

Storage and Immobilization of Photosystem II Reaction Centers Used in an Assay for Herbicides

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Significant improvements in a previously described method for detection of herbicides at nanogram per milliliter concentrations are described. The activity of photosystem II reaction centers from spinach thylakoids was extended from hours to over 6 months by rapid freezing and storage at -70°C in appropriate cryoprotection media. Freeze-dried reaction centers were also stable for at least 3 months at 4°C . Reaction centers immobilized in calcium alginate gels retained activity for 1 week at 4°C , and immobilization permitted optical and chemical interference by milk samples to be eliminated.

Analytical methods based on biorecognition are receiving increased attention from researchers seeking fast, sensitive, selective means of detecting chemical residues. Because biorecognition systems (e.g., antibodies, enzymes, receptors) are able to respond selectively to low concentrations of analytes in the presence of high concentrations of matrix species, they offer the possibility of eliminating the lengthy cleanup steps required with conventional trace analysis techniques. In addition, biorecognition assays can be implemented without the use of organic solvents, reducing the environmental and economic burdens associated with solvents. We have recently developed a biorecognition assay capable of detecting nanogram per milliliter levels of atrazine and related herbicides in biological matrices.¹ The assay is based on the catalytic photoreduction of a redox dye by the photosystem II reaction centers found in spinach thylakoids (a membranous substructure of the chloroplast²) as illustrated in Figure 1. A number of herbicides^{3,4} bind with very high affinity to the reaction center, blocking electron transfer from the excited state and preventing reduction^{5,6} of the redox dye from its normal blue form to its colorless reduced form. Herbicide concentration is determined by measuring the change in absorbance of the dye after illuminating a mixture of sample, thylakoids, redox dye, and electron donor (DPC) for a fixed period of time. The assay utilizes safe, inexpensive reagents and produces semiquantitative results in 5–10 min. When the assay is used in a microwell plate format, one analyst can process hundreds of samples per hour. The assay is well suited to field and laboratory screening of large numbers of food or water samples for violative levels of herbicide residues. Only samples giving positive assay results would require more complex and expensive chromatographic analysis, greatly reduc-

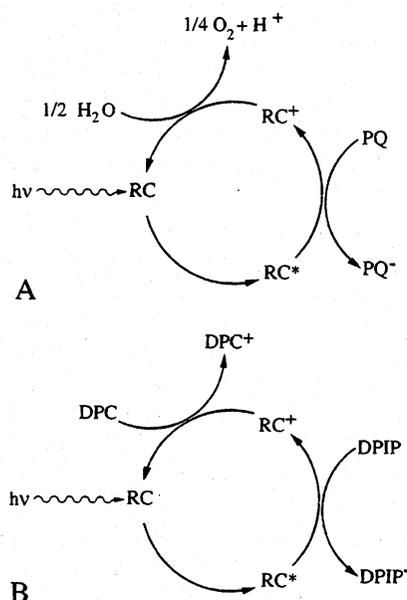


Figure 1. Simplified scheme for the photoreduction catalyzed by the photosystem II reaction center: (A) in vivo; (B) under assay conditions. RC, reaction center; P, primary electron donor; P*, excited primary donor; P⁺, oxidized primary donor; PQ, plastoquinone; PQ⁻, reduced plastoquinone; DPC⁺, oxidized DPC (diphenylcarbazide); DPIP⁻, reduced DPIP (dichlorophenolindophenol).

ing analysis time, cost, and solvent consumption. The photosystem II assay provides advantages in speed, simplicity, cost, and broad screening capability over immunoassays,¹ although detection limits are currently higher than those obtained with commercial immunoassays.

One barrier to practical implementation of this assay was the rapid loss in photoreduction activity of the reaction centers. After isolation from spinach leaves, suspensions of thylakoids at assay concentrations ($\sim 20 \mu\text{g}/\text{mL}$) had a useful lifetime of only 2–8 h. Problems were also encountered due to optical and chemical interference with the colorimetric measurement of dye reduction. Accurate absorbance readings could not be made in opaque or highly scattering samples such as homogenized meat; while milk samples could not be analyzed due to a direct reaction with the redox dye. This report describes the evaluation of the effects of freezing and freeze-drying on the activity and storage lifetime of spinach thylakoid reaction centers. Immobilization of thylakoids in calcium alginate gels and the use of the immobilized thylakoids to reduce interferences are also described.

