Streptavidin-Aequorin Fusion Protein for Bioluminescent Immunoassay

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The fusion protein of streptavidin to aequorin (STA-AQ) was highly purified from inclusion bodies in Escherichia coli cells and applied to a bioluminescent sandwich immunoassay. α-Fetoprotein (AFP), which is a serological marker of liver cancer, was used as a model analyte to test STA-AQ in an immunoassay. The measurable range of AFP by the sandwich immunoassay, using the complex of STA-AQ and the biotinylated anti-AFP antibody, was 0.02–200 ng/mL with an average coefficient of variation of 4.9%. The detection sensitivity with the complex of STA-AQ and the biotinylated anti-AFP antibody was similar to that with the complex of biotinylated aequorin, streptavidin and the biotinylated anti-AFP antibody. STA-AQ would be a useful reporter protein for immunoassays.

Key words: photoprotein; luciferase; fused protein; inclusion bodies; biotin

Aequorin is a member of the family of calcium-binding proteins and has been isolated from the luminous jellyfish, Aequorea aequorea.1,2) Aequorin is a stable complex of apoprotein (apoaequorin, 21.4 kDa protein) and 2-peroxycoelenterazine,3) and emits light (λmax at ~470 nm) when bound to Ca2+ ions (>10−7 M).4) The luminescence reaction of aequorin triggered by Ca2+ is fairly fast (within a few seconds), and the luminescence signal shows a high signal-to-noise ratio due to a low luminescence background. It has thus been used extensively as a calcium indicator4 and sensitive reporter protein for various assay methods including immunoassays.5) We have recently reported the preparation of aequorin with a reactive cysteine residue, whereby the aequorin-labeled antibody was applied to bioluminescent sandwich immunoassays.6) In addition, biotinylated aequorin has been prepared from highly purified recombinant aequorin, and the resulting conjugate was applied to the immunoassays.7−9) These conjugates could be used for detecting α-fetoprotein (AFP) which is a serological marker of liver cancer.6,9)

The streptavidin (avidin)-biotin complex obtained has an extremely high affinity for biotin (Kd = 10−14–10−16 M) and could conjugate chemically to other proteins or be labeled with various detecting reagents without significant loss of its biotin-binding ability.10) The chemical conjugate of aequorin with streptavidin has been reported and successfully used in various assays.11−13) The fusion protein of streptavidin with beetle luciferases has previously been prepared and applied to detect biotinylated IgG; click beetle luciferase14) and firefly luciferase15) were respectively fused to the carboxyl and amino terminus of streptavidin. These streptavidin-fused luciferases retained both the binding ability of biotinylated IgG and luminescence activity. Streptavidin-fused aequorin has also been reported for studying the bioluminescence resonance energy transfer between aequorin and enhanced green fluorescent protein.16) However, no practical results have been reported for the use of streptavidin-fused luciferases and streptavidin-fused photoproteins in bioluminescent sandwich immunoassays. In this study, we report the preparation of the highly purified fusion protein of streptavidin to aequorin (STA-AQ) from inclusion bodies expressed in Escherichia coli cells and its application to bioluminescent immunoassays. We evaluated the fusion protein of streptavidin to aequorin in an immunoassay system by comparing with the previously reported method using biotinylated aequorin and streptavidin complex.8)

