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Bioluminescent Immunoassay Using a Fusion Protein of Protein A and the Photoprotein Aequorin

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Aequorin is a photoprotein that emits light in the presence of Ca^{2+} ions. To develop a bioluminescent immunoassay based on the light emission property of aequorin, we have expressed the apoaequorin fusion protein with *S. aureus* protein A in *E. coli* by recombinant DNA techniques. The fusion protein expressed was purified by IgG-Sepharose affinity chromatography, gel filtration and HPLC procedures. The purified protein A-apoaequorin fusion protein has both the luminescent activity of aequorin and the IgG-binding ability of protein A. We compared results obtained using the protein A-aequorin fusion protein with those obtained using a protein A conjugated horseradish peroxidase based immunoassay, and found them to yield similar results.

Aequorin is a bioluminescent photoprotein isolated from the outer margin of the umbrella of the jellyfish, *Aequorea victoria* (1) and is present as a complex with apoprotein (apoaequorin) and coelenterazine through molecular oxygen (2, 3). The luminescent reaction is initiated by Ca^{2+}-binding to aequorin and yields light (λmax = 470 nm), CO_2, apoaequorin and coelenteramide (2). Aequorin can be reactivated by the incubation of apoaequorin with coelenterazine, dissolved molecular oxygen, a reducing agent and EDTA (4). Recently, cloning and sequence analysis of the cDNA for apoaequorin have revealed that aequorin is composed of 189 amino acid residues (M_r = 21,400) arranged in a single polypeptide chain (5, 6). The protein has three EF-hand structures characteristic of Ca^{2+}-binding sites (5, 7). It has been demonstrated that the assay of aequorin is a highly sensitive (detection of photon), non-hazardous (no use of radioactive compound), fairly rapid reaction (few seconds reaction) and simple reaction (one test tube reaction) (8, 9). We have already established an expression system for the apoaequorin cDNA in *E. coli* and purified the protein using a convenient procedure (10). The recombinant apoaequorin is a stable protein at low pH (10) and at high temperatures (11), and can be regenerated to aequorin possessing luminescent

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Abbreviations:
BAP, Bacterial alkaline phosphatase; kDa, kilodaltons; Fc, constant region of immunoglobulins; FPLC, fast protein liquid chromatography; HRP, horseradish peroxidase; IgG, Immunoglobin; rlu, relative light units; proA-apo protein, protein A-apoaequorin (apoprotein) fusion protein; proA-aeq protein, protein A-aequorin fusion protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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activity (10,11). Based on these properties, the apoprotein of aequorin may be a useful protein for immunoassay systems, analogous to conjugated proteins (enzymes) such as alkaline phosphatase (12) and horseradish peroxidase (13). Protein A, isolated from *S.aureus*, has a high specific affinity for IgG species and has been used for the purification of IgG and protein A conjugated enzymes are also used for the detection of IgG in immunoassays. Using this background information, we have tried to construct the bi-functional protein which has both the luminescent activity of aequorin and the IgG-binding ability of protein A by recombinant DNA techniques. This paper describes the expression and purification of the Protein A-apoaequorin fusion protein (proA-apo protein) in *E. coli*, and discusses the possibility of its use in a bioluminescent immunoassay.

MATERIALS AND METHODS

Materials. All restriction endonucleases and *E. coli* T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Toyama, Japan). Wakosil 5C4 column for HPLC was obtained from Wako Pure Chemicals (Osaka, Japan). Coelenterazine, 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-3, 7-dihydroimidazo [1,2-a]pyrazin-3-one, was synthesized as previously described (14). Immunoassay kit (goat anti-rabbit horseradish peroxidase labeled antibody) and protein A polyclonal antibody screening kit were products of Bio-Rad (Richmond CA, USA). Rabbit anti-goat IgG F(ab')2 was obtained from E.Y.Lab.(CA, USA), goat anti-rabbit IgG F(ab')2 from Jackson Immuno Research Lab. (PA, USA), and rabbit anti-protein A from Sigma (St.Louis MO, USA). Rabbit IgG fraction of polyclonal antibody to the recombinant apoaequorin was kindly provided by Dr.Tsuji F.I. (Osaka Bioscience Institute, Osaka, Japan). IgG-Sepharose 6 Fast Flow and Superose 12 were purchased from Pharmacia LKB (Uppsala, Sweden). All other chemicals were of the highest grade commercially available.

