SPONTANEOUS CHEMILUMINESCENCE OF SOYBEAN SEEDS

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1. Introduction

Chemiluminescence of seedlings and plant tissue extracts was first reported by Colli et al. [1]. Oxygen dependence of barley seedling chemiluminescence [2] and the relationship between radicle photoemission and cold-resistance in barley, wheat, corn, and cotton [3] was subsequently reported. This low level chemiluminescence is currently regarded as a measurement of oxidative free radical reactions [4] and related to lipid and organic peroxide formation [5], a topic of considerable interest in areas of plant physiology such as fruit ripening [6,7] and seed aging [8,9]. Chemiluminescence species are produced in side reactions of the main enzymatic pathway of lipoxygenase [10–13]. The enzyme and its substrates [14] are found in large amounts in soybean seeds, and moreover, the lipoxygenase reaction seems to account for an important fraction of the seed oxygen uptake [15].

Here we propose that the spontaneous chemiluminescence of the soybean seeds is originated mainly from the slow activity of the endogenous lipoxygenase.

2. Materials and methods

2.1. *Materials*

Soybean (glycine max. L. var. Hood) seeds recently harvested were used. Particles of soybean seed tissue (henceforth, particles) were obtained by treating the seeds for 30 s in a Waring blender and gently sieving the ground tissue through 2 layers of cheese cloth; av. particle size was ~0.2–0.5 mm. Tissue homogenates (henceforth, homogenate) were made in a glass–Teflon homogeneizer with 10 ml 50 mM phosphate buffer (pH 7.0).

2.2. *Chemicals*

Lipoxygenase (type III) and linolenic acid were purchased from Sigma Chemical Co. (St Louis, MO). Salicylhydroxamic acid (SHAM) was from Aldrich Chemical Co. (Milwaukee, WI). Other reagents were of analytical grade.

2.3. *Chemiluminescence measurements*

Chemiluminescence was measured in a Nuclear Chicago Scintillation counter with a EMI super S-11 photomultiplier in the 'out of coincidence' mode for 24 s or 60 s periods. Temperature of the phototube housing was kept at 10°C; this setting allowed a constant background of 30–60 counts/s. Seeds were placed into 25 mm diam. × 60 mm ht glass vials (25 × 60 mm). Seed particles were suspended immediately before measuring photoemission by shaking them for 3 s in 2 ml phosphate buffer at 37°C, contained in 13 mm diam. × 42 mm ht glass vials, then placed onto a small piece of insulating material inside the 25 × 60 mm glass vials and counted. Both, homogenate (diluted 1/20–1/50 in 2 ml phosphate buffer at 37°C) and lipoxygenase/linolenate mixtures were placed in the small vials and counted inside the larger vials as described. Temperature of the samples did not decrease during the measurement. Partial spectral analysis of the light emission was carried out with acetate filters, the transmittance of which was checked in a DK-2 Beckman spectrophotometer; the wavelength of maximal transmittance followed by the bandwidth at half-maximal transmittance are indicated.

