FEBS Letters 384 (1996) 193–197

Construction of a fusion protein between protein A and green fluorescent protein and its application to Western blotting

Takashi Aoki[a,*], Yasumitsu Takahashi[a], Katherine S. Koch[b], Hyam L. Leffert[b,c], Hiroyuki Watabe[a]

[a]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-02, Japan*
[b]*Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92036-0636, USA*
[c]*Center for Molecular Genetics, School of Medicine, University of California, San Diego, La Jolla, CA 92036-0636, USA*

Received 31 January 1996

Abstract ***Aequorea*** **green fluorescent protein (GFP) and protein A were fused and expressed in** ***Escherichia coli.*** **The fluorescent native fusion protein (PA-GFP) migrated at 47 kDa in SDS-PAGE. However, the non-fluorescent denatured PA-GFP migrated at 57 kDa which corresponds to the theoretical molecular mass. Although the reason(s) for this mobility shift between fluorescent and non-fluorescent molecules remains unclear, the small ring structure within the native molecules may affect their mobility. The cell extract, prepared from an** ***E. coli*** **strain producing PA-GFP, was used in Western and dot blots. The sensitivity and specificity of the PA-GFP detection were sufficient for rapid and easy screening.**

Key words: Green fluorescent protein; Protein A; Fusion protein; Western blotting; Dot blotting

1. Introduction

Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*, produces an intense and stable greenish fluorescence non-catalytically [1]. *Aequorea* GFP absorbs blue light maximally at 395 nm and emits green light (peak emission at 509 nm). GFP is a monomeric protein of 238 amino acids [2] with a molecular mass of 27–30 kDa [3,4]. The hexapeptide segment (starting at residue 64) functions as a fluorescent chromophore that is formed upon cyclization of the residues Ser dehydro-Tyr Gly within the hexapeptide, by post-translational modification [5]. Although the denaturation of GFP causes a loss of fluorescence under some conditions, it can often be restored by renaturation [6,7]. GFP is expressed as a fluorescent product in both prokaryotic and some eukaryotic cells, and its excitation and emission spectra are indistinguishable from those of native GFP [8]. GFP is conformationally very stable, and virtually no photobleaching occurs. Therefore, GFP may be useful for monitoring gene expression in living cells [9–12]. Moreover, Wang and Hazelrigg [13] have reported that a fusion protein between GFP and *exuperantia* protein emitted intense fluorescence in living and fixed cells during *Drosophila* oogenesis. Olson et al. [14] also analyzed the function of microtubule associated proteins (MAPs) in living cells using MAP-GFP fusion proteins. These results mean that GFP can be used as a fluorescent tag and as a new probe for intracellular protein localization in vivo.

However, GFP may also be a useful tool in vitro. Here we describe the application of GFP to Western and dot blotting, in which a labeled antibody or antibody-specific ligand (such as protein A or G) is required. Some enzymes (e.g., peroxidase, alkaline phosphatase, or β-galactosidase), gold particles, radioisotopes, or fluorochromes are usually used as labeling reagents. However, if protein A or G are tagged metabolically with GFP as fusion products, they may allow more simple and rapid detection of blotted proteins. In this study we constructed protein A-GFP fusion protein (PA-GFP) and evaluated it as a labeled antibody-specific ligand in blotting studies.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli HB101 and N4830-1 [15] were used as host strains for pRIT2T [16], a protein A gene fusion vector (Pharmacia Biotech, Sweden). The lambda right promoter in pRIT2T was induced by shifting the growth temperature from 30 to 42°C for 90 min in N4830-1, and HB101 was used for constitutive expression of the promoter. Plasmid TU#65 [8], containing *Aequorea* GFP cDNA, was provided by Dr. M. Chalfie (Columbia University, New York, USA). *E. coli* JM109 (pHTK503), a strain producing human neuron-specific enolase (NSE) [17], was used for blotting studies.