Construction of proA-apo fusion gene expression plasmid and expression in *E. coli*. An expression plasmid, pAAQ1, possessing the protein A gene and apoaequorin cDNA was constructed as shown in Fig.1. The protein A fusion vector pIRT5 (Pharmacia; 15) contained the promoter, the signal sequence and a truncated form of protein A gene (1-1103bp) including the region coding for the IgG-binding domains. The *Eco*RI fragment (565bp) of apoaequorin cDNA from piP-HE (10) was inserted into the *Eco*RI site of pRIT5. The fusion protein which was expressed in the periplasmic space of *E. coli* cells, consists of 462 amino acid residues lacking the N-terminal signal peptide sequence (16, 17). *E. coli* strain JM83 (18) was used for the expression experiments and was grown in LB medium (19) containing 50 μg/ml ampicillin at 37°C for 15 hrs with shaking.

Purification of proA-apo protein from *E. coli*. The purification was carried out at room temperature unless otherwise stated. The cells from 200 ml of culture suspension were harvested by centrifugation and resuspended in an adequate volume of 30 mM Tris-HCl (pH 7.6) containing 10 mM EDTA. Cell disruption was achieved by sonication with a Branson Model 250 Sonifier. After centrifugation at 18,000 x g for 30 min, the supernatant was stored at 4°C before use. The resultant supernatant (20 ml) was applied directly to a 1.5 x 10 cm IgG Sepharose 6FF column. After the column was washed with 100 ml of 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.05% Tween20 followed by 30 ml of 5 mM ammonium acetate (pH 5.0), the fusion protein was eluted with 100 mM glycine-HCl (pH 3.0). Flow rates were 2 ml/min for washing and 0.2 ml/min for elution, respectively. Peak fractions were pooled, adjusted to pH 7.6 with ammonia water and concentrated by Centricon (Amicon, MA, USA). A fifty microliter aliquot of the concentrated sample was subjected to gel filtration using a FPLC system equipped with a Superose 12 column (1 x 30 cm). The resulting fractions with the highest aequorin activity were pooled and applied onto a Wakosil 5C4 (0.46 x 10 cm) using a Beckman model 344 HPLC system with 163 variable wavelength detector. Elution was performed with a linear gradient of acetonitrile (20-80%) in the presence of 0.1% trifluoroacetic acid. Flow rate was 0.8ml/min.

Protein analysis Electrophoresis was performed essentially as described by Laemmli (20), using a separation gel of 12.5% polyacrylamide (0.1 x 9 x 6 cm) and run at 15 mA for 3 hr. Protein concentration was determined by the dye-binding method of Bradford (21),using reagents and protein standards supplied by Bio-Rad.

Western blot analysis After electrophoresis, the proteins were transferred electrophoretically to nitrocellulose sheets with an Atto transfer apparatus (AE-6670P/N, Tokyo,

