# Use of Recombinant Biotinylated Aequorin in Microtiter and Membrane-Based Assays: Purification of Recombinant Apoaequorin from *Escherichia coli*†

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ABSTRACT: Aequorin is a calcium-dependent bioluminescent protein isolated from the hydromedusan *Aequorea victoria*. The gene for aequorin has been cloned and overexpressed in *Escherichia coli* [Prasher et al. (1985) *Biochem. Biophys. Res. Commun. 126*, 1259; Prasher et al. (1987) *Biochemistry 26*, 1326]. Higher levels of expression have recently been obtained by subcloning aequorin cDNA into the pRC23 plasmid vector such that its expression is under control of the λ $P_L$ promoter [Cormier et al. (1989) *Photochem. Photobiol. 49*, 509]. Purification of recombinant apoaequorin from *E. coli* containing this new recombinant plasmid (pAEQ1.3) was accomplished by a two-step procedure involving gel filtration and anion-exchange chromatography on Sephadex G-100 and DEAE-Sepharose, respectively. Typically, 400–500 mg of recombinant protein was obtained from 100 L of fermentation culture. The purified recombinant apoaequorin could be converted to aequorin in high yield upon incubation with synthetic coelenterate luciferin, dissolved oxygen, and a thiol reagent with a photon yield similar to the native photoprotein. Detection of recombinant aequorin in the Dynatech ML1000 Microplate luminometer was linear between $10^{-18}$ and $10^{-12}$ mol, and little loss of specific activity was observed when the protein was derivatized with biotin. The biotinylated derivative was stable when frozen, lyophilized, or stored at 4 °C. The feasibility of using biotinylated aequorin as a nonradioactive tag was established by its application in a variety of solid-phase assay formats using the high-affinity streptavidin/biotin interaction. A microtiter-based bioluminescent immunoassay (BLIA) using biotinylated aequorin and the ML1000 luminometer was developed for the detection of subnanogram amounts of a glycosphingolipid (Forsmann antigen). In addition, nanogram to subnanogram quantities of protein antigens and DNA, immobilized on Western and Southern blots, respectively, were detected on instant and X-ray films using biotinylated aequorin.

Aequorin is a high-affinity $Ca^{2+}$-binding protein responsible for the bioluminescence produced in the circumoral ring of the hydromedusan *Aequorea victoria*. Native aequorin is a photoprotein (Shimomura et al., 1962) consisting of a single polypeptide chain of $M_r$ 21 000 containing 1 mol each of tightly bound coelenterate luciferin and oxygen. This complex is stable in the absence of calcium ion, and light emission is initiated upon the binding of 3 mol of $Ca^{2+}$/mol of aequorin (Allen et al., 1977; Charbonneau et al., 1985). In the presence of micromolar levels of free $Ca^{2+}$, aequorin catalyzes a single turnover event, the oxidation of luciferin to oxyluciferin with a concomitant flash of blue light ($\lambda_{max}$ = 469 nm) which persists for approximately 10 s.

The isolation of multiple aequorin cDNAs (Prasher et al., 1985; Inouye et al., 1985) and gel electrophoretic data indicate the presence of at least five aequorin isotypes in extracts of *Aequorea* tissue (Prasher et al., 1987). The available data suggest that aequorin is encoded by a multi-gene family consisting of a minimum of four genes (Cormier et al., 1990). A very high level of expression of recombinant apoaequorin was obtained when the AEQ1 cDNA (Prasher et al., 1985) was subcloned into the plasmid pRC23 (Crowl et al., 1985). In this new recombinant plasmid (pAEQ1.3) apoaequorin cDNA was linked to the λ $P_L$ promoter. *Escherichia coli* containing pAEQ1.3 were grown to the desired cell density, and the λ repressor was inactivated by a temperature shift and concomitant overexpression of apoaequorin. We now report the extraction and purification of recombinant apoaequorin and its conversion to the active photoprotein.

Due to the relatively high quantum yield of the reaction, aequorin can be detected at the attomole level ($10^{-18}$ mol) using commercially available luminometers (Ward, 1985; Bronstein & Kricka, 1990). Because of the ability to be detected at very low levels and its $Ca^{2+}$ requirement, aequorin has proven useful for monitoring levels of intracellular calcium in response to various stimuli (Blinks et al., 1982). The potential utility of using aequorin as a nonradioisotopic reporter molecule in various assay formats, however, has not been realized primarily due to limited quantities of the native protein. The availability of the recombinant protein will facilitate rapid development of mild labeling and conjugation procedures for aequorin such that it can be exploited for the sensitive bioluminescent detection of target molecules. In this report we describe conditions for the biotinylation of recombinant aequorin with minimal loss in activity and demonstrate the ability of the biotinylated derivative, when used in combination with streptavidin, to detect biotinylated targets immobilized on microtiter wells or membrane supports. Bioluminescent detection using biotinylated aequorin provides a sensitive and very rapid alternative to the enzyme and radioactive-based detection systems currently in use.

## Experimental Procedures

*Materials.* Aprotinin, leupeptin, phenylmethanesulfonyl fluoride, bovine serum albumin (BSA),[1] DEAE-Sepharose,

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Sephadex G-100, human transferrin, Polaroid 612 ASA 20000 instant film, and Kodak X-OMAT AR X-ray film were obtained from Sigma Chemical Co. (St. Louis, MO). The Bio-Rad protein assay kit, nitrocellulose, gelatin, Tween-20, biotinylated protein standards, goat anti-rabbit alkaline phosphatase, biotinylated goat anti-rabbit antibody, bromochloroindolyl phosphate (BCIP), and nitroblue tetrazolium (NBT) were obtained from Bio-Rad (Richmond, CA). Rabbit anti-human transferrin was from Dakopatts A/S (Denmark). Streptavidin was from Infergene (Benicia, CA). Sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) and immobilized avidin were purchased from Pierce Chemical Co. (Rockford, IL). Blocking reagent (gelatin) and DNase I were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Nytran nylon membrane was from Schleicher and Schuell (Keene, NH). Photoprobe biotin was obtained from Vector Laboratories (Burlingame, CA). Opaque Microlite 1 and 2 Removawell strips were obtained from Dynatech Laboratories (Chantilly, VA).

Synthetic coelenterate luciferin was prepared by Dr. Frank McCapra (University of Sussex, Brighton, U.K.). Forssman antigen and globoside were purchased from BioCarb (Accurate Chemical, Westbury, NY). The hybridomas M1/22.25.8.HL (ATCC TIB 121) and M1/87.27HLK (ATCC TIB 123) were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown under standard conditions, and the hybridoma supernatants containing the antibodies were stored at 4 °C in 0.004% $NaN_3$, and the monoclonal antibodies were used as hybridoma supernatants. Biotinylated goat anti-rat IgM was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). The monoclonal IgM directed against soybean calcium-dependent protein kinase (12G8/31) was kindly provided by Dr. Alice Harmon (University of Florida). The pTZR-Luc1 plasmid containing the 1.5-kb *Renilla* luciferase cDNA insert was a generous gift from Dr. Walter Lorenz (University of Georgia). All other chemicals and reagents were of the best grade available.

*Extraction and Purification of Recombinant Apoaequorin.* *E. coli* containing the recombinant plasmid pAEQ1.3 (Cormier et al., 1989) were grown to the desired cell density in a 100-L fermentor at 30 °C and then shifted to 42 °C for 2–3 h to inactivate the λ repressor and initiate overexpression of apoaequorin. The resulting cell paste (300–400 g) was immediately quick-frozen under liquid nitrogen in 50-g aliquots and stored at −80 °C. Prior to extraction, 50 g of *E. coli* cell paste was removed from the freezer and allowed to thaw at room temperature. The thawed cells were suspended in 150 mL of extraction buffer (0.05 M $NaH_2PO_4$, pH 7.2; 0.9% NaCl; 5 mM EDTA; 25% sucrose; 1% Triton X-100) and stirred slowly. Aprotinin (200 μL of 1.9 mg/mL), leupeptin (2 mg), and phenylmethanesulfonyl fluoride (0.4 g) were added to this suspension to inactivate endogenous proteases followed by the addition of 150 mg of lysozyme. After 15 min at room temperature, the cell suspension was frozen using liquid nitrogen. The cells were then thawed using a 37 °C water bath. This freeze–thaw cycle was repeated two more times with the exception that after the last freeze the cells were allowed to thaw slowly at 4 °C overnight.