2.2. Plasmid construction

A modified GFP cDNA was prepared from plasmid TU#65 by standard PCR using the following primers: 5′-CCCGAATTCCATGAGTAAAGGAGAAGAACTTTTCAC-3′ and 5′-AAAGGATCCCTATTATTTGTATAGTTCATCCA-3′ (*Eco*RI and *Bam*HI sites are underlined). The fragment was cloned into pRIT2T by standard procedures. Enzymes were purchased from Gibco BRL, USA, or from Nippon Gene Co., Japan. DNA was transformed into *E. coli* HB101 or N4830-1 with $CaCl_2$ [18], and transformants were selected on LB agar plates containing 50 μg ampicillin/ml. Recombinant plasmids were isolated as described by Birnboim and Doly [19] with slight modifications, then analyzed by agarose gel electrophoresis. DNA was sequenced using a fluorescence imaging analyzer FMBIO-100 (Takara Shuzo Co. Ltd., Japan).

2.3. Expression and purification of PA-GFP from E. coli

The HB101 strain harboring pPAGFP127 was grown on LB agar plates at 37°C. One day after plating, the incubation temperature was shifted to 25°C. Cells were collected from 19 plates after a 3-day incubation at 25°C. Cells (approximately 3.4 g, wet weight) were then suspended in 9.5 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 30 mM NaCl (TEN buffer), and disrupted by sonic oscillation. 2 ml of the supernatant, obtained by centrifugation at 27 000×*g*, was diluted 1:10 with TEN buffer and used as the 'PA-GFP working solution' for the blotting studies. The remaining supernatant was applied to a column (1.5×10 cm) containing rabbit IgG immobilized on Sepharose 6B. The column was washed with TEN

*Corresponding author. Fax: (81) (1332) 3-1669

Abbreviations: GFP, green fluorescent protein; PA-GFP, fusion protein between protein A and green fluorescent protein; CBB, Coomassie brilliant blue; POD, peroxidase; DAB, 3,3′-diaminobenzidine; R-NSE, recombinant human neuron-specific enolase.

