleic acid in the lipoxygenase reaction were both 32 μM measured by chemiluminescence and by O₂ uptake (Fig. 3B). Values for Vₘₐₓ, obtained from double reciprocal plots, yielded 0.28 nmol O₂/min-lipoxygenase unit from the measurements of O₂ uptake and 0.08 nmol O₂/min-lipoxygenase unit calculated from the measurements of chemiluminescence and using the above mentioned quantum yield.

Lipoxygenase activity can be inhibited by hydroxamic acids (14) and by quinols with antioxidant properties such as BHA, BHT, DTBQ, and PG (4, 16). The concentration-effect curves for the lipoxygenase inhibitors assayed with the pure enzyme and with the soybean embryonic axes are shown in Figures 4 and 5. With the purified enzyme, BHA was the most effective inhibitor among the antioxidant quinols; it inhibited O₂ uptake up to 90% at a 100 μM concentration. SHAM also inhibited lipoxygenase activity up to 90% but required a 5 mM concentration to reach such effect. Again with the soybean embryonic axis, BHA was the most effective inhibitor depressing oxygen uptake by 28% at a 100 μM concentration. SHAM at 5 mM inhibited embryo O₂ uptake by 38%.

**In Vitro Lipoxygenase Activity Estimated by the Sensitivity of Chemiluminescence and Oxygen Uptake to Inhibitors.** Two ways of estimating in vitro lipoxygenase activity were assayed. The first one involved the assumption that the light emission inhibited by infused BHA or by infused SHAM was due to lipoxygenase activity. Taking the case of the embryonic axes after 30 h of incubation, the BHA-sensitive light emission (about 300 cps/axis) divided by the quantum yield (3.3 × 10⁻¹⁴ counts/O₂ molecule) gave a value of 0.54 μL O₂/min-axis.

The lipoxygenase activity of the intact axes calculated in this way shows a 12-fold increase between 2 and 30 h of imbibition (Fig. 6). The second approach was based upon the assumption that the inhibition of O₂ uptake by 100 μM BHA was a measurement of lipoxygenase activity in the whole tissue, which gave a value of 1.2 μL O₂/min-axis for the embryonic axes after 30 h of imbibition with an about 5-fold increase between 2 and 30 h of incubation (Fig. 6).
Chemiluminescence of Soybean Embryonic Axes

Fig. 3. Lipoygenase activity measured as chemiluminescence and O₂ uptake. A, 1 mM linoleic acid. B, Substrate dependence of lipoygenase activity. 4000 units enzyme/ml.

Fig. 4. Effect of inhibitors on lipoygenase activity.

Fig. 5. Effect of inhibitors on the oxygen uptake of 2-h-imbibed soybean embryonic axes.

Fig. 6. Lipoygenase activity of soybean embryonic axes during imbibition; (●), estimated as the 100 μM BHA-sensitive oxygen uptake of the embryonic axes; (▲), calculated from the SHAM-sensitive chemiluminescence of the embryonic axes. Experimental conditions as in Figures 2 and 5.

and from the side reaction of the lipoygenase-catalyzed oxygenation of linoleic acid (reaction 3; in which \( \text{CH} \) represents the 13-carbon of linoleic acid and \( L \) denotes lipoygenase (7, 11).

\[
\text{CH} + \text{O}_2 + L \rightarrow \text{COO}^+ + \text{LH}^-
\]  

The primary and secondary peroxy-radicals produced in these chain reactions may yield in termination reactions either excited singlet oxygen (\('\text{O}_2'\)) (reaction 4; Ref. 17) or excited carbonyl groups (reaction 5).

\[
2 \text{ROO}^+ \rightarrow \text{RO} + \text{ROH} + \text{O}_2
\]  

\[
2 \text{ROO}^+ \rightarrow \text{RO}^* + \text{ROH} + \text{O}_2
\]

Moreover, excited carbonyls groups can be generated after decomposition of the dioxietane derivatives (12, 14) formed after addition of \('\text{O}_2'\) to unsaturated hydrocarbon chains (reaction 6; Refs. 13, 14) or after cyclization of unsaturated tertiary peroxy- and alkyl-biradicals (reaction 7).
H H H O → O H

 \[ \text{C} = \text{C} + {'}O_2 \rightarrow \text{C} = \text{C} \]

\[ \text{R} \quad \text{R} \quad \text{R} \quad \text{R} \]

\[ \text{H} \quad \text{H} \quad \text{H} \quad \text{H} \]