To the thawed cell suspension was added 0.6 mL of a DNase I stock (20 mg/mL in 50 mM Tris, pH 8). The suspension was stirred slowly for 15 min, and the extract was poured into eight 50-mL centrifuge tubes and centrifuged at 26890*g* for 15 min in a refrigerated centrifuge. The supernatants were poured off, leaving a pellet in the bottom of the centrifuge tubes. To each pellet was added 30 mL of wash solution (25% sucrose containing 1% Triton X-100). The pellet was thoroughly resuspended, centrifuged at 26890*g* for 15 min, and the supernatant was discarded. Any excess liquid was removed by aspiration. The pellets were washed as described above three additional times. Each pellet was thoroughly suspended in 10 mL of 1.75 M guanidine hydrochloride, placed on ice for 2 h, and centrifuged for 10 min at 26890*g*. The combined supernatants were dialyzed for 2 h at 4 °C against 2 L of dialysis buffer (25 mM Tris, pH 8; 5 mM EDTA; 0.5 M NaCl; 5 mM dithiothreitol) followed by overnight dialysis against an additional 2 L of dialysis buffer. The precipitate was removed by centrifugation at 26890*g* for 15 min. The supernatant was dialyzed as described above except that the NaCl concentration in the dialysis buffer was reduced to 0.01 M. Following dialysis, the supernatant was centrifuged as above to remove any insoluble material.

The resulting supernatant was applied to a 6.5 × 90 cm Sephadex G-100 column equilibrated at 4 °C with column buffer A (10 mM Tris, pH 7.5, containing 1 mM EDTA and 5 mM dithiothreitol) which had been purged with argon to prevent oxidation of dithiothreitol. After the column was loaded with the supernatant, column buffer A was applied to the column and 20-mL fractions were collected. The fractions comprising the apoaequorin peak were pooled and then applied to a 2.5 × 20 cm DEAE-Sepharose column which had been equilibrated at 4 °C with column buffer B (10 mM Tris, pH 7.0, containing 2 mM EDTA). The DEAE-Sepharose column was then washed with 100 mL of column buffer B followed by elution of apoaequorin using a 1-L linear salt gradient from 0 to 0.6 M NaCl in column buffer B and collection of 8-mL fractions. In both cases, fractions containing apoaequorin were identified by their ability to be converted to aequorin when regenerated with synthetic coelenterate luciferin. The various stages of purification of apoaequorin from the crude bacterial extract were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions (Laemmli, 1970).

*Conversion of Recombinant Apoaequorin to Aequorin.* Purified, recombinant apoaequorin was routinely converted to recombinant aequorin with synthetic coelenterate luciferin as follows: To the pooled apoaequorin fractions obtained after DEAE-Sepharose chromatography (in 10 mM Tris, pH 7.0, containing 2 mM EDTA and NaCl) was added a stock solution of dithiothreitol or β-mercaptoethanol such that the final concentration was 5 mM. Synthetic coelenterate luciferin was dissolved in argon-purged methanol and added directly to the apoaequorin solution at a level ranging from 5 to 20 μg of luciferin/200 μg of protein, which corresponds to a 1.3–5-fold molar excess of luciferin to apoaequorin. The regeneration of aequorin was conducted at 4 °C for 18–24 h in an open, wide-mouth vessel to allow the equilibration of the solution with oxygen in the air. Aequorin was then dialyzed exhaustively against Tris-buffered saline (10 mM Tris, 0.15 M NaCl, pH 8.0; TBS) containing 2 mM EDTA (TBS/E). The concentration of apoaequorin (within a range of 100–1000 μg/mL) or NaCl during regeneration had no effect on the resulting specific activity, provided that excess coelenterate lu-

[1] Abbreviations: BCIP, bromochloroindolyl phosphate; BLIA, bioluminescent immunoassay; BSA, bovine serum albumin; Cer, ceramide; EDTA, ethylenediaminetetraacetic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; $M_r$, relative molecular weight; NBT, nitroblue tetrazolium; NHS-LC-biotin, sulfosuccinimidyl 6-(biotinamido)hexanoate; RLU, relative light units; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBS/E, Tris-buffered saline containing EDTA; TBS/E/BSA, Tris-buffered saline containing EDTA and BSA; Tris, tris(hydroxymethyl)aminomethane.

ciferin was present. The crude bacterial extract and Sephadex G-100 fractions were regenerated under similar conditions.

*Absolute Specific Activity Determinations.* Recombinant aequorin and native aequorin specific activities were determined using a laboratory-built photometer (Wampler, 1975). The light-emission path through the sample holder was restricted by two aperture plates that were separated by a hollow spacer (Sparrow, 1973) to ensure a defined light-emission geometry. Scale attenuation was achieved by using sample holders with apertures of smaller diameter. The photometer was calibrated absolutely using the chemiluminescence of 3-aminophthalhydrazide (luminol). Both the aqueous luminol reaction (Lee et al., 1966)) and the luminol/DMSO reaction (Lee & Seliger, 1972) were used for calibration of the photometer and sample holders immediately before initiating these measurements. Daily calibration of the photometer was achieved using a tertiary $^{14}C$-activated radiophosphorescence standard which has an emission that closely approximates the corrected bioluminescence emission of aequorin (O'Kane & Lee, 1990).

The specific activity measurements were performed by diluting the aequorin samples into 0.5 mL of TBS/E containing 0.1 mg/mL bovine serum albumin (TBS/E/BSA). The dilutions were performed in 12 × 50 mm polypropylene assay tubes and then placed in a photometer. Light emission from the diluted aequorin samples was then initiated by syringe injection of 0.5 mL of 100 mM Tris, pH 8, containing 100 mM $CaCl_2$. The bioluminescence emission thus initiated was integrated over time until light emission ceased, which usually took about 10–15 s. The absolute specific activity was defined as the number of photons emitted per milligram of protein.

*Protein and Bioluminescent Measurements.* Protein concentrations were measured using a Bio-Rad protein assay kit on the basis of the method of Bradford (1976) with BSA as the standard. Aequorin was routinely diluted into TBS/E/BSA prior to light measurement. Aliquots of aequorin (5–100 μL) were placed in opaque Microlite wells and monitored for light activity using the Microlite ML1000 microplate luminometer (Dynatech Laboratories, Chantilly, VA). Light production was initiated by injecting 200 μL of 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ directly into the individual microtiter wells and was typically monitored for 0.5–1 s.

*Biotinylation of Recombinant Apoaequorin and Aequorin.* Apoaequorin or aequorin were biotinylated using a 5–60-fold molar excess of NHS-LC-biotin for 30 min at room temperature in 0.1 M $NaHCO_3$, 0.2 M NaCl, 0.5 mM EDTA, pH 8.6. Following addition of 1.5 M Tris, pH 8.8, to stop the reaction, excess biotinylating reagent was removed by passing the reaction mixture over prepacked Econo-Pak 10DG (Bio-Rad Laboratories) or Presto desalting (Pierce Chemical) columns in TBS/E. The concentration of biotinylated aequorin was determined using the Bradford assay as described above. BSA (1 mg/mL) was then added to the pooled fractions which were stored at 4 °C or frozen at either −20 or −80 °C.

*Assay for Biotinylation of Recombinant Aequorin.* Aliquots of biotinylated aequorin were tested for their ability to interact with streptavidin bound to microtiter wells coated with a "lawn" of biotinylated BSA. BSA was biotinylated using a 3–5-fold molar excess of NHS-LC-biotin in 0.1 M $NaHCO_3$, 0.2 M NaCl, 0.5 mM EDTA, pH 8.6, for 1.5 h at room temperature. The reaction mixture was desalted as described above, and the fractions containing the protein were pooled and the concentration was determined by measuring the absorbance at 280 nm. Opaque Microlite 2 wells were coated overnight at 4 °C with either BSA or biotinylated BSA at 10–25 μg/mL (100 μL) in TBS/E. The wells were then blocked with 1% gelatin (200 μL) for 30 min and then incubated with streptavidin in TBS/E at 1–10 μg/mL (100 μL) for 30 min. After being washed with TBS/E, aliquots of the biotinylated aequorin (100 μL) were placed on the wells for 30 min. The amount of streptavidin/biotinylated aequorin complex was measured after unbound material was washed away by injecting 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ directly into the wells and monitoring light production. Alternatively, biotinylated aequorin was tested for its ability to bind to avidin–agarose. An aliquot of the biotinylated aequorin preparation was applied to a column (0.5–1 mL) of immobilized avidin in TBS/E/BSA and several 1-mL fractions were collected. The amount of biotinylated aequorin bound to the column was estimated by subtracting the amount of light activity which was eluted from the column with that amount applied to the column.