EXPERIMENTAL SECTION

Apparatus.⁷ Homogenizations were performed with an Ultra Turrax T25 homogenizer (IKA Works, Cincinnati, OH). Cheese-

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cloth (Thomas Scientific, Swedesboro, NJ), 0.45 mm syringe filters (Cole Parmer Instrument Co., Chicago, IL), and 96 well microwell plates (Sarstedt, Newton, NC) were used as received. An EL 311s Microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by a Macintosh Plus computer (Apple Computer Inc., Cupertino, CA) running Δ Soft (BioMetallics Inc., Princeton, NJ) was used to read absorbance of microwell plate samples at 595 nm. Absorbance readings were also acquired with a Model DU-70 spectrophotometer (Beckman Instruments, Palo Alto, CA). Sorvall RC28S (DuPont, Wilmington, DE) and IEC Centra-8R (International Equipment Co., Needham Heights, MA) centrifuges were used as indicated below. A 500 W tungsten halogen floodlamp or a Model AF-3 slide projector with a 300 W bulb (Kodak, Rochester, NY) was used to illuminate liquid samples, and a locally constructed lightbox containing two 18 W U-shaped fluorescent bulbs with a $9.5 \times 13 \text{ cm}^2$ lighted area was used for illumination of immobilized samples. Light intensities were measured with an LX-101 luxmeter (Cole-Parmer Instrument Co., Chicago, IL).

Reagents.⁷ Dichlorophenolindophenol (DPIP), diphenylcarbazide (DPC), tricine, and sorbitol were from Sigma (St. Louis, MO) and used as received. Atrazine from Supelco, Inc. (Bellefonte, PA) and sodium alginate, SOBALG FD175 (Grindsted Products, Overland Park, KS), were used as received. DPC was stored under nitrogen at -10°C , and solutions were prepared weekly. Aqueous solutions were prepared with house-deionized water processed with a Nanopure water treatment system (Barnstead, Dubuque, IA). Spinach and milk were purchased locally.

Solutions. All buffers were adjusted to pH 7.8 with hydrochloric acid or sodium hydroxide. Homogenization buffer: 100 mM tricine, 400 mM sorbitol, and 10 mM NaCl. Resuspension buffer: 10 mM tricine and 10 mM NaCl. Analysis buffer: 100 mM sorbitol, 10 mM MgCl_2 , 10 mM NaCl, 10 mM tricine, and 1 mM NH_4Cl . Storage buffer: 400 mM sucrose, 50 mM tricine, 10 mM MgCl_2 , and 10 mM NaCl. DPIP stock: 0.2–0.3 mM DPIP in analysis buffer. DPC stock: 2 mM DPC in analysis buffer. Sodium alginate stock: 4% sodium alginate in analysis buffer, adjusted to pH 7.8. Gelling solution: 100 mM CaCl_2 in analysis buffer, adjusted to pH 7.8. Atrazine stock: atrazine dissolved in methanol or acetonitrile to give a $\sim 1 \text{ mM}$ solution of which this concentrate was diluted with analysis buffer to yield standard solutions containing appropriate concentrations of atrazine (organic solvent concentration in the final solutions was $<2\%$). Thylakoid stock: concentrated thylakoid suspension (5 mg/mL chlorophyll) diluted with analysis buffer to yield the desired final concentration (8–40 $\mu\text{g}/\text{mL}$).