(6)

\[ \text{H} \quad \text{H} \quad \rightarrow \text{CO}^* + \text{CO} \]

\[ \text{R} \quad \text{R} \quad \text{R} \quad \text{R} \]

\[ \text{H} \quad \text{H} \quad \rightarrow \text{CO}^* + \text{CO} \]

(7)

\[ \text{OO} \quad \text{R} \quad \text{C} = \text{C} \rightarrow \text{R} \quad \text{C} = \text{C} \]

\[ \text{H} \quad \text{R} \quad \text{R} \quad \text{R} \]

\[ \text{H} \quad \text{H} \quad \rightarrow \text{CO}^* + \text{CO} \]

(8)

Interestingly enough, \( {'}O_2 \) is able to generate excited carbonyl groups through reaction 6 and excited carbonyls groups are able to generate \( {'}O_2 \) after collisional quenching through reaction 8 (Ref. 13).

\[ \text{CO}^* + O_2 \rightarrow \text{CO} + {'}O_2 \]

Although at present there is no conclusive evidence on the identity of the emitting species, it is clear that the excited states are derived from the radical chain reaction of lipoperoxidation, that \( {'}O_2 \) is responsible for part of the photoemission, and that other chemiluminescent species are involved (8, 10).

The chemiluminescence of the soybean embryonic axes shows two distinct periods during imbibition. The first one occurs during the first 7 h with a rise (maximum at about 2 h) and a decline. This rise is insensitive to the lipoperoxidase inhibitor SHAM but sensitive to the free radical scavenger BHA. This difference suggests that the increase in photoemission might be the result of mobilization of peroxy-radicals of unsaturated fatty acids produced by lipoperoxidation during the storage of the seed and that become able to collide upon imbibition and so reactions 4 and 5 can take place. Alternatively, it could be considered that in the dry axis there is a small amount of free radicals which after hydration drastically increase the rate of reactions 1 and 2, increasing the steady-state level of ROOH, which after homolytic rupture yield RO' and HO', increasing the number of reaction centers and by reactions 4 and 5, the light emission. Concerning the decrease in light emission after 2 h of incubation, it might be speculated that there was either a depletion of the peroxy-radicals formed during dry storage or that a scavenging system developed after the start of imbibition. This rise in free radical activity in the first hours of imbibition, at a time when there is extensive membrane reorganization (3) and a defense mechanism like superoxide dismutase activity (19) may not be fully developed, might be of important consequence for the performance of the embryos. It is also in the early imbibition stages when some of the effects of aging may be also amplified or attenuated depending on the conditions in which the water uptake is carried out (20). The degree of involvement of the free radicals we have detected in those responses is an interesting point deserving further research.

The second rise in chemiluminescence observed between 7 and 30 h of incubation might be due to lipoperoxidase activity. This is suggested by the sensitivity of light emission to BHA and SHAM, both active inhibitors of lipoperoxidase. Moreover, assuming that the second rise in photoemission is due to lipoperoxidase and using the quantum yield of the lipoperoxidase reaction, the \( O_2 \) uptake calculated is about one-half of the BHA-sensitive \( O_2 \) uptake of the axes. However, both ways of estimating \textit{in vivo} lipoperoxidase activity were in qualitative agreement indicating a much increase between 2 and 30 h of imbibition. The quantitative discrepancy could be explained by photoemission quenching in the axis tissues and by incomplete photon capture by the photomultiplier due to the geometry of the measuring system. Therefore, it seems that it could be possible to estimate \textit{in vivo} lipoperoxidase activity by chemiluminescence measurements. However, the use of light emission to estimate \textit{in vivo} lipoperoxidase activity requires the consideration of the assay limitations. There are other sources of chemiluminescence besides lipoperoxidase (Fig. 2), but the use of lipoperoxidase inhibitors restricts the sources of error to other light emitters sensitive to the same inhibitors. There is also the possibility of an important difference between the \textit{in vivo} and the \textit{in vitro} counts to \( O_2 \) uptake ratio of the lipoperoxidase reaction, and there is not enough information available on the effect that pH, ion composition, type of substrate, etc. might have on such ratio. But even if the activity of the enzyme could not be expressed in absolute units, the detection of changes in activity in relative terms may be very helpful. Finally, it is also convenient to consider the absorption by the tissue of the emitted light. Preliminary work estimates that the measured light comes from the outer 50 to 100 µm of the tissue; consequently, the assay would have limited validity if considerable differences exist between the activity in the outermost cells and the rest of the tissue.

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