*Forssman Antigen Detection on Microtiter Wells.* Stock solutions of Forssman antigen and globoside in chloroform were prepared anaerobically and stored at 4 °C. Aliquots of the glycolipids diluted in methanol (usually 50 μL) were added to opaque Microlite 1 microtiter wells and allowed to bind to the surface of the wells by evaporating overnight at room temperature or for 1 h at 37 °C. The evaporation conditions did not appear to affect the results. After the glycolipid was bound, the wells were blocked with apoaequorin in TBS (0.1 mg/mL) for 30–60 min. Subsequent incubations with the hybridoma culture media containing the anti-Forssman monoclonal antibodies, biotinylated goat anti-rat IgM, streptavidin, and biotinylated aequorin were carried out for 60 min. All incubations were conducted at room temperature except that the incubation with biotinylated aequorin was carried out at 4 °C. The wells were washed with TBS following each incubation except that after the final incubation the wells were rinsed with TBS/E. Globoside, the direct biosynthetic precursor of Forssman antigen, was used as a glycolipid control, and the monoclonal IgM directed against soybean calcium-dependent protein kinase (12G8/31) was used as an antibody control.

*Protein Detection on Membranes.* The sample of interest was applied to either nitrocellulose or nylon membranes in a dot blot or by electrophoretic transfer following separation by SDS–PAGE (Laemmli, 1970). Nonspecific binding sites were blocked by incubating the membranes in 3% gelatin or 0.5% casein for 30 min. After the blots were exposed to the appropriate primary and biotinylated secondary antibody for 30 min, they were sequentially incubated for 30 min with streptavidin (1–10 μg/mL) and biotinylated aequorin (1 μg/mL). All incubations were conducted at room temperature, and all reagents were prepared in TBS/E/BSA or 3% gelatin in TBS/E. Washes between incubation steps were carried out with TBS/E. For the experiments conducted with biotinylated alkaline phosphatase or goat anti-rabbit alkaline phosphatase, the reagents were prepared in TBS containing 0.05% Tween-20 (TTBS), and the washes in between incubation steps were carried out with TTBS. For development, the blots were rinsed with TBS and the BCIP/NBT substrate mix was added according to the vendor's instructions. Incubation of blots with BCIP/NBT were conducted for a minimum of 10 min at room temperature.

*DNA Detection on Membranes.* Linearized pTZR-Luc1 plasmid containing cDNA encoding *Renilla reniformis* luciferase (Lorenz et al., 1991) was immobilized onto nitrocellulose or nylon membanes using standard protocols for dot blotting or after Southern transfer from an agarose gel

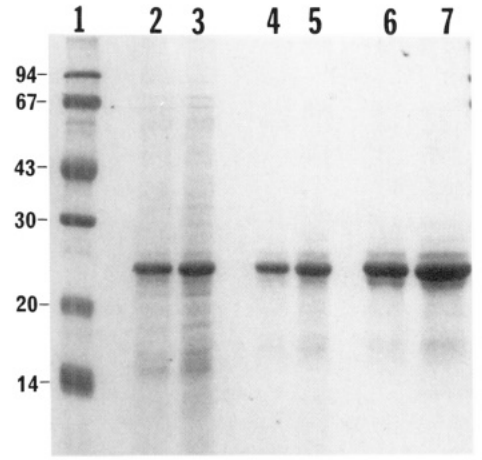


FIGURE 1: SDS–PAGE analysis during the purification of recombinant apoaequorin. Electrophoresis was performed using a 12.5% polyacrylamide gel in the presence of SDS and was stained with Coomassie blue. Lane 1 contained the following molecular mass markers (in kilodaltons): phosphorylase *b*, 94; BSA, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20; and α-lactalbumin, 14. Lanes 2 and 3 contained 10 and 20 μg of protein from the crude extract. Lanes 4 and 5 contained 10 and 20 μg of protein from the Sephadex G-100 pooled fractions. Lanes 6 and 7 contained 10 and 20 μg of protein from the DEAE-Sepharose pooled fractions.

(Maniatis et al., 1982). The target DNA was probed with the *Renilla* luciferase cDNA fragment which was generated by restriction enzyme digestion with *Sma*I and *Eco*RI and biotinylated with Photoprobe Biotin according to the vendor's instructions. After incubation in prehybridization buffer [6× SCC (0.09 M sodium citrate, pH 7, containing 0.9 M NaCl), 0.5% SDS, 0.1% Ficoll, 0.1% poly(vinyl pyrrolidone), 0.1% BSA, 0.1 mg/mL salmon sperm] for 4–6 h at 52 °C, the biotinylated *Renilla* cDNA fragment was added in hybridization buffer (prehybridization buffer containing 10 mM EDTA) at 100 ng/mL and incubated overnight at 52 °C. Following hybridization, the membranes were washed two times with 2× SSC, 0.1% SDS for 15 min at room temperature and then one time with 0.1× SSC, 0.1% SDS for 1 h at 52 °C. The membrane was then blocked and incubated sequentially with streptavidin and biotinylated aequorin as described above for the protein blots.

*Development of Protein and DNA Blots.* The streptavidin complex of biotinylated aequorin was visualized by developing the blots against polaroid instant or X-ray film by addition of calcium. This was accomplished by placing the membrane in a sandwich assembly against the shutter of the Dynatech camera luminometer (Chantilly, VA) containing instant film or directly against X-ray film. The sandwich consisted of a piece of plastic wrap to prevent wetting of the film, the moist membrane with the complex facing the film, and a moist piece of filter paper to prevent the blots from drying out. Light production was initiated by saturating a second piece of thicker, dry filter paper which lay on top of the sandwich with 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$. Calcium was added with a syringe in the darkroom to allow immediate exposure of the film. The films were exposed for 10–15 s and then developed according to the manufacturer's instructions.

## RESULTS

*Purification of Recombinant Apoaequorin.* As reported earlier (Cormier et al., 1989), a high level of expression of recombinant apoaequorin can be achieved by subcloning the apoaequorin cDNA into the plasmid pRC23 such that its expression is under control of the λ $P_L$ promoter (Crowl et al., 1985). Apoaequorin was overexpressed in *E. coli* containing this new recombinant plasmid, pAEQ1.3, and its purification from the crude bacterial extract was conducted as described under Experimental Procedures. As shown by SDS–PAGE analysis in Figure 1 (lanes 2 and 3), the most abundant protein in the crude extract was apoaequorin as identified by its relative mobility ($M_r \sim 22\,000$). For this reason, only two steps were required to purify apoaequorin to greater than 95% homogeneity. Gel filtration on Sephadex G-100 served to eliminate traces of contaminating proteins (lanes 4 and 5). Subsequent anion-exchange chromatography on DEAE-Sepharose (lanes 6 and 7) removed a significant amount of nonprotein (nucleic acid/peptide) contaminants (data not shown), which likely interfered with the protein determination of the crude and Sephadex G-100 fractions. Thus, in the case of the crude and G-100 fractions, the actual amount of protein loaded onto the gel in Figure 1 may have been slightly underestimated (lanes 2–5) relative to that amount loaded following the DEAE step (lanes 6 and 7), resulting in the differences observed in the intensity of the stained apoaequorin bands.

Table I: Summary of Purification of Recombinant Apoaequorin

| fraction | volume (mL) | protein (mg) | total light ($h\nu \times 10^{-15}$) | sp act.[a] [($h\nu$ mg$^{-1}$) $\times 10^{-15}$] |
|---|---|---|---|---|
| crude | 94 | 342 | 1265 | 3.7 ± 0.44 |
| Sephadex G-100 | 375 | 124 | 260 | 2.1 ± 0.20 |
| DEAE-Sepharose | 155 | 64 | 230 | 3.6 ± 0.29 |
| purified native aequorin | | | | 4.2 ± 0.43 |

[a] Mean ± standard deviation; $n$ = 4–6.