SSDI S0014-5793(96)00289-X

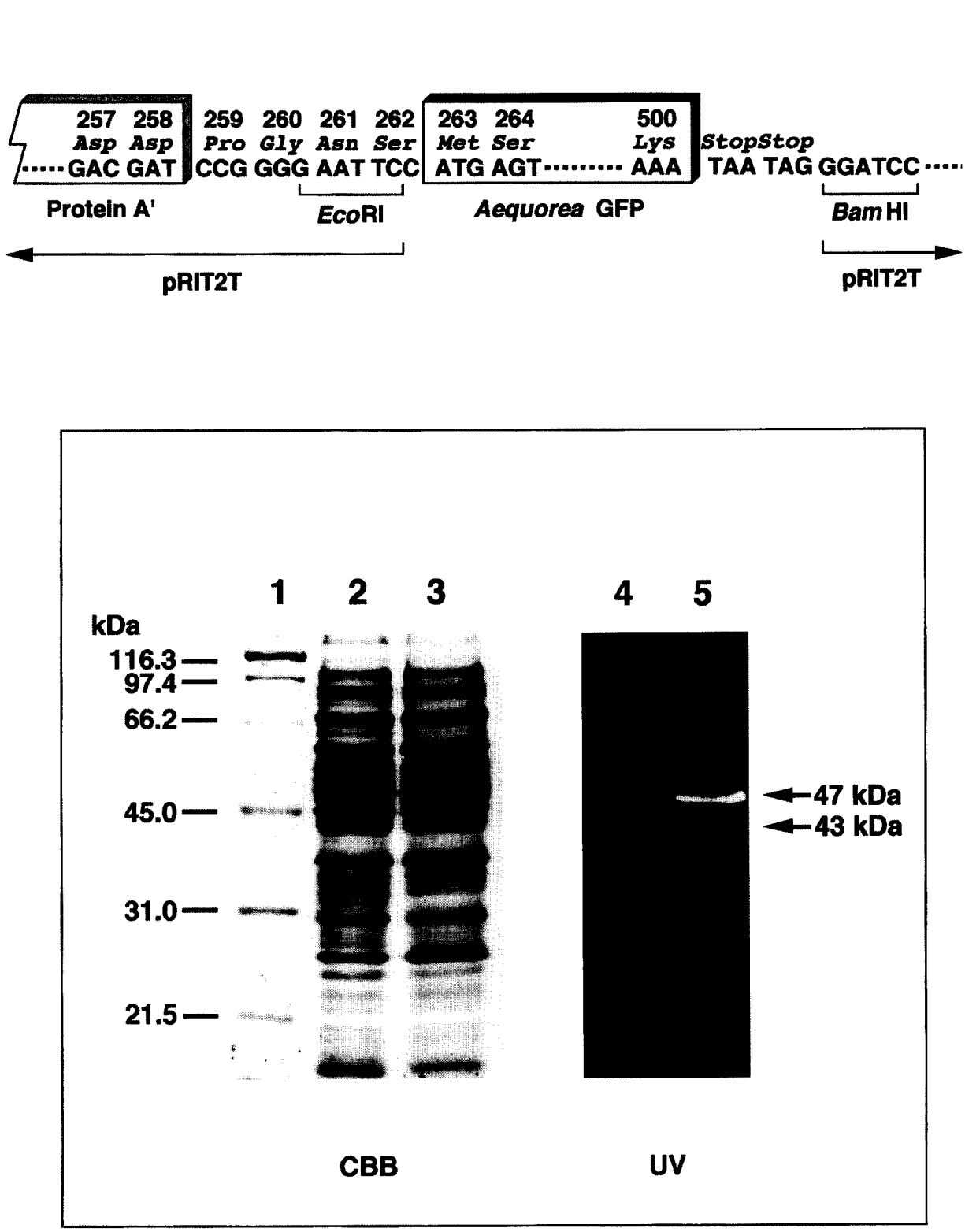

Fig. 1. Construction of the recombinant plasmid pPAGFP127 and SDS-PAGE of PA-GFP expressed in *E. coli*. (a) A 0.75 kb *Aequorea* GFP cDNA was modified and amplified by PCR, then digested with *Eco*RI and *Bam*HI, and cloned into pRIT2T. Transcription of the fusion protein (PA-GFP) was controlled by the lambda right promoter in pRIT2T. (b) Strains harboring pRIT2T or pPAGFP127 were grown on LB agar plates at 37°C (1 day), followed by 25°C (3 days). Cells collected from 2 plates (about 0.3 g) were suspended in 1 ml of TEN buffer and sonicated. Supernatant (1 μl) obtained after centrifugation was resolved by SDS-PAGE. The cell extract, prepared from strains harboring pRIT2T (lanes 2 and 4) or pPAGFP127 (lanes 3 and 5), were stained with CBB (lanes 2 and 3) or irradiated by UV light at 365 nm (lanes 4 and 5). Molecular mass markers are shown in lane 1.

buffer and the bound materials were eluted with the same buffer containing 9 M urea. Fractions emitting green fluorescence under a UV light at 365 nm (MANASLULIGHT, Manaslu Chemical Industry Co. Ltd., Japan) were pooled and dialyzed against TEN buffer.

2.4. Electrophoresis and immunoblotting

Proteins were separated on 0.1% SDS-14% polyacrylamide gels according to Laemmli [20]. SDS-PAGE molecular mass standards kit was purchased from Bio-Rad Laboratories, USA. The separated proteins were stained with 0.25% Coomassie brilliant blue (CBB) R-250, or electroblotted onto nitrocellulose membranes. Proteins were dot blotted using the Easy-Titer™ ELIFA system (Pierce Chemical Co., USA).

The blotted proteins were visualized by means of conventional color detection or fluorescent detection using PA-GFP. After blocking (1 h at 25°C) with 3% BSA and an incubation (1 h at 37°C) with rabbit antiserum to human NSE (Eiken Chemical Co. Ltd., Japan), the bound antibody was visualized after an incubation for 20 min in PA-GFP working solution. The greenish fluorescent bands or spots were detected under a UV light at 365 nm, and recorded on Polapan 3200B (Polaroid Corp., USA) using a green filter. For color detection, the bound antibody was visualized with POD-labeled sheep antibody against rabbit IgG and DAB (Sigma Chemical Co., USA) as described by Hawkes et al. [21].

3. Results and discussion

3.1. Construction and expression of PA-GFP

The *Aequorea* GFP cDNA was modified by PCR using the plasmid TU#65 [8] as a template. The amplified fragment (0.75 kb), containing *Eco*RI or *Bam*HI site at each end, was digested with these enzymes and cloned into the protein A fusion vector pRIT2T. The resulting plasmid, pPAGFP127, carried a fused protein A-GFP cDNA encoding a protein of about 60 kDa composed of 500 amino acids (except for the initiating methionine residue) downstream from the lambda right promoter (Fig. 1a). The cell extracts prepared from *E. coli* strains harboring pRIT2T or pPAGFP127 were analyzed by SDS-PAGE (Fig. 1b). PA-GFP was not detected as a major band by CBB staining (lane 3). However, a strong greenish fluorescent band of 47 kDa, and a weak 43-kDa band were visualized by UV-irradiation only in the sample from the strain harboring pPAGFP127 (lane 5). The major (47 kDa) and minor (43 kDa) fluorescent bands were thought to be PA-GFP and its degradation product, respectively; but the molecular mass differed considerably from the theoretical value (60 kDa).

