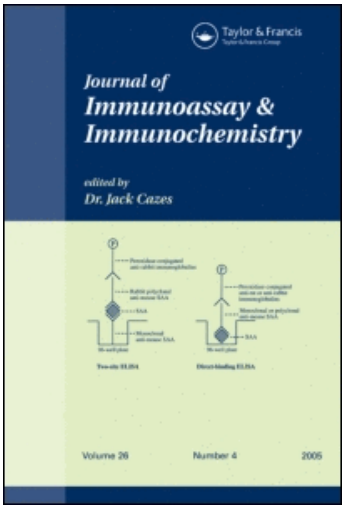

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A SIMPLE AND RAPID IMMUNOASSAY SYSTEM USING GREEN FLUORESCENT PROTEIN TAG

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ABSTRACT

A fusion protein between green fluorescent protein (GFP) and neuron-specific enolase (NSE) was expressed in *Escherichia coli*. The GFP-NSE fusion protein migrated at 62 kDa in SDS-PAGE and retained the fluorescence under non-heating conditions. However, heat-denatured GFP-NSE was non-fluorescent and migrated at 74 kDa corresponding to the theoretical value. This suggests that the special structure of GFP, which is not denatured by SDS, influences its mobility in SDS-PAGE under non-heating conditions. The fluorescence intensity of GFP-NSE was measurable over a wide range by spectrophotometry or densitometry. The competitive immunoassay for NSE was performed using GFP-NSE as labeled antigen. Under our assay conditions, the working range of this system was about 2 - 60 ng. This simple and rapid fluorescence immunoassay (FIA) using GFP-tagged antigen may be applicable to many protein markers.

(KEY WORDS: green fluorescent protein, GFP-tagging, fusion protein, neuron-specific enolase, fluorescence immunoassay)

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INTRODUCTION

Green fluorescent protein (GFP), isolated from the jelly fish *Aequorea victoria*, is a new tool in cell biology. *Aequorea* GFP is a protein of 238 amino acids (1) with a molecular mass of 27-30 kDa in SDS-PAGE (2,3). Excitation of GFP with long-wave UV or blue light (maximally at 395 nm) leads to bright greenish fluorescence (peak emission at 508 nm). The GFP chromophore is formed by cyclization of Ser65, Tyr66 and Gly67, and by oxidation of the hydroxybenzyl side chain of Tyr66 (4,5). This modification proceeds post-translationally within several hours after synthesis, and this process is probably non-catalytic (5). Although GFP has many unique physical properties (e.g., high stability to denaturing reagents or proteases) (6), the most useful is its fluorescence which occurs without co-factors (7). The latter property allows GFP fluorescence in the non-native organisms in which it is expressed. Although GFP is a relatively large molecule as a fusion tag, GFP-tagged proteins often retain their original functions. Therefore, GFP is now widely used in many studies as a reporter for gene expression, a tracer of cell lineage, or a fusion tag to investigate protein localization and secretion systems in living cells (8). GFP is also a useful tool for *in vitro* studies. For example, the fusion proteins to protein A (9) or streptavidin (10) have been reported; these proteins have a potentially wide range of *in vitro* applications. Here, for example, we describe a new application, the immunoassay of GFP-tagged protein.

Neuron-specific enolase (NSE) was used as the model of GFP-tagged antigen. Enolase is a glycolytic dimeric isoenzyme that is composed of two of three subunits (α, β and γ) (11,12). The tissue specificities and mechanisms of organ-specific expression of enolase isoforms are of genetic and biochemical interest. Of the five enolase isoforms ($\alpha\alpha$, $\beta\beta$, $\gamma\gamma$, $\alpha\beta$ and $\alpha\gamma$), $\gamma\gamma$-enolase is the neuron-specific enolase (13). NSE is also of medical interest as a tumor marker of neuroblastoma (14,15) and small-cell lung carcinoma (SCLC) (16,17). We have purified the recombinant human NSE (R-NSE) from *E. coli* and adapted it or its derivatives to enzyme immunoassay (EIA) and radioimmunoassay (RIA) (18,19). In this study, we use the fusion protein GFP-NSE as a model GFP-tagged protein to evaluate a fluorescence immunoassay (FIA) system based on competition between an antigen and GFP-tagged antigen.