Materials and Methods

Isolation of the streptavidin gene from Streptomyces avidini. The streptavidin gene was isolated from Streptomyces avidini (IFO13429) which was obtained from Japan Chemical Innovation Institute. The culture was grown in a liquid medium containing 2 g of yeast extract (Difco Lab., Detroit, MI, USA) and 10 g of soluble starch (Wako Pure Chemicals, Osaka, Japan) per liter (pH 7.3) at 28°C for 2 d. The cells were collected from 50 mL of the cultured medium by centrifugation at 5,000 g for 5 min and suspended in 5 mL of 20 mM Tris–HCl (pH 7.5) containing 75 mM NaCl and 25 mM EDTA, and were then lysed by adding 5 mg of lysozyme (Seikagaku Kogyo, Tokyo, Japan) at 37°C for 30 min. The reaction mixture was treated with 2 mg of Protenease K (Wako Pure Chemicals) in the presence of 1% SDS at 55°C for 2 h. The lysed solution was mixed by inversion with 10 mL of chloroform for 30 min at room temperature. After centrifugation, to the water layer was added 7.5 mL of isopropanol, and the precipitate of chromosomal DNA was taken up with a glass bar and rinsed with 2 mL of 70% ethanol. The resulting chromosomal DNA was dissolved in 600 μL of Tris–HCl (pH 7.6)-2 mM EDTA and used as a template for PCR. The streptavidin gene (GenBank accession no. X03591) without the signal peptide sequence was isolated17) by gene amplification in a Perkin-
Elmira DNA thermal cycler (30 cycles: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) with primer sets of ST-7 (5' ggc gaa cct GAC CCC TCC AAG GAC TCG AAG GCC CAG 3'; HindIII site underlined) and ST-8 (5' ggc gaa ttc CTA CTG CTG AAC GCC TGC GAG CGG GTT GCC 3'; EcoRI site underlined), using LA-Taq polymerase (Takara-Bio, Kyoto, Japan). The amplified DNA fragment was digested with HindIII/EcoRI and then inserted into the HindIII/EcoRI site of pLC2107 to give pSTA51. The nucleotide sequence of the streptavidin gene in pSTA51 was confirmed by an analysis with a 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Expression of the fusion protein of streptavidin to apoaequorin in E. coli cells. To express the fusion protein of streptavidin to apoaequorin in E. coli cells, the pCold-STA-AQ expression vector was constructed in two steps. Firstly, the fragment of the streptavidin gene was obtained by the PCR procedure (25 cycles: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) with primer sets of ST-12 (5' ggc gac gtc GAC CCC TCC AAG GAC TCG AAG 3', SacI site underlined) and ST-13 (5' ggc gtc gtt CTA CTG CTG AAC GCC TGC GAG 3', XhoI site underlined) using pSTA51 as a template. Secondly, the HindIII/XhoI fragment containing apoaequorin cDNA (GenBank accession no. L29571) was inserted into the pCold-I vector. The bacterial strain BL21 (Novagen, Madison, WI, USA) was used as the host strain and streptavidin was expressed in soluble form in the cytoplasm of E. coli cells. Secondly, the HindIII/XhoI fragment containing apoaequorin cDNA (GenBank accession no. L29571) was inserted into the HindIII/XhoI site of pCold-STA to give pCold-STA-AQ (Fig. 1A).

Purification of the streptavidin-aequorin fusion protein (STA-AQ). The seed culture of the bacterial strain BL21 possessing the pCold-STA-AQ vector was grown in 10 mL of Luria-Bertani (LB) broth containing ampicillin (50 µg/mL) at 37°C for 18 h. The seed culture was transferred into 400 mL of LB broth in a 3-L flask, and the solution was transferred into 400 mL of LB broth in a 3-L flask, and the solution was incubated for 2.5 h and then cooled for 1 h in an ice-water bath. After adding IPTG (Wako Pure Chemicals) at a final concentration of 0.2 mM to the culture medium, the bacterial cells were incubated at 15°C for 17 h. To isolate STA-apoAQ from the inclusion bodies, the cells were harvested by centrifugation at 3,000 g for 5 min from 80 mL of the culture medium. The resulting pellet was suspended in 80 mL of 50 mM Tris–HCl (pH 7.6) and then disrupted by sonication in a 250 sonifier (Branson, Danbury, CT, USA) 2 times for 3 min on ice. After centrifugation at 12,000 g for 10 min, the precipitate containing STA-apoAQ was suspended in 45 mL of 6 M guanidine-HCl (Wako Pure Chemicals) in 50 mM Tris–HCl (pH 8.0) (A-buffer). The soluble fraction obtained by centrifugation was applied to a nickel chelate column (1.5 x 4.3 cm; GE Healthcare, Uppsala, Sweden), equilibrated with A-buffer. After washing with 100 mL of A-buffer and then by 20 mL of 8 M urea in 50 mM Tris–HCl (pH 8.0) (B-buffer), the adsorbed proteins were eluted with 40 mL of 0.1 M imidazole in B-buffer. An SDS–PAGE analysis showed the purity of STA-apoAQ (from the nickel chelate column to be below 60% (data not shown). To prepare highly purified STA-AQ, a peak fraction containing STA-apoAQ (4 mL), 16.4 µg of proteins, corresponding to 150 µL of cultured cells was diluted in 200 mL of 30 mM Tris–HCl (pH 7.6)–15 mM EDTA containing 40 mg of dithiothreitol (Wako Pure Chemicals), and STA-apoAQ was regenerated to STA-AQ by incubation with 0.4 mg of coelenterazine (Chisso Co., Yokohama, Japan) at 4°C for 18 h. The solution containing regenerated STA-AQ was adjusted to a final concentration of 1.2 mM (NH4)2SO4 and applied to a butyl Sepharose 4 Fast Flow column (1.5 x 5.5 cm, GE Healthcare), equilibrated with 10 mM Tris–HCl (pH 7.6)–2 mM EDTA containing 1.2 mM (NH4)2SO4 (C-buffer). The column was washed with 30 mL of C-buffer and STA-AQ on the gel was eluted with 10 mM Tris–HCl (pH 7.6)–2 mM EDTA containing 0.4 mM (NH4)2SO4. The peak fractions with luminescence activity were combined and stored at −80°C.