The lambda right promoter is commonly induced by shifting the growth temperature from 30 to 42°C for 90 min in *E. coli* N4830-1, which produces a temperature sensitive repressor [15]. However, fluorescent PA-GFP was not expressed by this standard induction procedure (data not shown). Instead, fluorescent PA-GFP was expressed most efficiently in *E. coli* HB101 constitutively at 25°C (or below) on agar rather than in liquid medium. The fluorescence intensity increased gradually, reaching a maximum at 3–5 days, at low temperature under oxygen rich conditions on plates, rather than by the faster expression at higher temperatures. Although the mechanism is still unclear, GFP must be modified post-translationally to acquire fluorescent activity. Inouye and Tsuji [22] have reported that the fluorescent intensity of intact *Aequorea* GFP expressed in *E. coli* cells increases in harvested cells stored overnight at room temperature. Lim et al. [23] have also reported the thermosensitive formation of fluorescent GFP in *Saccharomyces cerevisiae*. These reports and our results suggest that many parameters of cell growth (e.g., temperature, oxygen, and expression time) are important for the formation of fluorescent GFP.

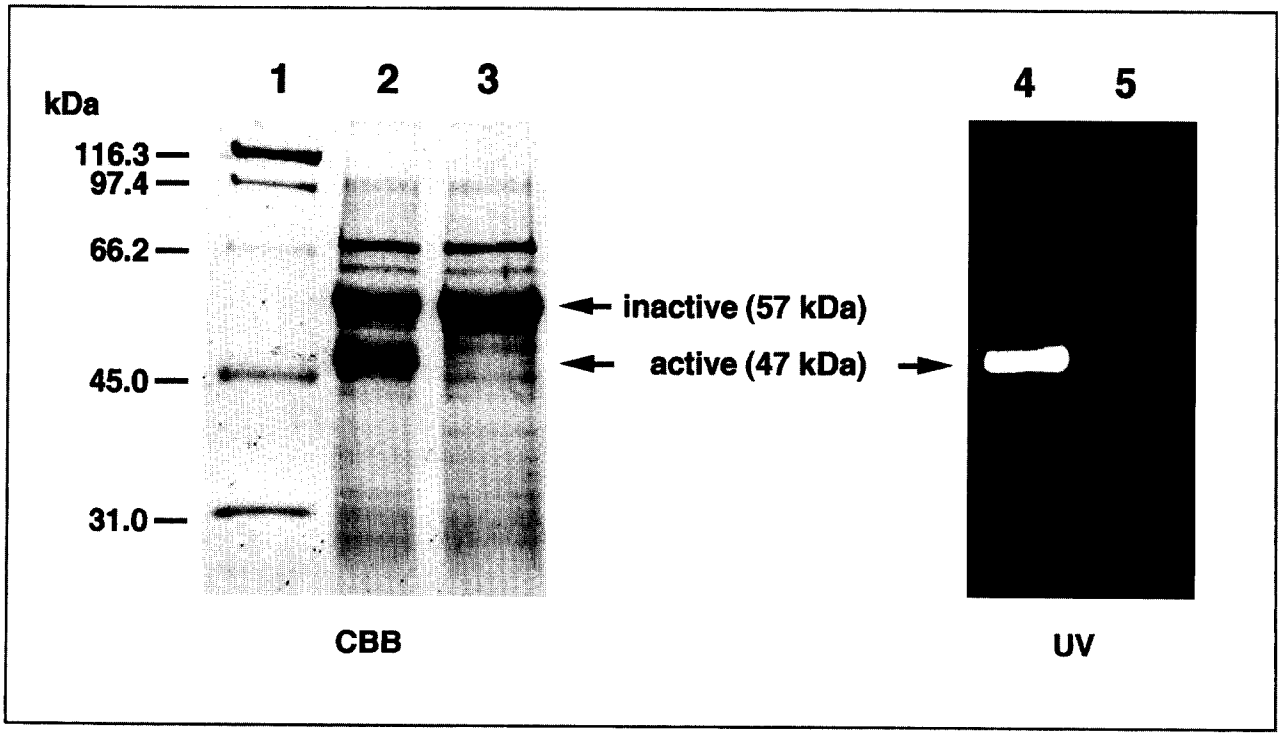

Fig. 2. SDS-PAGE of purified PA-GFP. PA-GFP was purified as described in section 2. After dialysis, the fraction containing PA-GFP (5.2 ml) was resolved by SDS-PAGE. Purified PA-GFP (20 μl) was directly loaded on the gel (lanes 2 and 4) or after heating of 95°C for 5 min (lanes 3 and 5). PA-GFP was stained with CBB (lanes 2 and 3) or irradiated by UV light at 365 nm (lanes 4 and 5). Fluorescent and non-fluorescent PA-GFP bands are indicated as 'active' and 'inactive', respectively. Molecular mass markers migrated in lane 1.