Thylakoid Storage. Spinach thylakoids were isolated, and the chlorophyll concentration was determined using the procedures described previously.¹ After final pelleting, thylakoids to be stored were resuspended in storage buffer at a concentration of $\sim 600 \mu\text{g}/\text{mL}$, and small aliquots were placed in 1.5 mL screw-cap polyethylene tubes. The tubes were immersed in liquid nitrogen or dry ice until frozen and transferred to a refrigerator or freezer held at the desired temperature. Freeze-drying was performed by placing open tubes containing frozen thylakoids in the freeze-drying apparatus for 24 h, with dry ice cooling of the vacuum vessel during the last 8 h to ensure that the thylakoids were not

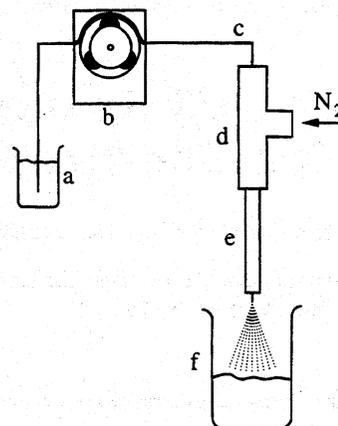


Figure 2. Atomizer for production of small calcium alginate beads. a, alginate solution reservoir; b, peristaltic pump; c, $1/16$ in. i.d. Teflon liquid feed tube; d, $1/8$ in. i.d. brass tee; e, $1/8$ in. i.d. copper tubing; f, gelling solution reservoir.

exposed to elevated temperatures. The tubes were then capped and transferred to a refrigerator or freezer held at the desired temperature. For activity determinations and assays, a tube of frozen material was thawed in cold water, and the contents were diluted to a chlorophyll concentration of $\sim 20 \mu\text{g}/\text{mL}$ with analysis buffer. Freeze-dried samples were reconstituted with analysis buffer to a chlorophyll concentration of $\sim 20 \mu\text{g}/\text{mL}$.

Immobilization. Thylakoid/alginate solutions were prepared by mixing appropriate volumes of thylakoid and alginate stock solutions with analysis buffer. The thylakoid/alginate solution was dropped from a Pasteur pipet into ice-cold gelling solution to obtain large (2–4 mm diameter) beads. Beads were incubated in gelling solution for 15–30 min after preparation, washed with analysis buffer, and stored in closed containers at 4°C . For small (0.1–0.5 mm diameter) beads, the thylakoid/alginate solution was sprayed into the gelling solution using a simple atomizer as shown in Figure 2. The flow rate of gas and liquid and the position of the liquid feed tube were adjusted to give beads of the desired size. Bead diameter was determined using a microscope with a graduated reticule.

Activity Determination. Equal volumes of ice-cold thylakoid stock, DPIP stock, DPC stock, and analysis buffer were combined. The mixture was pipetted into three matched quartz cuvettes and illuminated for 1 min at a light intensity of 200 000 lx. The average change in absorbance at 590 nm was used to determine the amount of DPIP reduced, and activity (millimoles reduced per hour per milligram of chlorophyll) was calculated on the basis of the concentration of chlorophyll and time of illumination.

Atrazine Assay with Immobilized Thylakoids. All solutions were kept on ice during the procedure. Nine calcium alginate beads containing immobilized thylakoids were selected from a single preparation of beads. Three beads were incubated in the sample solution for 10 min, washed with analysis buffer, and blotted dry with tissue. The three sample-treated beads and six control beads were incubated for 10 min with a 50:50 mixture of DPC stock and DPIP stock and blotted dry with tissue. The sample-treated beads and three of the control beads were placed in a Petri dish directly over the fluorescent light box. The other three control beads were kept in the dark. At the end of the illumination period, the color of the sample-treated beads was compared to the illuminated (light control) and unilluminated

(7) Reference of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1. Effect of Preparation and Storage Temperature on Relative Thylakoid Activity

preparation	$T_{\text{stor}}/^{\circ}\text{C}$	rel activity/%	
		7 days	90 days
frozen	-10	57	25
frozen	-70	100	97
freeze-dried	-10	94	89
freeze-dried	-70	95	90
alginate gel	4	70	

(dark control) beads and the extent of DPIP photoreduction assessed.