Protein analysis. SDS–PAGE analysis was carried out under reducing conditions using a 12% separation gel as described by Laemmli. Protein concentration was determined by the dye-binding method of Bradford. Using a commercially available kit (Bio-Rad, Richmond, CA, USA) bovine serum albumin as a standard (Pierce; Rockford, IL, USA).

Determination of the luminescence activity of STA-AQ. The luminescence activity of STA-AQ was determined as follows. After STA-apoAQ was regenerated to STA-AQ by incubation with 1 µg of coelenterazine (1 µg/mL in absolute methanol) in 1 mL of 30 mM Tris–HCl (pH 7.6)–10 mM EDTA containing 1 µL of 2-mercaptoethanol at 4°C for 2 h, the mixture was placed in a 96-well white plate (Nunc, cat. no. 236108). The initial maximum intensity of luminescence (Imax) was determined by injection with 100 µL of 50 mM CaCl2 in 50 mM Tris–HCl (pH 7.6) using a Centro 960 lumiluminometer (Bertold Technologies, Bad Wildbad, Germany) in 0.1 s intervals for 10 s.

Biotinylated sandwich immunoassay using streptavidin-fused apoaequorin (STA-AQ). The biotinylated anti-AFP antibody 1D5 was prepared by the chemical conjugation of anti-AFP antibody 1D5 (Japan Clinical Lab., Kyoto, Japan) with (+)-biontynyl-3-maleimidopropiona- midyl-3,6-dioxoacetamidene (PEO-maleimide activated biotin; Pierce Biotechnology, Inc., Rockford, IL, USA). For preparation of biotinylated apoaequorin at the amino residues (AQ-N-biotin), the chemical conjugation was carried out using succinimidyl-6-(biotina- mido)hexanoate (NHS-LC-biotin, Pierce). The procedures for sandwich immunoassays were as follows. The anti-AFP antibody 6D2 (100 µL of 5 µg/mL; Japan Clinical Lab., dissolved in 50 mM sodium bicarbonate buffer (pH 9.5) was incubated in the wells of a 96-well white plate (Nunc, cat. no. 437796) for 18 h at 30°C. After removing the capturing antibody, the wells were post-coated with 200 µL of 1% bovine serum albumin (Fraction V, Sigma, St. Louis, MO, USA) in 20 mM Tris–HCl (pH 7.6)–150 mM NaCl (TBS) containing 2 mM EDTA and 0.05% NaN3 for 18 h at 4°C. The wells were washed three times with 340 µL of TBS containing 0.05% (v/w) Tween 20 (Sigma) and 2 mM EDTA (TBST-E). A series of AFP solutions (12.5 µg/mL to 250 ng/mL; DakoCytomation, Denmark) diluted with 0.4% (v/w) Block Ace (Daninppon Pharmaceutical Co., Osaka, Japan) containing 5 mM EDTA were prepared, and 50 µL of each AFP solution was then added to the anti-AFP 6D2 coated wells. Subsequently, 50 µL of biotinylated Ab1D5 (37.3 ng/mL) was added to the wells containing the anti-AFP 6D2–AFP complex. After incubation at 30°C for 1 h, the wells were washed three times with 340 µL of TBST-E, and 100 µL of STA-AQ (125 ng/mL) or 100 µL of a mixture of AQ-N-biotin and streptavidin at 1:1 ratio (115 ng/mL) was added and further incubated at 30°C for 30 min. The wells were washed three times with 340 µL of TBST-E, and Imax was measured by injection of 100 µL of 50 mM CaCl2 in 50 mM Tris–HCl (pH 7.6) using a Centro 960 lumiluminometer in 0.1 s intervals for 10 s.