The summary of purification of apoaequorin (Table I) shows the specific activities for the crude bacterial extract and the pooled fractions obtained after Sephadex G-100 and DEAE-Sepharose chromatography. These values were determined by converting the apoaequorin to aequorin by regeneration with coelenterate luciferin and measuring total light production, as outlined under Experimental Procedures. As shown in Table I, the specific activity of recombinant apoaequorin was not notably enhanced as a result of the purification protocol. Given that the bulk of the protein (~95%) in the crude bacterial extract was apoaequorin and that there are errors associated with both protein and specific activity measurements, one would not expect a large difference in the absolute specific activities obtained from the crude, Sephadex G-100, or DEAE-Sepharose fractions. The data shown in Table I and Figure 1 also indicate that, in order to eliminate the minor protein and nonprotein contaminants from crude apoaequorin, the overall yield of apoaequorin was compromised. A possible explanation for the observation that the specific activity of the G-100 fraction was less than that of the crude and DEAE fractions is that this step may have resulted in some denaturation of apoaequorin. In this case, the Sephadex G-100 pool may exist as a mixture of denatured and active forms which the DEAE-Sepharose column was able to resolve.

It has been reported that approximately 90% of native apoaequorin can be converted to aequorin under the regeneration conditions used here (Shimomura & Johnson, 1975). Since the absolute specific activity values obtained for recombinant aequorin approached 90% of that reported for native aequorin, it appears that the regeneration reaction was very efficient (Table I). In addition, the value of $4.2 \times 10^{15}$ $h\nu$ mg$^{-1}$, found for the specific activity of native aequorin, is in good agreement with the value of $4.5 \times 10^{15}$ $h\nu$ mg$^{-1}$ reported earlier (Shimomura & Johnson, 1975).

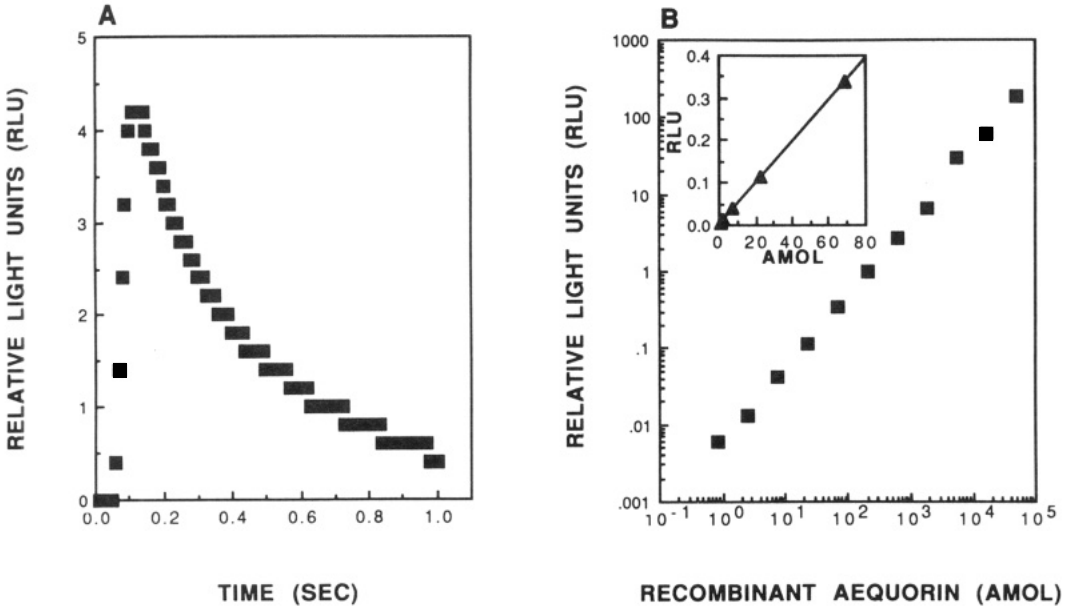


FIGURE 2: Kinetics and standard curve of recombinant aequorin light production using ML1000 Microplate luminometer. Panel A: Approximately 6 fmol (130 pg) of recombinant aequorin was placed in a microtiter well. Light production was monitored for 1 s upon injection of 200 $\mu$L of 100 mM Tris, pH 8, containing 100 mM $CaCl_2$. Panel B: Aliquots (10 $\mu$L) containing different amounts of recombinant aequorin were placed in the wells of a microtiter plate. Light production was monitored for 0.5 s upon injection of 200 $\mu$L of 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$.

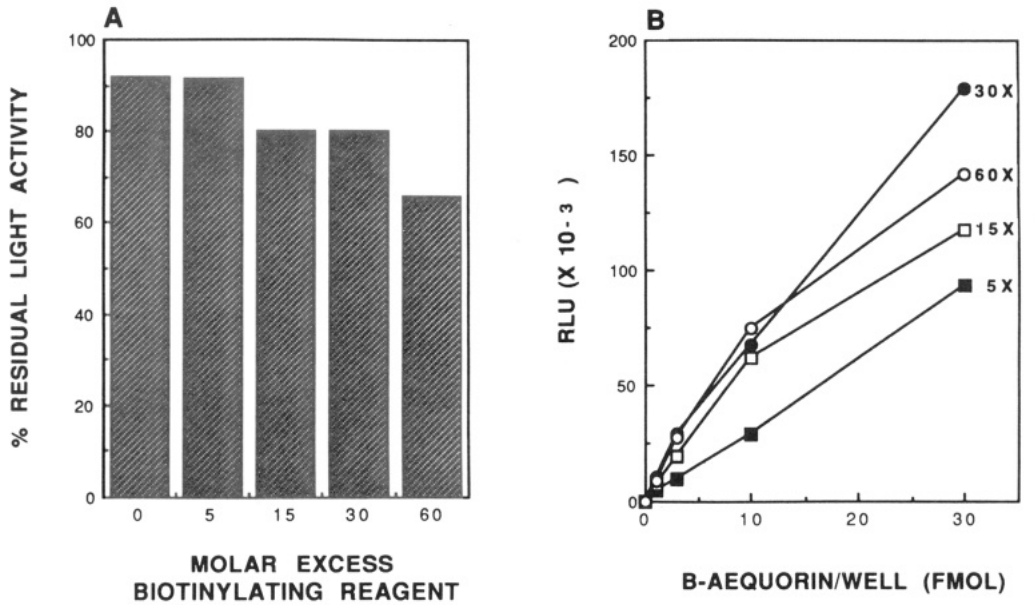


FIGURE 3: Effect of biotinylation on the activity of recombinant aequorin and its ability to bind to biotinylated BSA. Panel A: Aequorin was biotinylated at room temperature for 30 min with increasing concentrations of NHS-LC-biotin. The samples were monitored for light activity prior to and after incubation with biotinylating reagent. Panel B: Different amounts of aequorin biotinylated at different molar ratios were incubated in microtiter wells previously coated with biotinylated BSA and streptavidin. After washing, biotinylated aequorin bound to the wells was monitored upon injection of 200 $\mu$L of 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ in the ML1000 luminometer.

*Detection of Recombinant Aequorin in Microtiter Wells Using the Microlite ML1000 Luminometer.* The Dynatech Microlite ML1000 luminometer (Gehle & Lazar, 1990) is capable of injecting calcium directly into the wells while simultaneously measuring light production above the wells. Opaque, white microtiter wells are used in the luminometer to prevent possible cross-talk between the wells and to minimize absorption of light during the measurement. The kinetic profile of light generated upon addition of 100 mM calcium to approximately 6 fmol of aequorin in a microtiter plate is shown in Figure 2A. Each point on the profile represents a light measurement at 10-ms intervals for a total time of 1 s. The relative light units (RLU) are expressed as full integral values which represent the mean of the values of all the readings over the time of the measurement. Using recently expanded software developed by Dynatech for the ML1000 luminometer, it is now possible to obtain actual integrated values. The measurement of light production by recombinant aequorin in the ML1000 luminometer exhibited a wide range of linearity as shown in Figure 2B. The standard curve is linear over 7 log units of aequorin concentration from 1 amol to 10 pmol. From this standard curve, the specific activity of recombinant aequorin is approximately 200 RLU/ng.