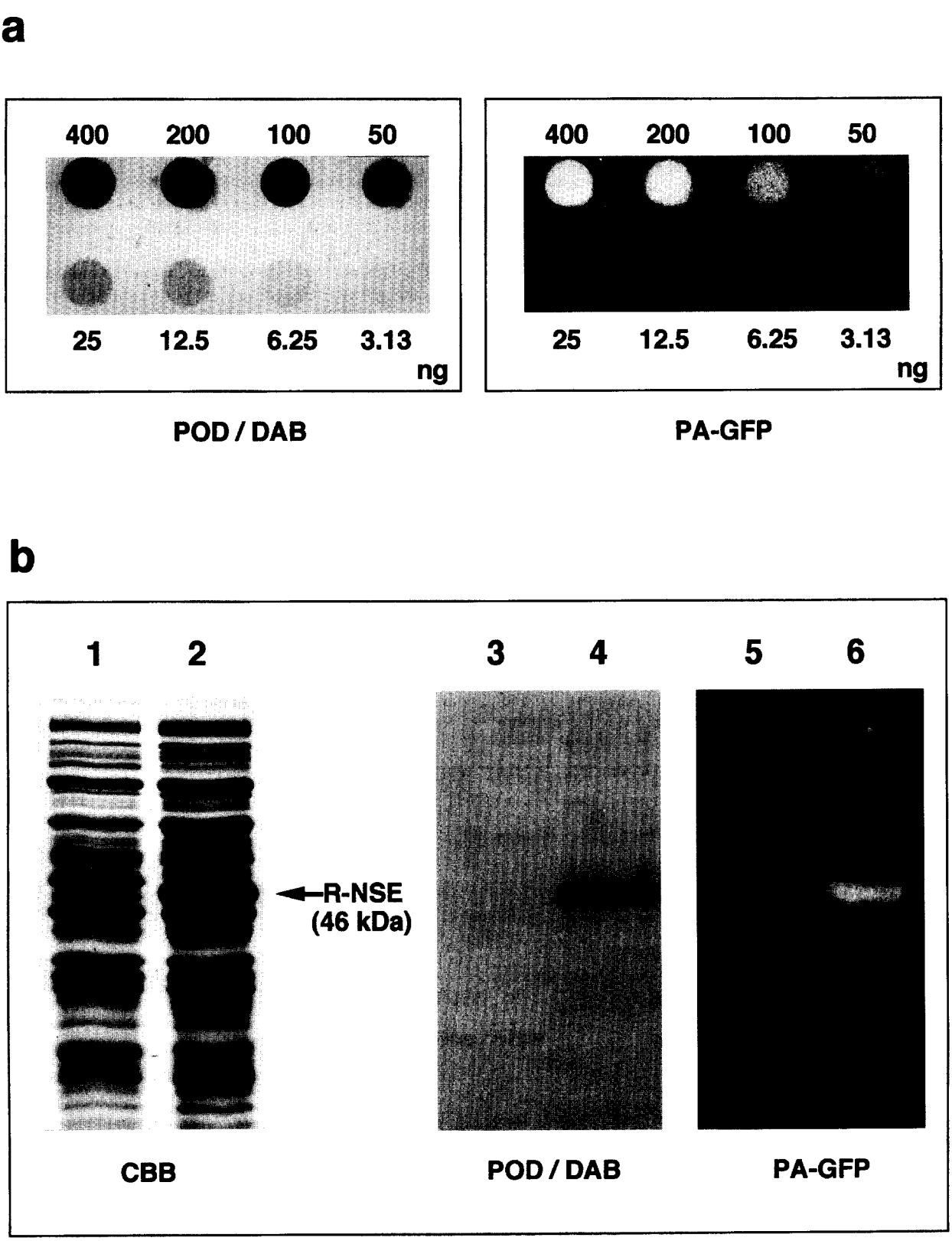

Fig. 3. Comparison of PA-GFP and conventional POD/DAB detection. (a) Purified R-NSE [17] (3.13–400 ng) was blotted onto a nitrocellulose membrane as 4 mm spots. (b) The *E. coli* cell extract prepared from the R-NSE non-producing strain, harboring pKK223-3 (lanes 1, 3 and 5), or the R-NSE producing strain, harboring pHTK503 (lanes 2, 4 and 6), were resolved by SDS-PAGE and electroblotted as described [17]. The separated proteins were stained with CBB (lanes 1 and 2), and the R-NSE band was detected with POD/DAB system (lanes 3 and 4) or PA-GFP (lanes 5 and 6) as described in section 2.

3.2. Electrophoretic analysis of purified PA-GFP

To clarify molecular properties of fluorescent PA-GFP in *E. coli* cells, purified PA-GFP was prepared from cell extracts of the strain harboring pPAGFP127 using a column containing rabbit IgG coupled to Sepharose 6B. As shown in Fig. 2, purified PA-GFP migrated as major bands at 47 kDa and 57 kDa; but only the former band was fluorescent. When the sample was denatured by heating, only one non-fluorescent band was detected at 57 kDa position, corresponding to the upper band in lane 2. The theoretical molecular mass of PA-GFP is about 60 kDa. Therefore, these results indicate that the upper band in lane 2 is non-fluorescent (inactive) PA-GFP, and that the lower band is fluorescent (active) PA-GFP although it did not correspond to the theoretical molecular mass. It is still unclear whether inactive PA-GFP is an artifact of purification and/or it is an extant cellular component.

Intact *Aequorea* GFP expressed in *E. coli* cell migrated at about 40 kDa as a greenish fluorescent band in SDS-PAGE [22]. This did not correspond to the theoretical molecular mass (28 kDa), but heat-denatured (non-fluorescent) GFP migrated at the position predicted for its theoretical mass. Therefore, when active GFP molecules were inactivated, the mobility in SDS-PAGE shifted from the