Atrazine Assay with Frozen Thylakoids. Thylakoids were thawed and diluted as described above. Into individual wells of 96 well microwell plates were pipetted 50 μL each of thylakoid solution, DPC stock solution, DPIP stock solution, and atrazine standard solution. Four replicates were used at each atrazine concentration (0, 10, 50, 100, and 500 ppb). The initial absorbance of each well was recorded with the microplate reader, the plate was illuminated for 5 min at $\sim 100\,000$ lx, and the final absorbance was recorded. Absorbance data were transferred to an Excel (Microsoft, Redmond, WA) spreadsheet for subtraction of initial absorbance and calculation of average ΔA values, theoretical curves, and standard deviations. Parameters for the theoretical curves were generated by fitting the data to the Hill equation⁸ using a nonlinear least-squares program.⁹

RESULTS AND DISCUSSION

Thylakoid Storage and Stability. The activity of freshly prepared thylakoid reaction centers stored at 4 $^{\circ}\text{C}$ at high chlorophyll concentrations ($>300\ \mu\text{g}/\text{mL}$) declined continuously after preparation, reaching $<50\%$ of initial activity after 24 h, and 20% or less after 48 h of storage. Dilute solutions were considerably less stable and were generally unusable after 4–8 h. Successful storage of frozen spinach thylakoids at liquid nitrogen temperatures has been reported recently,^{10–12} and we used a similar cryoprotectant solution to study the stability of frozen thylakoids at various temperatures. We also explored the feasibility of freeze-drying thylakoids for storage at refrigerator temperatures. The relative activity of frozen and freeze-dried thylakoid reaction centers as a function of storage conditions is summarized in Table 1. Relative activity is defined as the percent of activity at the indicated time relative to the activity 2 h after preparation. While the initial activity of various preparations ranged from 105 to 200 $\mu\text{mol h}^{-1} \text{mg}^{-1}$, the decline in relative activity shown in Table 1 was representative of all thylakoid preparations studied (at least two preparations for each storage regime). The data of Table 1 and additional studies indicate that useful activity is retained for at least 6 months under appropriate conditions. Frozen thylakoids stored at $-70\ ^{\circ}\text{C}$ were most stable, but the most convenient means of storage appeared to be freeze-drying with subsequent storage at $-10\ ^{\circ}\text{C}$. Freeze-dried material retained high activity for months at normal freezer temperatures, and

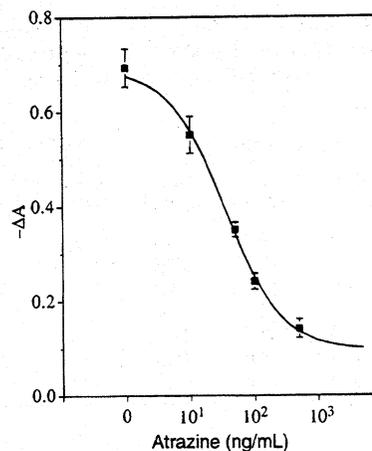


Figure 3. Assay response for atrazine in water using frozen thylakoids. Squares and error bars represent the experimental data; the solid line is a fit of the data to the Hill equation. Each sample contained 5 $\mu\text{g}/\text{mL}$ chlorophyll, 0.5 mM DPC, 80 μM DPIP, and the indicated concentration of atrazine. Illumination time, 5 min. Error bars represent 1 SD about the mean of four replicates.

storage in the refrigerator at 4 $^{\circ}\text{C}$ for at least 1 month was feasible. A calibration plot for atrazine using frozen thylakoids is shown in Figure 3. The detection limit and 50% inhibition value for the frozen material were equivalent to those obtained with freshly prepared thylakoids.¹ We are currently investigating the possibility of preparing microwell plates containing freeze-dried aliquots of thylakoids, DPC, DPIP, and standards. The user would only need to add water to the standards and sample to the test wells in order to conduct an analysis.