*Biotinylation of Recombinant Aequorin.* Recombinant aequorin can be modified at its amino groups with commercially available *N*-hydroxysuccinimide ester derivatives of biotin with minimal loss in activity. Figure 3A shows the effect of increasing the molar ratio of the biotinylating reagent,

sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin), to aequorin during the biotinylation reaction on light activity. Even at a 60-fold molar excess of biotinylating reagent, aequorin retains 70% of its original activity. To assess the efficiency of biotinylation, dilutions of the biotinylated derivatives were applied to microtiter wells coated with a "lawn" of biotinylated BSA which had also been incubated with blocking protein and streptavidin. As shown in Figure 3B, lower levels of bound biotinylated aequorin are detectable on the wells when prepared at higher molar ratios of the biotinylating reagent. However, biotinylation above a 30-fold molar excess of biotinylating reagent did not afford any additional sensitivity. Biotinylation of aequorin was also confirmed by demonstrating the ability of the different preparations to bind to immobilized avidin and by SDS–PAGE in which the biotinylated derivative ran with a slightly slower mobility relative to unmodified aequorin (data not shown).

Biotinylation of apoaequorin prior to regeneration with luciferin at a 15-fold molar excess of NHS-LC-biotin offered no advantage over biotinylating aequorin directly under the same conditions. In both cases, the same specific activities (in RLU per nanogram) were obtained, and both preparations were equally efficient in their ability to bind to biotinylated BSA coated microtiter wells (data not shown). When the biotinylation reaction was routinely conducted at 100–200 $\mu$g of aequorin/mL, less than 500 amol of biotinylated aequorin bound to biotinylated BSA coated wells could be detected. The biotinylation reaction was predictably more efficient when the concentration of aequorin was high ($\sim$1 mg/mL), permitting detection of less than 50 amol of aequorin bound to the biotinylated BSA coated wells (data not shown).

*Properties and Stability of Biotinylated Recombinant Aequorin.* Biotinylated aequorin was stable when it was stored under a variety of different conditions. After three weeks of storage in TBS/E at 4 °C with a carrier protein such as BSA, biotinylated aequorin retained 80% of its original activity. After two months under these conditions, biotinylated aequorin lost approximately 60% of its original activity. Biotinylated aequorin could withstand many cycles of freeze–thaw with minimal loss of activity in the presence of carrier BSA. The biotinylated derivative was very stable when stored frozen at −20 °C; after 6 months, greater than 75% of the original activity remained. Greater than 85% of the activity of biotinylated aequorin was routinely obtained after lyophilization from TBS/E containing 5 mg/mL BSA and resuspension in water. The lyophilized powder was stored at −80 °C, at −20 °C, and at room temperature for three weeks with no loss in activity. Only 35% of the original light activity was lost when the lyophilized powder was stored for three weeks at temperatures ranging from 25 to 37 °C. Storage of the lyophilized powder at 4 °C resulted in more significant losses in activity (up to 60%) after the three-week period, probably due to the presence of moisture. Therefore, it is recommended that the lyophilized biotinylated aequorin be stored frozen in the presence of a desiccant such as anhydrous calcium sulfate. Biotinylated aequorin was stable in a variety of buffers other than TBS/E including HEPES, pH 8, and PBS, pH 7.4, containing EDTA. Although biotinylated aequorin was stable in sodium bicarbonate, pH 8.6, containing EDTA for several hours, it lost significant activity after 24 h. Therefore, after the biotinylation reaction was carried out in this buffer, biotinylated aequorin was immediately placed in TBS/E by gel filtration or dialysis.

Given that recombinant aequorin was successfully derivatized with biotin, the feasibility of using the biotinylated derivative, in combination with streptavidin, as a sensitive biochemical tag in a variety of solid-phase assays was investigated. Many different configurations are possible for the detection of molecules of interest in microtiter wells or membrane supports using streptavidin and biotinylated aequorin. The only requirement is that there be available a specific biotinylated-detecting reagent such that a streptavidin/biotinylated aequorin complex can be constructed and subsequently monitored upon addition of excess calcium by a luminometer or appropriate film. Figure 4 illustrates the specific assay configurations that are discussed in this report. The bioluminescent immunodetection of a specific glycolipid immobilized directly to microtiter wells using a primary antibody coupled with a biotinylated secondary antibody is shown in panel A. In panel B, protein targets transferred to membrane supports are detected by the appropriate primary and biotinylated secondary antibodies. In the case of nucleic acid detection, the DNA of interest is immobilized onto membranes and then certain sequences are specifically identified by hybridization with an appropriate biotinylated DNA probe as depicted in panel C.

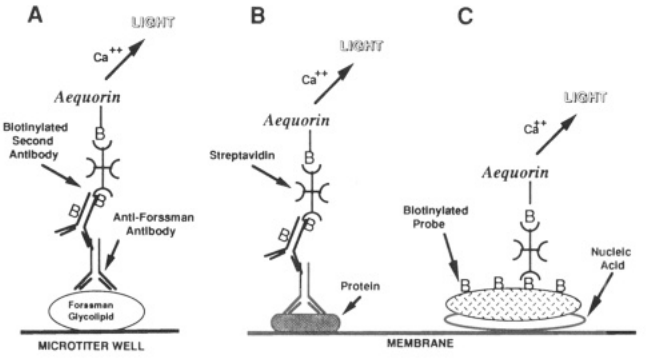


FIGURE 4: Schematic representation of various assay configurations for the bioluminescence detection of glycolipids, protein antigens, and nucleic acids immobilized on microtiter wells or membrane supports using biotinylated aequorin.

*Use of Biotinylated Aequorin in the Detection of Forssman Glycolipid.* The application of biotinylated aequorin in a microtiter plate-based assay for the nonradioactive immunodetection of a glycosphingolipid known as the Forssman antigen (GalNAc$\alpha$1–3GalNAc$\beta$1–3Gal$\alpha$1–4Gal$\beta$1–4Glc$\beta$Cer) was investigated. The bioluminescent assay developed for the Forssman glycolipid was based on the specificity of *Helix pomatia* lectin and two monoclonal antibodies for the Forssman oligosaccharide (see Figure 4A). Preliminary experiments were conducted using biotinylated *H. pomatia*, a snail lectin which recognizes $\alpha$1,3-linked GalNAc (*N*-acetylgalactosamine) residues. In this assay configuration, Forssman glycolipid immobilized onto microtiter wells was recognized by the biotinylated lectin, which was subsequently identified using streptavidin and biotinylated aequorin. The assay was found to be specific for the Forssman glycolipid since relatively little light activity bound to wells coated with globoside, the immediate precursor to the Forssman antigen which has terminal $\beta$1,3-linked GalNAc and interacts poorly with biotinylated *H. pomatia*. In addition, if the incubation of Forssman-coated wells with the biotinylated lectin was conducted in the presence of GalNAc, which inhibits the interaction, very little light activity was bound by the wells (data not shown).

In addition to using biotinylated *H. pomatia*, it was possible to use two monoclonal antibodies against the Forsmann antigen in a bioluminescence immunoassay (BLIA) with comparable results. The rat IgM antibodies, obtained from the culture supernatants of hybridomas M1/87 and M1/23 from the

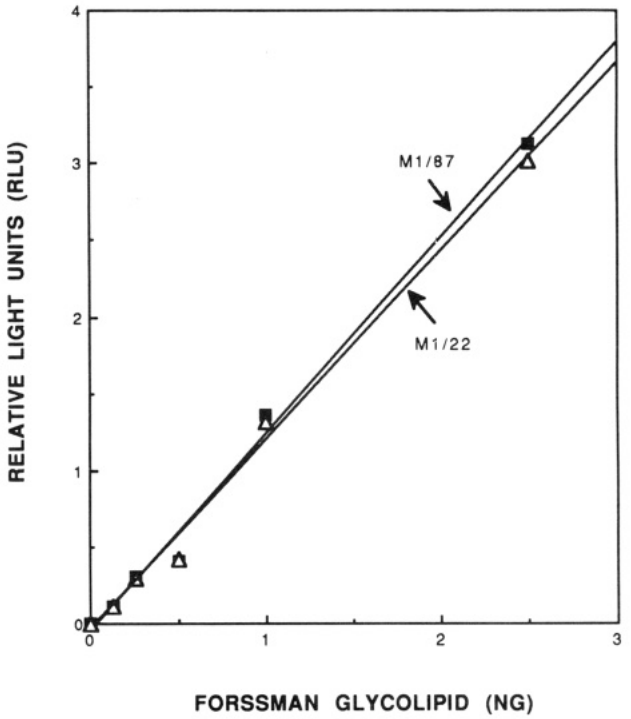


FIGURE 5: Detection of Forssman glycolipid using a bioluminescent immunoassay. Different amounts of Forssman glycolipid were immobilized onto microtiter wells and detected using two different anti-Forssman monoclonal antibodies (M1/22 and M1/87). Following sequential incubation with biotinylated anti-rat antibody, streptavidin, and biotinylated aequorin, bound aequorin was determined upon injection of 200 μL of 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ in the ML1000 luminometer.