MATERIALS AND METHODS

Plasmid construction

The cDNA for human NSE was modified by standard PCR using the following primers: 5'-TTTGAATTCGATGTCCATACAGAAGATCTG-3' and 5'-GGGAAG-CTTTTATCAATGGTGATGGTGATGGTGCAGCACACTGGGATTA-CGGA-3' (*Eco*RI and *Hin*dIII sites are underlined). Plasmid pHTK503 (18), containing a complete human NSE cDNA, was used as a template. The modified NSE cDNA was digested with *Eco*RI and *Hin*dIII and cloned into the *E. coli* expression vector pKK223-3 (Pharmacia Biotech). The resulting plasmid was designated pNSE.H6. The modified *Aequorea* GFP cDNA was prepared from plasmid TU#65 (7) by PCR using the following primers: 5'-TTTGAATTCATGAGTAAAGGAGAAGAACTTT-TC-3' and 5'-TTTGAATTCGCTTTGTATAGTTCATCCATG-3' (*Eco*RI sites are underlined). This fragment was digested with *Eco*RI and inserted into pNSE.H6. The final construct was designated pGFPNSE2. Enzymes were purchased from Gibco BRL or from Nippon Gene Co., Japan. DNA was transformed into *E. coli* JM109 with $CaCl_2$ (20), and transformants were selected on LB agar plates containing 50 μg ampicillin/ml. Recombinant plasmids were isolated as described by Birnboim and Doly (21), then analyzed by agarose gel electrophoresis. DNA was sequenced using a fluorescence imaging analyzer FMBIO-100 (Takara Shuzo Co. Ltd., Japan).

Expression and purification of GFP-NSE from *E. coli*

The *E. coli* strain producing GFP-NSE fusion protein was grown on 9 cm LB agar plates at 37℃. After plating, 0.6 ml of 10 mM IPTG was added to the plate. Following complete IPTG penetration, the incubation temperature was shifted to 25℃. After incubating for one more day, cells collected from 27 plates (2.8 g, wet weight) were suspended in 15 ml of buffer A (50 mM Na-phosphate buffer, pH 8.0, containing 0.1 M KCl, 0.1% Tween 20 and 10 μM PMSF) and disrupted by sonic oscillation. Following centrifugation at 27,000 $\times g$, 1 ml of the supernatant was diluted with the same volume of glycerine and stored at -20℃. This solution was used for immunoassays without purification as the "non-purified GFP-NSE". The remaining supernatant was applied to a Ni-chelate affinity column (2.8 x6 cm;

QIAGEN Inc.) equilibrated with buffer A. The column was washed with the same buffer, and the bound materials were eluted with a linear gradient of imidazole from 0 to 0.1 M in buffer A. Fractions emitting green fluorescence under UV light at 365 nm (NLMS-20E; UVP, Inc.) were pooled, dialyzed against 5 mM Na-phosphate buffer (pH 7.0) and diluted with the same volume of glycerine. This solution was used for immunoassays as the "purified GFP-NSE".

Protein analysis

Fluorescence of GFP-NSE was measured using a fluorescence spectrophotometer (F-4500; Hitachi Ltd., Japan). GFP-NSE was excited at 395 nm, and the emission at 508 nm was measured. The protein concentration was measured with a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as the standard. SDS-PAGE was performed by standard procedures, and the separated proteins were stained with 0.25% Coomassie brilliant blue (CBB) R-250. The green fluorescent band was detected under UV light at 365 nm before the CBB-staining and recorded on Polapan 3200B (Polaroid Corp.) using a green filter.

Immunoassay

Immunoassay employed a double-antibody technique. Specific antibodies to NSE or rabbit IgG were purified by immunoadsorption (22) with the column containing NSE or rabbit IgG immobilized on Sepharose 6B. The affinity columns were prepared as follows. The Sepharose gels were activated by BrCN and suspended in 0.1 M $NaHCO_3$. Then, 10 mg of NSE or 200 mg of rabbit IgG were added to the gels and the mixtures were kept for 12 h at 4℃ with gentle stirring. The gel-NSE and gel-rabbit IgG conjugates were washed with phosphate-buffered saline (PBS) and packed in 1.5 x 5 and 1.5 x 40 cm column, respectively. The specific antibodies bound to the columns were eluted with 0.1 M glycine-HCl buffer, pH 2.8, and dialyzed against PBS. The "purified GFP-NSE" (100 ng/0.1 ml) or "non-purified GFP-NSE" producing equivalent fluorescence intensities were used as labeled antigens. Assays were performed with the Easy-Titer ELIFA system (Pierce Chemical Co.). Briefly, 50 μg of sheep antibody against rabbit IgG was blotted on a nitrocellulose membrane as a 4 mm spot and blocked with 5% bovine serum albumin. The labeled antigen (0.1 ml), 0.1 ml of standard NSE

solution (0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 or 100 ng/0.1 ml), and 0.1 ml of anti-NSE rabbit IgG (14 μg/0.1ml) were mixed in a 0.5 ml microtube (Eppendorf). After incubating for 1 h at 37℃, the reaction mixtures were filtered through immobilized anti-rabbit IgG spots on membranes at a flow rate of 35 μl per min. The membranes were washed, and green fluorescence signals were recorded on Polapan 3200B. The photographs were digitized by scanner (ScanJet 3c, Hewlett-Packard Co.), and the signal intensities were quantified using densitometry software ("FM analysis", Takara Shuzo Co. Ltd., Japan).