Immobilization of Thylakoids. The assay essentially involves two independent reactions: binding of herbicide to the reaction center and photoreduction of redox dye by uninhibited reaction centers. In the usual assay protocol, both reactions took place in the same solution, and sample components which interfered optically or chemically with the detection reaction prevented successful analysis. By carrying out the binding reaction separately from the detection reaction, however, this interference could be eliminated. Such a two-step assay requires a means of separating the reaction centers (with bound herbicide) from the sample solution and washing off any interfering matrix species prior to conducting the detection reaction. Immobilization of the reaction centers in a porous matrix was explored as a convenient means of achieving this separation. Several reviews are available which discuss entrapment of chloroplasts and thylakoids in a wide variety of polymer gels and foams.^{13–18} Because acrylamide gels and polyurethane foams require exposure of thylakoids to reactive chemical species, and agar gels require exposure to relatively high temperatures, calcium alginate gels were chosen for this study. Calcium alginate gels can be formed over a wide temperature and

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Table 2. Time (min) Required for Photoreduction of DPIP by Immobilized Thylakoids as a Function of Chlorophyll Concentration, Alginate Level, and Storage Time

storage time/days	5 g/mL chlorophyll			20 g/mL chlorophyll		
	0.5% alginate	1% alginate	2% alginate	0.5% alginate	1% alginate	2% alginate
1	3.5	9.9	9.0	2.5	4.5	5.5
2	6.0	8.5	12.5	2.0	3.0	4.5
3	7.0	12.5	16.5	3.0	3.5	5.0
6	7.5	12.0	16.0	4.0	6.5	7.0
7	8.0	12.5	17.0	5.0	7.0	7.0
8	9.0	14.0	18.5	6.0	7.5	8.0
9	10.0	16.5	19.0	6.5	8.0	9.0

pH range by addition of sodium alginate solutions to solutions containing calcium ion. The gels are readily formed into small beads and can be redissolved by addition of agents which complex or precipitate calcium, such as EDTA or phosphate.

Calcium alginate gels containing various concentrations of alginate and chlorophyll were evaluated qualitatively for features such as ease of preparation and handling, rate of photoreduction, and ability to detect changes in DPIP absorbance. Beads containing high concentrations of alginate were difficult to prepare due to the high viscosity of cold sodium alginate solutions and gave low rates of photoreduction but the beads were quite rigid and easy to handle. Beads with low concentrations of alginate were easily prepared and gave faster photoreduction, but did not maintain their shape and tended to stick to each other and to surfaces quite strongly. Beads with higher chlorophyll concentrations (15–20 $\mu\text{g}/\text{mL}$) gave higher rates of photoreduction, but their dark color made detection of DPIP absorbance changes difficult. Optimum results were obtained with beads containing 1% alginate and 5 $\mu\text{g}/\text{mL}$ chlorophyll. The stability of thylakoids immobilized in calcium alginate gels was enhanced significantly over that of thylakoids stored in buffer (Table 2). In these experiments, the time required for complete reduction of DPIP was used to assess activity since instrumental absorbance measurements could not be made with beads. After 9 days, im-

mobilized thylakoids stored at 4 °C had lost approximately 50% of their initial activity but were still sufficiently active for herbicide determinations. Thylakoids stored in buffer had lost virtually all activity after 24 h.

The utility of immobilized thylakoids in eliminating interferences was demonstrated by assaying milk samples with and without added atrazine. The beads exposed to unspiked milk lost all blue color after illumination, while beads exposed to milk containing 20 $\mu\text{g}/\text{mL}$ atrazine remained dark blue, clearly indicating that matrix interferences could be eliminated while retaining sensitivity to herbicide. Similar results were obtained with small beads (0.1–0.5 mm diameter) using shorter incubation times, but attempts to measure absorbance changes of beads in microwell plates were of limited success due to scattering. Beads could be dissolved in warm EDTA to give nonscattering solutions, but back-reaction of reduced DPIP with atmospheric oxygen during dissolution prevented acquisition of accurate absorbance measurements.

CONCLUSIONS

The major barrier to practical implementation of a rapid method for herbicide detection has been removed. Reaction centers in spinach thylakoids remained active for months when frozen at -70 °C or when freeze-dried and stored at -10 °C. Immobilization in calcium alginate gels stabilized reaction centers and offers a simple and effective means to eliminate interferences. The availability of stable, storable preparations of the bioreagent will permit evaluation of the method in more laboratories and with a wider variety of matrices and analytes than was previously possible. Immobilized reaction centers may also find use in biosensors and in affinity procedures for isolation and concentration of herbicides from complex matrices.