American Type Culture Collection, were used in combination with a biotinylated anti-rat antibody for the identification of the Forssman glycolipid. The resulting biotinylated second antibody/anti-Forssman antibody/glycolipid complex was detected using streptavidin and biotinylated aequorin. In Figure 5, dilutions of Forssman antigen were allowed to bind to the wells of an opaque microtiter plate. After the appropriate incubations and washes were conducted, the presence of specifically bound biotinylated aequorin was quantified in the Dynatech ML1000 luminometer upon injection of calcium. The data indicate that the assay was linear and easily capable of detecting less than 0.1 ng (~40 fmol) of the antigen using either anti-Forssman monoclonal antibody. Wells coated with up to 5 ng of globoside bound background levels of biotinylated aequorin similar to that obtained when the wells were coated with the carrier solvent. In addition, very low levels of light activity were found associated with Forssman-coated wells when a nonspecific primary antibody, in this case directed against soybean calcium-dependent protein kinase, was substituted for the Forssman monoclonals.

*Use of Biotinylated Aequorin in the Detection of Proteins and DNA on Membranes.* We have also investigated the application of biotinylated aequorin in the detection of biotinylated protein and nucleic acid targets immobilized on nitrocellulose and nylon membranes. Streptavidin/biotinylated aequorin complexes were detected on the membranes by saturating the blots, which were placed directly against either instant (Polaroid 612 ASA 20000) or X-ray film, with calcium-containing buffer. Preliminary studies in the dot blot format indicated the feasibility of using biotinylated aequorin in such applications (Stults et al., 1991). Immunodetection of human transferrin in dot blots was acomplished by sequential incubation with rabbit anti-human transferrin,

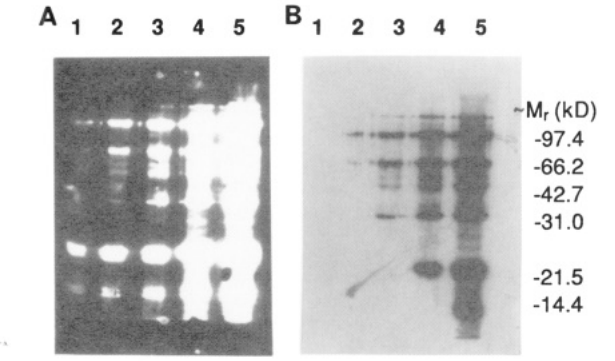


FIGURE 6: Bioluminescent detection of biotinylated proteins immobilized on nitrocellulose blots. Different amounts of biotinylated proteins were subjected to SDS–PAGE and transferred to nitrocellulose membranes, and the blots were incubated sequentially with streptavidin and biotinylated aequorin. The bands were visualized by saturating the blots with 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ while juxtaposed to polaroid instant film (panel A) and X-ray film (panel B). Lanes 1–5 were loaded with 3, 10, 29, 83, and 260 ng of total protein, respectively. The position and approximate molecular masses (in kilodaltons) of the biotinylated protein standards are indicated.

biotinylated goat anti-rabbit antibody, streptavidin, and biotinylated aequorin (see Figure 4B). It was possible to detect between 6 and 60 pg of human transferrin in dot blots on both types of membranes. The sensitivity compared favorably with that obtained with a goat anti-rabbit antibody conjugate of alkaline phosphatase using the substrate pair BCIP/NBT (data not shown). Bioluminescent detection of proteins in the Western blot format was also examined using the instant film and standard X-ray film (Kodak X-OMAT AR). In Figure 6, different amounts of commercially available biotinylated protein standards were separated by SDS–PAGE and then transferred to nitrocellulose. The blots were probed by sequential incubation with streptavidin and biotinylated aequorin and developed against instant and X-ray film. The instant film was more sensitive where the best results were obtained when 10–30 ng of total protein was loaded onto the gel (lanes 2 and 3). The best pattern on the X-ray film corresponded to 80 ng of total protein (lane 4). The instant film was routinely about 2–3-fold more sensitive than the X-ray film in the detection of biotinylated aequorin. Preflashing the X-ray film was found to lower the detection limit by approximately 2-fold, thereby making its sensitivity comparable to that of the instant film (data not shown).

The question of whether biotinylated aequorin could be used to detect different antigens in a complex mixture after Western blotting was addressed in Figure 7. Rabbit serum and human serum were fractionated by SDS–PAGE, transferred to nitrocellulose, and probed using the appropriate antibody reagents in combination with streptavidin and biotinylated aequorin for the presence of immunoglobulin G and transferrin, respectively. The heavy (~55 kDa) and light (~25 kDa) chains of the immunoglobulin were visualized in lanes containing both purified rabbit IgG and rabbit serum using biotinylated aequorin (Figure 7A). A band corresponding to transferrin (~78 kDa) was observed in lanes loaded with purified transferrin and human serum (Figure 7B). A second transferrin blot was probed in parallel with biotinylated alkaline phosphatase and BCIP/NBT (Figure 7C). Biotinylated aequorin detection showed comparable, if not greater, sensitivity to the results obtained with the enzyme detection system.

Detection of DNA using biotinylated aequorin was successful in both dot blot and Southern blot formats (see Figure 4C). Figure 8 shows an example of a Southern blot hybridization experiment in which different amounts of the linearized

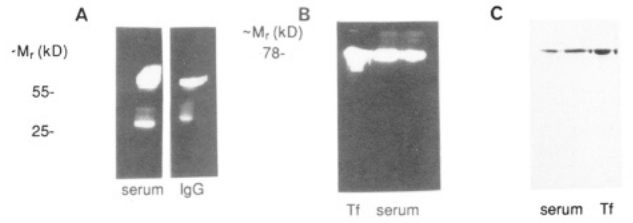


FIGURE 7: Bioluminescent immunodetection of rabbit IgG and human transferrin after Western blotting. The purified proteins and appropriate sera were subjected to SDS–PAGE and transferred to nitrocellulose. Panel A: Blots with lanes containing purified rabbit IgG (12.5 $\mu$g) or rabbit serum (10 $\mu$L of a 1/8 dilution) were incubated sequentially with biotinylated goat anti-rabbit antibody, streptavidin, and biotinylated aequorin. Panels B and C: Blots with lanes containing purified human transferrin (320 ng) and human serum (5 and 10 $\mu$L of 1/20 dilution) were incubated sequentially with rabbit anti-transferrin antibody, biotinylated goat anti-rabbit antibody, streptavidin, and biotinylated aequorin (B) or biotinylated alkaline phosphatase (C). In panels A and B, the bands were visualized by saturating the blots with 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ while juxtaposed to polaroid instant film. In panel C, the bands were visualized by incubating with BCIP/NBT to generate the purple reaction product.