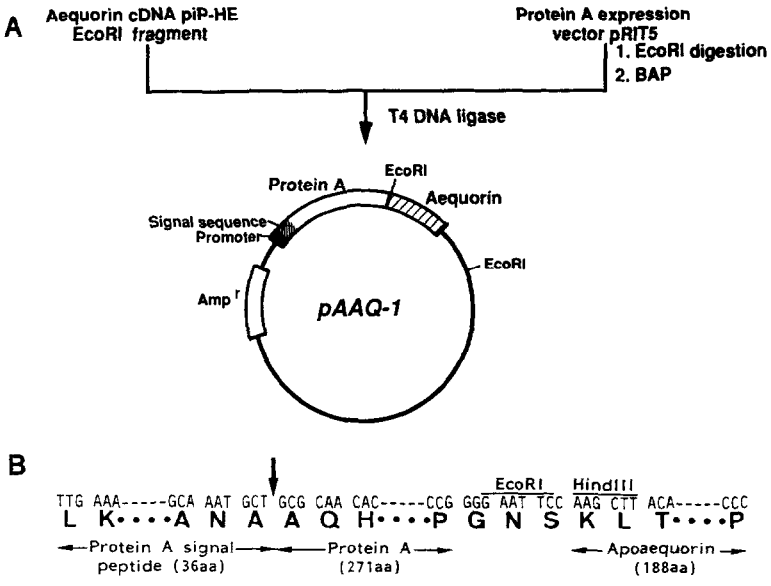

Figure 1. Scheme for the construction of the proA-apo protein expression plasmid. (A) The strategy for the construction of pAAQ1. The coding region of apoaequorin and protein A genes are shown as the shaded box and the open box, respectively. Amp^r represents the ampicillin resistant gene. (B) An outline of the protein A signal peptide, the protein A and the apoaequorin fusion protein. Arrow shows the possible cleavage site of the protein A signal peptide in *E. coli*.

Japan) at 70 mA for 12 hrs at 4°C (22). The nitrocellulose sheets were blocked with 3% gelatin in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl (TBS) for 30 min, washed 2 times with TBS containing 0.05% Tween 20 (TTBS) on a shaker and then incubated with polyclonal anti-apoaequorin or anti-protein A antibodies at room temperature for 1 hr. After washing again, the sheets were incubated with HRP labeled anti-rabbit IgG for 2 hr at room temperature and then washed with TTBS and TBS, respectively. The specific bound antibodies were visualized by incubation with 0.3% 4-chloro-1-naphtol (Bio-Rad) and 0.1% hydrogen peroxide in TBS. The proteins transferred to nitrocellulose sheets were also checked by staining in 0.1% Amido-Black 10B (Bio-Rad) dissolved in methanol-acetic acid-water (30:10:60, v/v/v).

Assay for the luminescent activity of proA-aeq protein. The proA-aeq protein was regenerated from proA-apo protein by adding 1 µl of 2-mercaptoethanol, 4 µl of coelenterazine (2 µg/ml, in methanol) and 177 µl of 30 mM Tris-HCl (pH 7.6) containing 10 mM EDTA to 20 µl of the sample solution, and allowing the mixture to stand for 2 hrs at 4°C. The above mixture was placed in a Laboscience Model TD4000 lumiphotometer (Tokyo, Japan) and was injected with 100 µl of 30 mM $CaCl_2$/30 mM Tris-HCl (pH 7.6). The luminescence was recorded and the values were presented in relative light units (rlu) (10). One rlu is equivalent to the luminescent activity from 2.5 pg of native aequorin.

Assay for horseradish peroxidase (HRP) activity HRP activity was determined by a colormetric procedure using 2,2'-azino-di[3-ethylbenzthizoline sulfonate] as a substrate, as described by Caniarero *et al.* (23). The assay was performed at 37°C for 1 hr and the activity was measured at 420 nm. One unit is defined as that amount protein yielding 1 mol of chromophore.

Bioluminescent Immunoassay As a model system, IgG $F(ab')_2$ fragment and anti-IgG $(Fab')_2$ were used as antigen and primary antibody, respectively. The proA-aeq protein or HRP-conjugated protein A were used for the detection of the primary antibody. Polystyrene tubes (10 x 65 mm, Falcon, NJ, USA) were washed with 2 N NaOH at 60°C for 30 min before use. Procedures were as follow: First, 100 µl of goat anti-rabbit IgG $F(ab')_2$ (26 µg/ml) was added into the polystyrene tube and incubated at 37°C for 1 hr. After unbound $F(ab')_2$ was removed, the polystyrene tubes were washed 5 times with TBS and completely filled with blocking solution (1% BSA in TBS), followed by incubation at 37°C for 1 hr. The blocking solution was then discarded and the tubes were washed 5 times with TTBS. The tubes then received 100 µl of various concentrations (0-100 µg/ml diluted in TTBS containing 1% BSA) of rabbit anti-goat IgG