RESULTS AND DISCUSSION

Construction and purification of GFP-NSE fusion protein

To facilitate purification, six histidine residues were attached at the C-terminal of human NSE. As shown in Fig.1a, the human NSE cDNA was modified by PCR and cloned into pKK223-3, and then the modified *Aequorea* GFP cDNA was inserted 5' to the NSE cDNA. The resulting plasmid, pGFPNSE2, carried the fused GFP-NSE cDNA encoding a protein of about 74 kDa composed of 680 amino acids (except for the initiating methionine residue). DNA sequencing confirmed the predicted plasmid structure.

Expression of GFP-NSE fusion protein was induced by the addition of IPTG, and the fluorescent GFP-NSE was expressed most efficiently at 25℃ on agar plate rather than in liquid medium. Under this condition, most GFP-NSE molecules did not form inclusion bodies and existed as soluble protein. On the other hand, under the normal expression procedure in liquid medium, almost all GFP-NSE molecules formed inclusion bodies not only at 37℃ but also at 25℃, and these molecules were non-fluorescent. To acquire fluorescent activity, GFP must be modified post-translationally. Studies have shown that oxygen and temperature are important for the formation of fluorescent GFP (5,23), and that GFP expressed in *E. coli* as an inclusion body is non-fluorescent (24). Our findings support these reports.

The cell extract prepared from the *E. coli* strain harboring pGFPNSE2 was analyzed by SDS-PAGE (Fig.1b). The GFP-NSE band was not visible by CBB staining, but a strong green fluorescent band of 62 kDa was visualized by UV irradiation. This fluorescent band was thought to be GFP-NSE, but the molecular

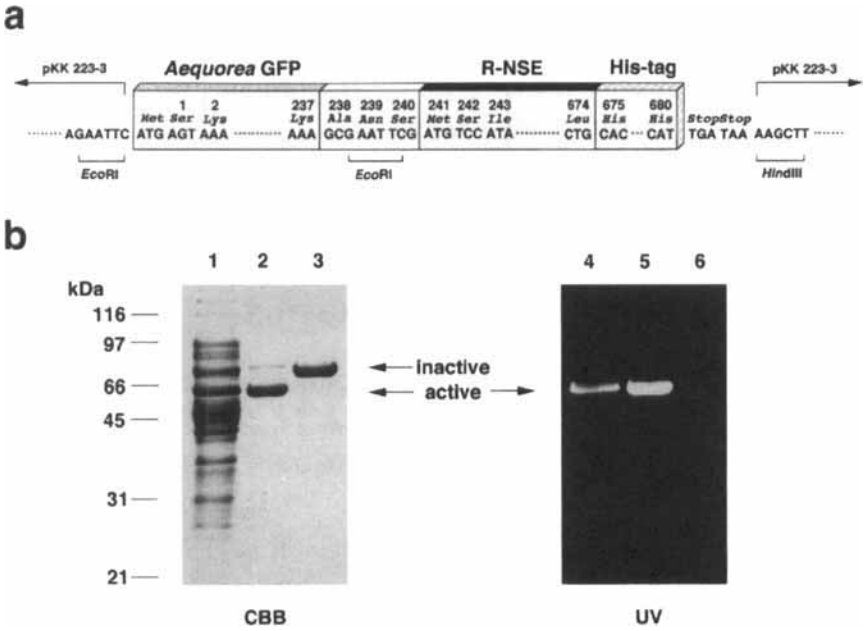

FIGURE 1. **Construction of the recombinant plasmid pGFPNSE2 encoding a GFP-NSE fusion protein.** (**a**) A modified human NSE cDNA containing histidine-tag was first cloned into pKK223-3, and then *Aequorea* GFP was inserted 5' to the NSE cDNA. (**b**) The cell extract prepared from the strain harboring pGFPNSE2 and purified GFP-NSE were resolved by a 0.1% SDS-12% polyacrylamide gel. GFP-NSE was expressed and purified as described in the text. Cell extracts were loaded on the gel without heating (lanes 1 and 4); purified GFP-NSE was loaded without heating (lanes 2 and 5) or after heating at 95℃ for 5 min (lanes 3 and 6). Gels were irradiated by UV light at 365 nm (lanes 4 - 6) before CBB-staining (lanes 1 - 3). Fluorescent and non-fluorescent GFP-NSE bands are indicated as "active" and "inactive", respectively. The relative positions of molecular weight markers are shown at the left.