3. Results

3.1. *Chemiluminescence of soybean seeds*

Air-dry soybean seeds show spontaneous light

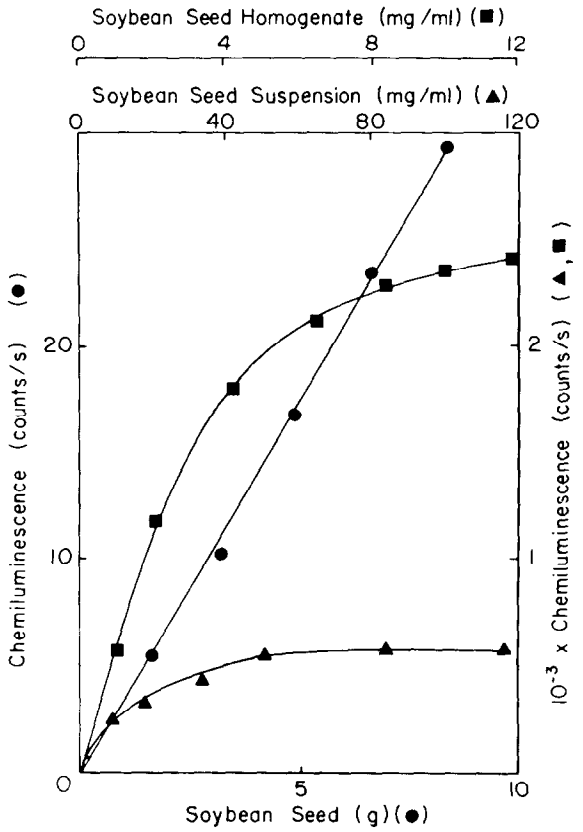

Fig.1. Chemiluminescence of soybean seeds (•), suspensions of particles (▲), and homogenate (■) from soybean seeds. Particles and homogenate were diluted in 10 mM phosphate buffer (pH 6.6); temp. 37°C.

emission which was linearly related to the number of seeds in the vial (fig.1). The detected chemiluminescence was about 3.5 counts . s^{-1} . g $seed^{-1}$. Suspensions of soybean particles showed a photoemission that saturated at ~21 mg seed material/ml. Chemiluminescence was a linear function of the amount of seed tissue up to 8 mg/ml, yielding ~10 counts . s^{-1} . mg $tissue^{-1}$. Consequently, ground tissue was ~2800-times more effective in yielding light emission.

Since the seeds are ~8 mm diam. spheres, it could be considered that light arises from an active volume of a depth a such that the ratio of total seed volume to emitting volume should be equal to the ratio of tissue to seed chemiluminescence (eq. (1)]:

$$[4/3\pi r^3] : [4/3\pi r^3 - 4/3\pi(r-a)^3] = 2800/(A \,.\, B) \quad (1)$$

[A and B are correction factors for the seed surface exposed in the vial (A = 4) and the increase in chemiluminescence after soaking (B = 4)]. The imbibing of H_2O rapidly converts the seed from an almost quiescent body into a dynamic organism [16]; moistening the seeds for ~1 min increased photoemission 4 times. Resolution of eq. (1) for r = 4 mm shows that seed chemiluminescence would be generated in cells located up to ~0.01 mm from the surface.

The tissue homogenate also showed a saturation behaviour with maximal light emission below 4 mg seed material/ml and with a light emission of ~200 counts . s^{-1} . mg $seed^{-1}$ (fig.1). Chemiluminescence of the homogenate was ~20-times higher than that of the particles. It is apparent that in the intact tissue, lipoxygenase is shielded from its substrate by some structural barrier which is lost after homogeneization. The chemiluminescence signal of either particles or homogenate reached its maximal yield within 30–40 s, decaying afterwards to the background level within 5–10 min.

3.2. *Effects of cyanide and salicylhydroxamic acid on chemiluminescence of soybean seeds*

The effects of cyanide and SHAM were tested on the whole seeds, particles, homogenate, and lipoxygenase (fig.2). SHAM inhibited chemiluminescence of seeds by 15%, of particles by 40%, of homogenate by 63%, and of enzyme by 80%. The increasing inhibitory effect of SHAM was correlated

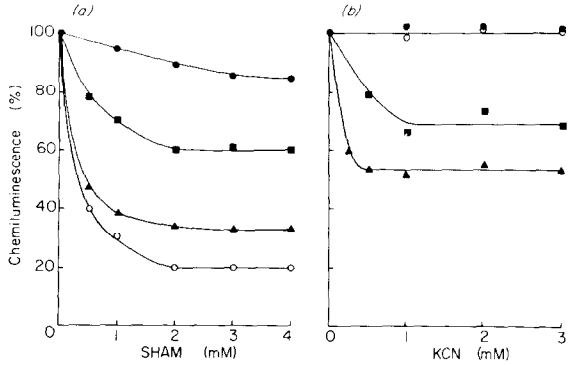

Fig.2. Effect of inhibitors on chemiluminescence of soybean seed tissue. Seeds (•) were imbeded with 10 mM phosphate buffer (pH 6.6) for 10 min at 30°C, containing SHAM or KCN at the indicated concentrations. Moistened seed emitted about 12 counts . s^{-1} . g $seed^{-1}$. Particles (■), homogenate (▲), and lipoxygenase (○) were used as indicated in table 1; temp. 30°C.

to an increasing fractionation level, perhaps due to a better access of the inhibitor to the source of light emission. Cyanide was inactive with the seeds and the enzyme, but inhibited particles and homogenate chemiluminescence by 32% and 43%, respectively. The lack of effect of cyanide on lipoxygenase or seeds was predictable because of the insensitivity of the enzyme to cyanide [14] or the low enzymatic activity of the seeds. The inhibition exerted by cyanide on particles and homogenate light emission accounts for a source of light emission other than the lipoxygenase reaction, like the cyanide-sensitive chemiluminescence arising from hemoprotein-catalyzed hydroperoxide breakdown [17], that might be triggered after exposure of the material to air and fractionation procedures.