F(ab')2 and were incubated at 37°C for 1 hr. After the antibody was removed and the tubes were washed 5 times with TTBS, 100 µl of proA-aeq protein (96 ng/ml) or protein A-HRP conjugate (100 ng/ml) prepared by the method of Nakane and Kawaoi (13) was added, the tubes were incubated at 37°C for 1 hr, and then washed 10 times with TTBS. Aequorin or HRP activities were determined as described above.

RESULTS AND DISCUSSION

A protein A fusion expression system resulting in accumulation of the fusion protein in the periplasmic space of *E. coli* has been described (15). We succeeded in expressing the proA-apo protein using this system. The level of the production of proA-apo protein, calculated from the specific activity of proA-aeq protein (Table1), was approximately 40 µg/ml in cultured cells. Western blot (Fig.2) and SDS-PAGE (Fig.3) analyses of the fusion protein revealed two polypeptides with M_r 46 kDa and 47 kDa. The smaller form seemed to be the result of proteolysis in *E. coli* cells. The proteolysis occurred in the N-terminal region of the protein A as described by Hellebust *et al.* (24) and the C-terminal region of apoaequorin is essential for the luminescent activity (25).

The results of the purification of the fusion protein are summarized in Table 1. The decrease in specific activity from 5.93 to 3.24 before the Superose 12 step was caused by non-specific adsorption to the Centricon filter membrane. Similar adsorption was found using various supports such as Sephadex (unpublished results). In our purification procedures, it was difficult to separate the two molecular weight forms of the fusion protein (Fig.3). However, the purified proteins from acidic reversed phase HPLC retained the ability to bind IgG and their luminescent activity. This important characteristic of the fusion protein is advantageous for the present application. Furthermore, the luminescent activity of the fusion protein was almost identical to that of the recombinant aequorin. The specific activity of the recombinant aequorin was approximately 4×10^8 rlu/mg protein (10) and that of the fusion protein was 2.1×10^8 rlu/mg protein (Table 1). The loss of the luminescent activity for the fusion protein was small when computed on a molar basis. These results suggest that the protein fusion to the N-terminus of aequorin is possible without the loss of activity. This information suggest that aequorin might be used as a reporter protein in other cases.

Table 1. Purification of the proA-apo protein from the culture cells

Steps	Total protein		Total Activity		Specific Activity	Total Volume
	(mg)	(%)	($\times 10^7$rlu)	(%)	($\times 10^7$rlu/mg)	(ml)
1.IgG Sepharose	288	(100)	180	(100)	0.63	20.0
	5.12	(1.8)	31	(17)	5.93	29.0
2.Superose 12	3.30	(100)	10.7	(100)	3.24	0.05
	0.54	(16)	7.9	(74)	14.6	1.60
3.Wakosil 5C4	0.096	(100)	1.41	(100)	14.7	0.10
	0.030	(31)	0.62	(44)	21.0	0.05