mass differed considerably from the theoretical value of 74 kDa. GFP-NSE, purified by Ni-chelate affinity chromatography, also migrated to the 62 kDa position as a fluorescent band. However, when this sample was denatured by heating, a single non-fluorescent band was detected at the 74 kDa position corresponding to the theoretical mass of GFP-NSE. These findings indicate that fluorescent (active) and non-fluorescent (inactive) GFP-NSEs differ in mobility on SDS-PAGE. A similar mobility shift on SDS-PAGE has been reported with intact GFP (25) and GFP-protein A fusion protein (9). These phenomena may be due to the special structure of the GFP molecule (26). GFP is very stable in denaturing reagents (e.g., urea, guanidine and SDS) and resistance to protease degradation. Without heating, the native structure of GFP may not be fully destroyed in SDS-PAGE, and this partially (or non-) denatured active GFP migrates differently from the heated inactive GFP.

Competitive immunoassay for NSE using GFP-NSE

The fluorescence excitation and emission spectra of purified GFP-NSE were indistinguishable from those of native *Aequorea* GFP. The fluorescence intensity of GFP-NSE solution was measured by a fluorescence spectrophotometer at the maximum excitation wave length (395 nm) or by densitometry of the fluorescence signal, excited by UV light at 365 nm, on nitrocellulose membrane. As shown in Fig.2, a linear relationship between GFP-NSE concentration and its fluorescence intensity was obtained spectrophotometrically over a broad range. Although the linearity of the densitometric measurement above 100 ng/spot was inferior to that of spectrophotometry, the speed of densitometry facilitated the following immuno-assay. For the development of this assay, the appropriate concentration of the anti-NSE antibody was determined by titration with 100 ng GFP-NSE. The densitometrically-measured fluorescent signal of bound GFP-NSE correlated with added antibody (Fig.3a), and the antibody concentration forming complexes with about 60% of GFP-NSE was used for the assay. The standard inhibition curve obtained from 100 ng of "purified GFP-NSE" with standard NSE is shown in Fig.3b. The inhibition curve obtained from 100 ng of "non-purified GFP-NSE" was almost identical (data not shown). Under these conditions, 2 ng of standard NSE were quantifiable, and the working range of this assay was about 2 - 60 ng/0.1

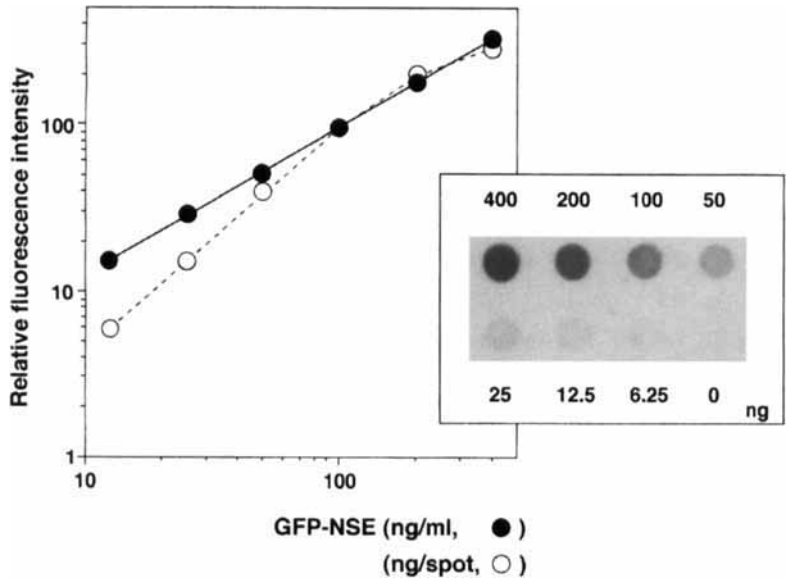

FIGURE 2. **GFP-NSE concentration and its fluorescence intensity.** The fluorescence intensity of purified GFP was measured by fluorescence spectrophotometry (●). GFP-NSE solutions (6.25 - 400 ng/ml) were excited at 395 nm, and the emission at 508 nm was measured. The fluorescence intensity was then quantified by densitometry (○). GFP-NSE was blotted on a nitrocellulose membrane as a 4 mm spot; the fluorescence signal excited by UV light at 365 nm was recorded on Polapan 3200B. The photograph was scanned, and the signal intensity was calculated as described in Methods. The attached photographic image was generated on a video printer (VY-5000R, Hitachi Ltd., Japan). All amplitudes were arbitrarily normalized: the fluorescence intensity of 100 ng/ml or 100 ng/spot of GFP-NSE was taken as 100.