Results and Discussion

Expression and purification of the fusion protein of streptavidin to apoaequorin in E. coli cells.

To express the fusion protein of streptavidin to apoaequorin in E. coli cells, we first isolated the streptavidin gene from S. avidinii by the PCR procedure and then constructed the pCold-STA-AQ expression vector (Fig. 1A) from the pCold-STA vector as described in the Materials and Methods section. The gene expression was under the control of the cold shock protein A (cspA) promoter and the lac repressor, and the fused protein consisted of streptavidin (159 amino acid residues) and apoaequorin (188 amino acid residues) with a histidine-tagged sequence at the N-terminus for nickel-chelate affinity chromatography (Fig. 1B). When the fusion protein of streptavidin to apoaequorin (STA-apoAQ) was expressed in E. coli cells by using the cold induction system, the fusion protein was mainly expressed as inclusion bodies. The inclusion bodies in E. coli cells were solubilized by 6 M guanidine-HCl, and the bound biotin derived from bacteria was dissociated from the streptavidin moiety of STA-apoAQ. The soluble fraction of STA-apoAQ was applied on a nickel-chelate column, and the STA-apoAQ fraction
eluted with 0.1 M imidazole was regenerated to STA-AQ by incubation with coelenterazine. The highly purified STA-AQ was obtained by hydrophobic chromatography with a butyl-Sepharose column. The yield of recombinant STA-AQ was 0.75 mg from 150 mL of cultured cells with >95% purity (Fig. 1C).

**Bioluminescent sandwich immunoassay using streptavidin-fused aequorin (STA-AQ)**

α-Fetoprotein (AFP) was used as a model analyte to evaluate STA-AQ for the streptavidin-biotin complex in a bioluminescent sandwich immunoassay (Fig. 2A). The sandwich immunoassay for AFP was carried out by using the capturing antibody of anti-AFP antibody 6D2 and biotinylated anti-AFP antibody 1D5 (Fig. 2A).6,9) The procedure for the immunoassay was essentially the same as that previously described.6,9) The relationship between the amount of STA-AQ and the luminescence intensity was investigated. The linearity of luminescence intensity was observed in a range covering over 10,000-fold, similar to that for the aequorin-antibody conjugate and biotinylated aequorins of AQ-N-biotin and AQ-S-biotin.9)

To compare the detection sensitivity of STA-AQ with that of the complex of streptavidin and biotinylated aequorin (AQ-N-biotin),8) the concentration of AFP was determined by the sandwich immunoassay (Fig. 2B). Luminescence values greater than the means of the blank value plus 3SD (blank + 3SD) were taken to be significant, and the detection limit of AFP with STA-AQ was estimated to be 0.02 ng/mL. The coefficient of variation (%CV, n = 6) was also determined between 0.024 and 12.5 ng/mL of AFP, and the average CV value and the correlation coefficient for STA-AQ were 4.9 and 0.9993, respectively. On the other hand, the average CV value and the correlation coefficient for AQ-N-biotin between 0.024 and 12.5 ng/mL of AFP were 2.2 and 0.9996, respectively (Fig. 2B). The background value for STA-AQ was ten times higher than that for the complex of streptavidin and AQ-N-biotin under our conditions. However, the measurable range of AFP by the sandwich method, using STA-AQ and the complex of streptavidin and AQ-N-biotin, was almost the same from 0.02 to 200 ng/mL. The immunoassay method using STA-AQ had the advantage of being able to perform the assay without preparing the biotinylated aequorin and streptavidin complex (Fig. 2A).

In conclusion, the highly purified streptavidin-aequorin fusion protein was prepared and shown to be useful as a reporter protein in a bioluminescent immunoassay. The detection sensitivity was similar to that of the methods using the complex of biotinylated aequorin and streptavidin,9) and the aequorin-labeled antibody.6)

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References