3.3. *pH dependence and partial spectral analysis of chemiluminescence*

The maximal light emission from the spontaneous chemiluminescence of the homogenate was found at pH ~7.8; a second maximum was found at pH 6.6 (fig.3). It is worth noting that the seed internal pH seems to be close to this latter value, since particle suspension (20–100 mg/ml) in distilled water have pH 6.5. A series of filters were assayed on the photoemission of the homogenate. Red light (620 ± 20 nm) seems more important at pH 6.5, whereas blue light (460 ± 35 nm) was predominant at pH 7.5–8.5. Green light (520 ± 30 nm) appears more important than blue-green light (500 ± 20 nm) over the whole pH range. Table 1 shows a partial spectral analysis of the photoemission of soybean seeds at pH 6.5. Red and blue light are predominant in seeds, particles, homogenate, and the lipoxygenase reaction. Emission was higher in the green band than in the blue-green band, in all cases.

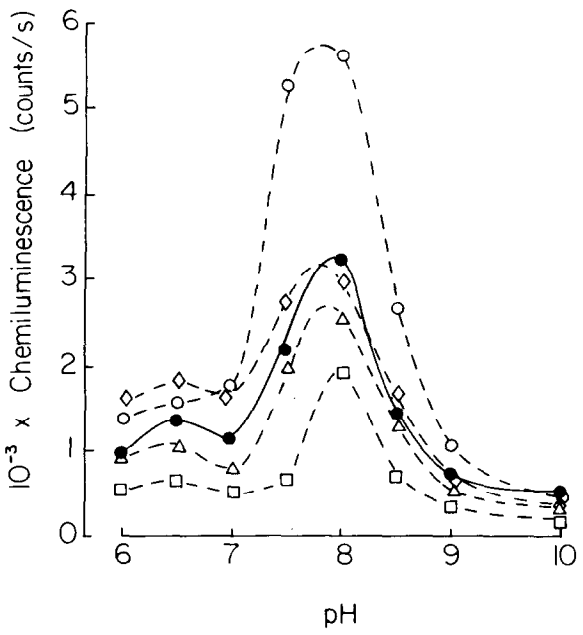

Fig.3. Chemiluminescence of soybean seed homogenate (pH 6.0–8.0); 50 mM phosphate buffer (pH 8.0–10.0); 50 mM glycine/50 mM NaCl/NaOH buffer. Temp. 37°C. (●) No filter; (◇) red (620 ± 20 nm) filter; (○) blue (460 ± 35 nm) filter; (△) green (520 ± 30 nm) filter; (□) blue-green (500 ± 20 nm) filter.

Table 1
Partial spectral analysis of soybean seed chemiluminescence

	Filter			
	Blue (460 ± 35 nm)	Blue-green (500 ± 20 nm)	Green (520 ± 30 nm)	Red (620 ± 20 nm)
(a) Whole seeds	26	8	25	51
(b) Particles	37	7	12	44
(c) Homogenate	31	12	21	35
(d) Lipoxygenase	30	9	18	43

The values are expressed as arbitrary units and have been corrected for filter transmittance and phototube sensitivity. (a) 10.5 g whole seeds (60 seeds); (b) 77 mg particles suspension/ml; (c) 4.2 mg homogenate/ml; (d) 8000 units lipoxygenase/ml and 180 μM linolenic acid. In (b), (c) and (d) 50 mM phosphate buffer (pH 6.6) was used. Final vol. 2 ml; temp. 37°C

4. Discussion

The spontaneous light emission of soybean seeds might be identified as originating from the lipoxygenase reaction on the basis of:

(1) A similar, on qualitative basis, effect of SHAM, an effective inhibitor of lipoxygenase [16], that was found to inhibit chemiluminescence from enzyme, homogenate, particles, and seeds, although in the last case only up to 15%. A limited access of the inhibitor along with a low turnover rate may explain this lower effect;