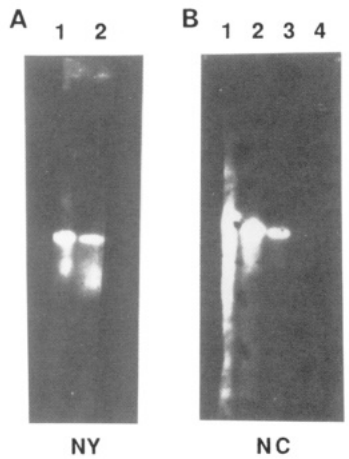


FIGURE 8: Bioluminescent detection of DNA after Southern blotting. Different amounts of the linearized pTZR-Luc1 plasmid containing *R. reniformis* luciferase cDNA was subjected to agarose gel electrophoresis and transferred to nylon (panel A) and nitrocellulose (panel B) membranes. Following prehybridization, the target DNA was probed with the biotinylated *Renilla* luciferase cDNA fragment. Following hybridization and washing, the blots were blocked and incubated sequentially with streptavidin and biotinylated aequorin. The bands were visualized by saturating the blots with 100 mM Tris, pH 7.5, containing 100 mM $CaCl_2$ while juxtaposed to polaroid instant film. In panel A, lanes 1 and 2 were loaded with 250 and 125 ng of target DNA. In panel B, lanes 1–4 were loaded with 500, 125, 31, and 8 ng of target DNA, respectively.

pTZR-Luc1 plasmid containing the *Renilla* luciferase cDNA insert were subjected to agarose gel electrophoresis, transferred to nitrocellulose and nylon membranes, and then probed with the biotinylated *Renilla* cDNA fragment generated by restriction enzyme digestion of the plasmid. After hybridization, the blot was incubated sequentially in blocking protein, streptavidin, and biotinylated aequorin. A single band of target DNA corresponding to about 5 kb was seen in all lanes on both membranes. There is a very faint, but discernible band on the nitrocellulose blot in lane 4 of panel B corresponding to 8 ng of target DNA. This level of sensitivity was comparable to that obtained with biotinylated alkaline phosphatase and BCIP/NBT in a parallel Southern blot (data not shown).

## Discussion

The demand for more sensitive labels for the detection and identification of biological molecules has led to the rapid development of bioluminescent and chemiluminescent assay technology over the last 10 years [reviewed by Kricka (1990)]. That bioluminescent and chemiluminescent reactions be exploited for this purpose is due to the relatively high quantum yields of light production and the availability of instrumentation to detect light at very low levels. Such interest has led to the cloning of various bioluminescent proteins including luciferases from marine bacteria (Boylan et al., 1989), firefly (de Wet et al., 1985), and *Renilla* (Lorenz et al., 1991), which exhibit "glow kinetics", and the calcium-dependent photoprotein, aequorin, which exhibits "flash kinetics". The luciferases have been used primarily as reporters for gene expression and promoter activity in both prokaryotic and eukaryotic systems and, in the case of firefly luciferase, as a sensitive marker for ATP. In general, the subunit complexity, instability, and absolute cofactor requirements of the bacterial and firefly enzymes make them less suitable for direct biochemical markers such as alkaline phosphatase or horseradish peroxidase. However, *Renilla* luciferase, which is a single polypeptide chain of $M_r$ 36 000 requiring only coelenterate luciferin for activity, has been successfully derivatized with biotin and used in membrane-based applications (Stults et al., 1991).

Aequorin cDNA has been expressed in *E. coli* using a number of different plasmid vector constructs (Prasher et al., 1985, 1986, 1987; Inouye et al., 1985, 1986). Using the pAEQ1.3 recombinant temperature-sensitive plasmid discussed in this report, high levels of expression in *E. coli* were obtained (4–5 mg/L of fermentor culture), and a simple protocol was developed for the extraction and purification of the apoprotein (Figure 1). The recombinant apoprotein was found to be fully functional as it was readily converted to aequorin, which had a specific activity similar to that of native aequorin (Table I). Additional improvements in the expression levels of recombinant aequorin have been recently reported. Apoaequorin cDNA (AQ440) has been fused to the signal peptide coding sequence of the outer membrane protein A (*omp*A) of *E. coli* (Inouye et al., 1989). Subsequent expression of this cDNA in *E. coli* results in a high-level production of apoaequorin and its release into the culture medium (7.4 mg/200 mL). Further optimization of this expression system has resulted in a further 8-fold increase in the production of apoaequorin (Inouye et al., 1991). In addition, a fusion protein consisting of aequorin and an antibody has been constructed and expressed in mammalian cells (Casadei et al., 1990). Galactose-dependent expression of aequorin in yeast has also been accomplished with the goal of monitoring intracellular calcium levels in vitro (Nakajima-Shimada et al., 1991).

Aequorin has been traditionally used by cell biologists and physiologists for monitoring levels of intracellular calcium. The availability of recombinant aequorin makes possible the widespread use of this protein as a calcium indicator and, with appropriate derivatization, a sensitive tag for the detection of many compounds of biological importance such as enzymes and antigens. The sensitivity with which it can be measured, coupled with the linearity of light emission over many log units of concentration (Figure 2), makes aequorin an ideal marker for any assay configuration which can accommodate flash kinetics. Aequorin can be biotinylated with minimal loss in activity (Figure 3), and the biotinylated derivative can be stored under a variety of conditions for long periods of time. Although sequential incubations are described here for the detection of biotinylated targets with streptavidin and biotinylated aequorin, it is possible to use a preformed complex, thereby reducing the amount of time required to conduct the

assay. Direct conjugates of recombinant aequorin with antibodies or lectins would facilitate even faster detection of biological targets of interest by eliminating the avidin/streptavidin/biotin interaction entirely. We have demonstrated the feasibility of using antibody conjugates of aequorin in the detection of Salmonella antigen (Smith et al., 1991) and protein antigens immobilized on membrane supports (Stults et al., 1991).

The sensitivity of detection of Forssman glycolipid in the BLIA (Figure 5) is at least an order of magnitude better than the radioactive overlay technique for glycolipid detection on thin-layer chromatographic plates (Magnani et al., 1980; Smith, 1983) or its adaptation on microtiter wells (Torres et al., 1988). The BLIA exhibits a wide range of linearity and has the added advantage of being nonradioactive and significantly faster, requiring less than 1 s per well for the measurements of light activity. It would be predicted, on the basis of the success of using biotinylated aequorin for the detection of proteins and nucleic acids immobilized on membrane supports, that a similar bioluminescence assay could be conducted on thin-layer chromatograms using film detection or luminographic scanning instrumentation. The current sensitivity of the BLIA for Forssman antigen, however, has not reached the theoretical limit of sensitivity for the detection of aequorin (see Figure 2B). Optimization of the various parameters which influence the sensitivity of the assay, e.g., degree of biotinylation of the lectin or secondary antibody, factors contributing to nonspecific binding, and the incubation conditions, will be required to further enhance the sensitivity of the assay. Several glycolipid antigens, such as the 19-9 antigen present in colorectal carcinoma (Basso et al., 1988), have been reported to be associated with malignancy (Hakomori, 1983; Nudelman et al., 1988) and are currently under study for their reliability as diagnostic markers. The availability of a rapid nonradioactive screening method such as the Forssman BLIA described here should facilitate the establishment of the diagnostic relevance of such glycolipid antigens.

The biotinylated derivative of aequorin can be also used for the detection of protein and nucleic acid targets immobilized on membrane supports after Western and Southern blotting (Figures 6–8) using either instant or X-ray film. The sensitivity of detection of protein antigens immobilized on both nitrocellulose and nylon supports using biotinylated antibody reagents coupled with streptavidin and biotinylated aequorin (Figures 6 and 7) was comparable to, if not more sensitive than, alkaline phosphatase detection with BCIP/NBT. In addition, hybridization of biotinylated DNA probes to DNA targets could be identified by the presence of the bound streptavidin/biotinylated aequorin complex on the membranes (Figure 8). Bioluminescent detection offers a rapid and sensitive alternative to radioactive and enzyme detection systems currently employed for the development of Western and Southern blots. It is not necessary to expose the film for long periods of time, as is the case with radioactive probes, or to wait for the accumulation of product which is a requirement of the enzyme-based assays. In addition, it does not involve the use of radioactive or toxic substrates and a permanent, nonfading record of the blots is obtained. With further development of conjugates and sensitive films as well as optimization of the detection protocols, it is anticipated that bioluminescent detection using recombinant aequorin will offer additional convenience and sensitivity.

A variety of chemiluminescent detection systems have been exploited for the subnanogram detection of nucleic acids immobilized on membrane supports including the phosphorylated dioxetanes using alkaline phosphatase (Schaap et al., 1987, 1989; Bronstein et al., 1990), acridinium esters (Septak, 1989), and enhanced chemiluminescence using horseradish peroxidase and luminol derivatives (Thorpe & Kricka, 1986). More recently, some of these chemiluminescent reagents have been adapted to protein detection on membranes (Gillespie & Hudspeth, 1991a,b) and are also currently being developed for immunoassays [reviewed by McCapra et al. (1989)]. While both bioluminescent and chemiluminescent labels can theoretically facilitate the nonradioisotopic, ultrasensitive detection of biological analytes, each of these detection systems possesses its own unique advantages such that its performance will prove superior in certain, but not all, assay configurations. With further investigation, it will be possible to ascertain the label of choice for a given research or diagnostic application.