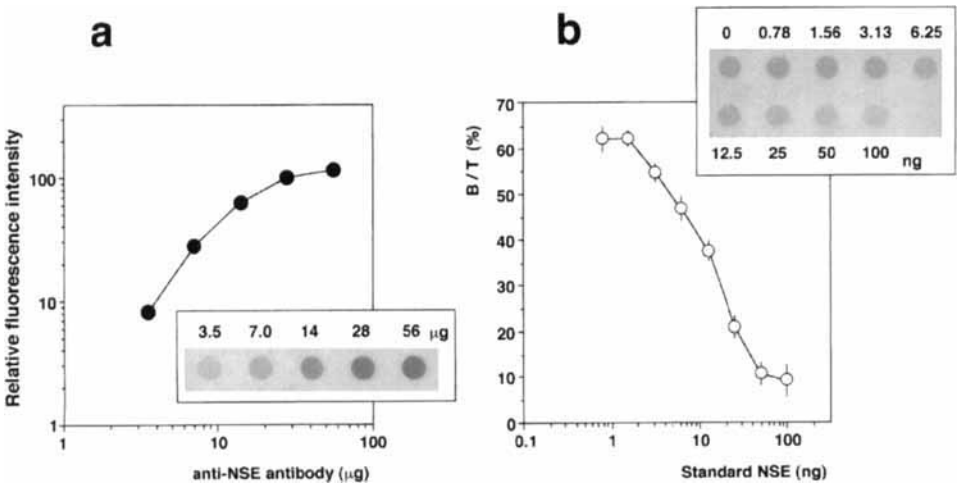

FIGURE 3. **Competitive immunoassay for NSE using GFP-NSE as the labeled antigen.** (**a**) Determination of the amount of anti-NSE antibody required for assay. Fifty μg of sheep IgG against rabbit IgG was blotted onto nitrocellulose membranes as 4 mm spots. The membranes were then blocked with bovine serum albumin. GFP-NSE (100 ng/0.1 ml) and 0.1 ml of anti-NSE antibody (3.5 - 56 μg/0.1 ml) were mixed in a microtube. After incubating for 1 h at 37°C, the reaction mixtures were filtered through the sheep IgG spots at a flow rate of 35 μl per min, and the fluorescence signals were recorded. The fluorescence intensity of 100 ng of the GFP-NSE spot, shown in Fig. 2, was taken as 100. (**b**) Fluorescence immunoassay. Each point represents the mean ± S.D. of 4 individual experiments. The fluorescence intensity of 100 ng of the GFP-NSE spot was used as the total intensity for the B/T (bound/total) index.

ml sample. However, if less GFP-NSE and anti-NSE antibody were used in the system, the sensitivity increased. Fischbach et al. (27) reported that the serum concentration of NSE in healthy subjects was 7.0 ± 1.2 ng/ml, and that in patients with SCLC, it was 55.7 ± 11.4 ng/ml by RIA. Although many alterations are necessary, this assay has the possibility to detect NSE levels in serum and to be used for rapid screening. In this study, the wild-type GFP of *Aequorea* was used as the fluorescent tag. However, several GFP mutants with red-shifted excitation

spectra and with increased fluorescence signals have been recently reported (24,28,29). It may be possible to construct a more sensitive assay system using these variants as the tag source.

The subject of this study is to prove the possibilities of a new immunoassay system using GFP-tagged antigen. The GFP-NSE fusion protein was used as a model antigen. The principle of this system is potentially applied to many other protein markers. At present, this assay system is hard to apply to weak markers, such as serum NSE. However, as described above, the improved system will expand the application range. This assay may be performed with commonly used laboratory equipment. Although special and expensive equipment is not required, the computer-controlled fluorescence scanning system facilitates both speed and sensitivity. Important features of this FIA system are: generally, (i) this type of system may be applicable to many protein markers; (ii) chemicals and isotopes for assay are not required; therefore, the procedure is simple, safe and inexpensive; and, specifically, in this case, (iii) the fluorescence does not decrease when stored in 50% glycerol solution at least several months at -20℃; and (iv) in many cases, since purification of GFP-tagged antigen may not be required for the assay, the crude cell extract containing GFP-tagged protein can be applied directly without purification. In conclusion, although further comparative studies of assay conditions are needed, this simple and rapid system using GFP-tagged antigen shows significant potential for a useful FIA.

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