(2) A similar spectral distribution, since the 3 main emission bands of the chemiluminescence of the lipoxygenase reaction (around 450 nm, 550 nm, and 630–640 nm) [11–13] are present in comparable proportion in seeds, particles, homogenate, and enzyme. Partial spectral analysis is then consistent with the lipoxygenase reaction as responsible for the spontaneous chemiluminescence of soybean seeds. Free radicals ($R^{\cdot}$), dissociated from the lipoxygenase–linolenate (ES) complex, autoxidize to $ROO^{\cdot}$, which upon cancelation reactions [18], generate several possible excited species:

(i) carbonyl groups (emission at 450 and 550 nm [19,20]);

(ii) singlet oxygen (dimol emission at 630–640 nm [21]);

(iii) dioxetane intermediates [20];

(3) A similar oxygen/count ratio. The ratio of light emitted [10 counts . s^{-1} . mg $tissue^{-1}$ (fig.1)] to O_2 consumed (4×10^{12} O_2 molecules/s) [15] in tissue preparations is $\sim 4 \times 10^{11}$ O_2 molecules/count, and this value is similar to the one for the lipoxygenase reaction (10^{11} O_2 molecules/count) [13]. Taking into account that particles are the closest biological level of organization to whole seeds (they only involve grinding, sieving, and a 30 s suspension in buffer), it seems that photoemission of soybean seeds is mainly related to the lipoxygenase reaction.

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References

[1] Colli, L., Fachini, U., Guidotti, G., Dugnani Lonati, R., Orsenigo, M. and Sommariva, O. (1955) Experientia 11, 479–481.

[2] Gasanov, R. A., Mamedov, T. T. and Tarusov, B. N. (1963) Dokl. Biol. Sci. Sect. 150, 645–647.

[3] Gasanov, R. A., Mamedov, T. G. and Tarusov, B. N. (1963) Dokl. Biol. Sci. Sect. 153, 1480–1482.

[4] Boveris, A., Cadenas, E., Reiter, R., Filipkowski, M., Nakase, Y. and Chance, B. (1980) Proc. Natl. Acad. Sci. USA 77, 347–351.

[5] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605.

[6] Biale, J. (1960) Adv. Food Res. 10, 293–354.

[7] Brennan, T. and Frenkel, C. (1977) Plant Physiol. 59, 411 416.

[8] Villiers, T. A. (1973) in: Seed Ecology (Heydecker, W. ed) pp. 265–288, Butterworths, London.

[9] Parrish, D. J. and Leopold, A. C. (1978) Plant Physiol. 61, 365–368.

[10] Finazzi Agro, A., Giovagnoli, C., De Sole, P., Calabrese, L., Rotilio, G. and Mondovi, B. (1972) FEBS Lett. 21, 183–185.

[11] Mamedov, T. G., Konev, V. V. and Popov, G. A. (1973) Biophysika 18, 685–691.

[12] Nakano, M. and Sugioka, K. (1977) Arch. Biochem. Biophys. 181, 371–383.

[13] Boveris, A., Cadenas, E. and Chance, B. (1980) Photobiochem. Photobiophys. submitted.

[14] Tappel, A. L. (1963) Enzymes 8, 275–283.

[15] Parrish, D. J. and Leopold, A. C. (1978) Plant Physiol. 62, 470–472.

[16] Parrish, D. J. and Leopold, A. C. (1977) Plant Physiol. 59, 1111–1115.

[17] Cadenas, E., Boveris, A. and Chance, B. (1980) Biochem. J. 186, in press.

[18] Russell, G. A. (1957) J. Am. Chem. Soc. 79, 3871–3877.

[19] Faria-Oliveira, O. M. M., Huan, M., Duran, N., O'Brien, P. J., O'Brien, C., Bechara, E. and Cilento, G. (1978) J. Biol. Chem. 253, 4707–4712.

[20] Zinner, K., Vidigal-Martinelli, C., Duran, N., Marsaioli, A. J. and Cilento, G. (1980) Biochem. Biophys. Res. Commun. 92, 32–37.

[21] Khan, A. V. and Kasha, M. (1963) J. Chem. Phys. 39, 2105–2106.