In summary, the availability of recombinant aequorin, which can be detected at attomole levels, and the ability to derivatize the photoprotein with minimal loss in light activity provide the basis for a new bioluminescent technology. The sensitivity, speed, and ease-of-use of biotinylated aequorin in the detection of Forssman antigen as well as protein and nucleic acid targets point to its general utility as a universal reagent which can theoretically replace any biotinylated label currently used in combination with avidin/streptavidin in ELISA or membrane-based assays. Derivatives of aequorin can be applied to the detection of other classes of biologically important molecules on various solid-phase supports. For example, biotinylated aequorin and antibody conjugates of aequorin have been successfully substituted in a capture immunoassay for Salmonella antigen as the detection reagent (Smith et al., 1991) and in solid-phase assays for glycosyl transferases and glycoproteins (Mengeling et al., 1991; Zatta et al., 1991a,b).

## References


Allen, D. G., Blinks, J. R., & Prendergast, F. G. (1977) *Science (Washington, D.C.) 195*, 996–998.

Basso, D., Fabris, C., Del Favero, G., Panucci, A., Plebani, M., Angonese, C., Leandro, G., Dodi, G., Burlina, A., & Naccarato, R. (1988) *Oncology 45*, 24–29.

Blinks, J. R., Wier, W. G., Hess, P., & Prendergast, F. G. (1982) *Prog. Biophys. Mol. Biol. 40*, 1–114.

Boylan, M., Pelletier, J., & Meighen, E. A. (1989) *J. Biol. Chem. 264*, 1915–1918.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–252.

Bronstein, I., & Kricka, L. J. (1990) *Am. Clin. Lab. (Jan/Feb)*, 33–37.

Bronstein, I., Voyta, J. C., Lazzari, K. G., Murphy, O., Edwards, B., & Kricka, L. J. (1990) *BioTechniques 8*, 310–314.

Casadei, J., Powell, M. J., & Kenten, J. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2047–2051.

Charbonneau, H., Walsh, K. A., McCann, R. O., Prendergast, F. G., Cormier, M. J., & Vanaman, T. C. (1985) *Biochemistry 24*, 6763–6771.

Cormier, M. J. (1978) in *Bioluminescence in Action* (Herring, P., Ed.) pp 75–108, Academic Press, London.

Cormier, M. J., Prasher, D. C., Longiaru, M., & McCann, R. O. (1989) *Photochem. Photobiol. 49*, 509–512.

Crowl, R., Seamans, C., Lomedico, R., & McAndrew, S. (1985) *Gene 38*, 31–38.

de Wet, J. R., Wood, K. V., Helinski, D. R., & DeLuca, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7870–7873.

Gehle, W. D., & Lazar, B. S. (1990) *Am. Biotechnol. Lab. 8*, 42–45.

Gillespie, P. G., & Hudspeth, A. J. (1991a) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2563–2567.

Gillespie, P. G., & Hudspeth, A. J. (1991b) *J. Cell Biol. 112*, 625–640.
Hakomori, S. (1984) *Annu. Rev. Immunol. 2*, 103.
Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T., & Tsuji, F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3154–3158.
Inouye, S., Sakaki, Y., Goto, T., & Tsuji, F. (1986) *Biochemistry 25*, 8425–8429.
Inouye, S., Aoyama, S., Miyata, T., Tsuji, F., & Sakaki, Y. (1989) *J. Biochem. 105*, 473–477.
Inouye, S., Zenno, S., Sakaki, Y., & Tsuji, F. I. (1990) VIth International Symposium on Bioluminescence and Chemiluminescence, Cambridge, England, September 10–13, 1990.
Kricka, L. J. (1990) *Am. Clin. Lab. (Nov/Dec)*, 30–32.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lee, J., & Seliger, H. H. (1972) *Photochem. Photobiol. 15*, 227–237.
Lee, J., Wesley, A. S., Ferguson, J. F., & Seliger, H. H. (1966) in *Bioluminescence in Progress* (Johnson, F. H., & Haneda, Y., Eds.) pp 35–43, Princeton University Press, Princeton, NJ.
Lorenz, W. W., McCann, R. O., Longiaru, M., & Cormier, M. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4438–4442.
Magnani, J. L., Smith, D. F., & Ginsburg, V. (1980) *Anal. Biochem. 109*, 399–402.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McCapra, F., Watmore, D., Sumun, F., Patel, A., Beheshti, I., Ramakrishnan, K., & Branson, J. (1989) *J. Biolumin. Chemilumin. 4*, 51–58.
Mengeling, B. J., Smith, P. L., Stults, N. L., Smith, D. F., & Baenziger, J. U. (1991) *Anal. Biochem.* (in press).
Nakajima-Shimada, J., Iida, H., Tsuji, F. I., & Anraku, Y. (1991) *Biochem. Biophys. Res. Commun. 174*, 115–122.
Nudelman, E. D., Levery, S. B., Stroud, M. R., Salyan, M. E. K., Abe, K., & Hakomori, S. (1988) *J. Biol. Chem. 263*, 13942–13951.
O'Kane, D. J., & Lee, J. (1990) *Photochem. Photobiol. 52*, 723–734.
Prasher, D., McCann, R. O., & Cormier, M. J. (1985) *Biochem. Biophys. Res. Commun. 126*, 1259–1268.
Prasher, D. C., McCann, R. O., & Cormier, M. J. (1986) *Methods Enzymol. 133*, 288–298.
Prasher, D. C., McCann, R. O., Longiaru, M., & Cormier, M. J. (1987) *Biochemistry 26*, 1326–1332.
Schaap, A. P., Sandison, M. D., & Handley, R. S. (1987) *Tetrahedron Lett. 28*, 1159–1162.
Schaap, A. P., Akhavan, H., & Romano, L. J. (1989) *Clin. Chem. 35*, 1863.
Septak, M. (1989) *J. Biolumin. Chemilumin. 4*, 351–356.
Shimomura, O., & Johnson, F. H. (1975) *Nature 256*, 236–238.
Shimomura, O., Johnson, F. H., & Saiga, Y. (1962) *J. Cell. Comp. Physiol. 59*, 223–240.
Smith, D. F. (1983) *Biochem. Biophys. Res. Commun. 115*, 360–367.
Smith, D. F., Stults, N. L., Rivera, H., Gehle, W. D., Cummings, R. D., & Cormier, M. J. (1991) *Proceedings of the VIth International Symposium on Bioluminescence and Chemiluminescence*, Cambridge, England, September 10–13, 1990, pp 529–532.
Sparrow, E. M. (1973) in *Handbook of Heat Transfer* (Rohsenow, W. M., & Hartnett, J. P., Eds.) section 15-29–15-52, McGraw-Hill, New York.
Stults, N. L., Stocks, N. A., Cummings, R. D., Cormier, M. J., & Smith, D. F. (1991) *Proceedings of the VIth International Symposium on Bioluminescence and Chemiluminescence*, Cambridge, England, September 10–13, 1990, pp 533–536.
Thorpe, G. H. G., & Kricka, L. J. (1986) *Methods Enzymol. 133*, 331–353.
Torres, V., McCrumb, D. K., & Smith, D. F. (1988) *Arch. Biochem. Biophys. 262*, 1–11.
Wampler, J. E. (1975) in *Analytical Applications of Bioluminescence and Chemiluminescence* (Chappelle, E. W., & Picciolo, G. L., Eds.) pp 105–115, National Aeronautics and Space Administration, Washington, DC.
Ward, W. (1985) in *Chemi- and Bioluminescence* (Burr, J. G., Ed.) pp 321–358, Marcel Dekker, New York.
Zatta, P. F., Nyame, K., Cormier, M. J., Mattox, S. A., Prieto, P. A., Smith, D. F., & Cummings, R. D. (1991a) *Anal. Biochem. 194*, 185–191.
Zatta, P. F., Smith, D. F., Cormier, M. J., & Cummings, R. D. (1991b) *Proceedings of the VIth International Symposium on Bioluminescence and Chemiluminescence*, Cambridge, England, September 10–13, 1990, pp 537–540.