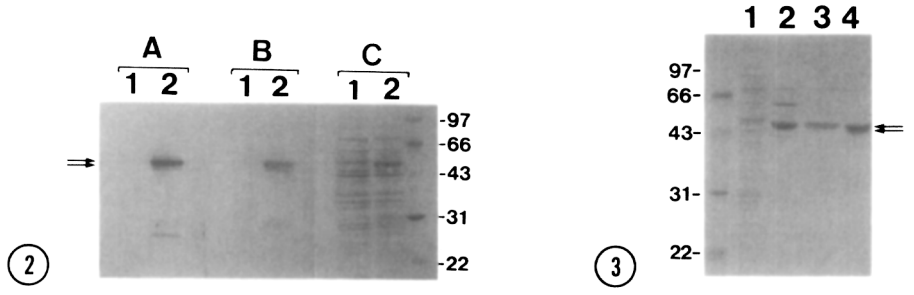

Figure 2. Western blot analysis of the proA-apo proteins expressed in *E. coli*. The blot was performed using rabbit anti-apoaequorin (A) or rabbit anti-protein A(B). An identical nitrocellulose sheet was stained with Amido Black 10B(C). Lane 1 (control), 10 µl of over night culture of JM83 strain; lane 2, 10 µl of over night culture of JM83 strain with pAAQ1. An arrowhead shows the location of the expressed proA-apo protein. The numbers on the right indicate the molecular weights in kDa of the protein standard markers (Bio-Rad).

Figure 3. SDS-PAGE analysis of the proA-apo protein in the various purification steps. The protein sample was dissolved in Laemmli's sample buffer and heated for 5 min at 95°C before being applied to the gel. The gels were stained in 0.1% Coomassie brilliant blue R250 (Eastman Kodak, Rochester, USA) dissolved in 50% trichloroacetic acid-water mixture (w/v) for 1 hr at room temperature. Destaining was carried out by gently shaking in a mixture of ethanol-acetic acid-water (10:10:80, v/v/v) at 50°C. Lane 1, cell extracts (20 µl) ; lane 2, IgG Sepharose 6FF fraction; lane 3, Superose 12 FPLC fraction; lane 4, Wakosil 5C4 HPLC fraction. Arrowheads show the location of proA-apo protein.

As described in the Materials and Methods, we applied the fusion protein in an immunoassay as a model system. As shown in Fig.4, a linear dependence was obtained and the proA-aeq protein could be used to detect the IgG fragment just as effectively as protein A-HRP conjugate. Recently, Casadei *et al.* (26) reported that a chimera of aequorin and a specific antibody against the 4-hydroxy-3-nitrophenacetely group was expressed in myeloma cells and could be used in an immunoassay system. In this paper, we also demonstrated the possibility of IgG detection

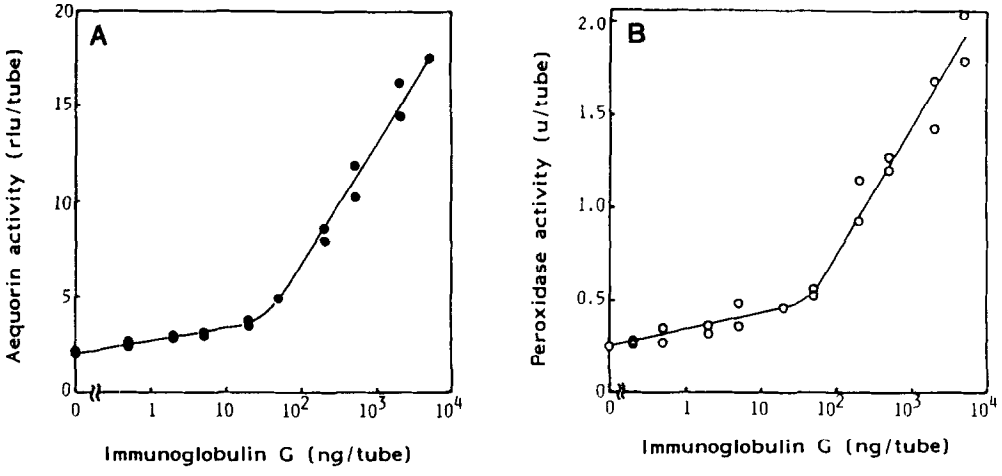

Figure 4. Immunoassay of rabbit IgG with pro-aeq protein (A) and protein A-HRP conjugate (B). Closed circles represent aequorin activity in the test tube. Open circles represent peroxidase activity in the test tube.

using proA-aeq protein expressed in *E. coli*. Other applications to various assay systems using the IgG-binding ability of proA-aeq protein are expected, but further improvement and validation of the assay system is necessary.

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