upper (40 kDa) to the lower position (28 kDa). In contrast with this report, our results indicate that protein A-fused GFP molecules shifted from the lower (47 kDa) to the upper position (57 kDa) when the active molecules were denatured. This may be caused by the fusion, but is difficult to explain. However, at least, from these results, we speculate as follows: (i) in SDS-PAGE, the formation of an imidazolone ring within GFP influences the mobility of the molecule; (ii) PA-GFP is not denatured completely by SDS alone owing to the ring structure; and (iii) such partially denatured PA-GFP migrates at the different position from the PA-GFP denatured completely by SDS and heating, because both PA-GFPs differ in part of conformation and amount of SDS binding to the molecules.

3.3. Application of PA-GFP to immunoblotting

Dot and Western blots were performed using cell extracts prepared from the *E. coli* strain harboring pPAGFP127, and the sensitivity and specificity were compared with those of the standard POD/DAB system. Dot blots of purified recombinant human NSE (R-NSE) [17] are shown in Fig. 3a. The POD/DAB system was slightly more sensitive than PA-GFP detection. However, the sensitivity of PA-GFP detection was clearly sufficient for rapid and easy screening. The cell extract prepared from the R-NSE producing strain [17] was then examined by Western blotting. As shown in Fig. 3b, the specificity of PA-GFP detection was identical to that of the POD/DAB system. Though further comparative studies are needed, it is clear that PA-GFP can be used in these blotting studies.

The useful properties of PA-GFP detection are: (i) chemicals for detection are not required, therefore the procedure is simple, rapid and inexpensive; (ii) the reaction is controlled easily by monitoring the fluorescent band under UV light; (iii) the fluorescence is stable to UV-irradiation for at least 30 min; (iv) the fluorescence and binding activity do not decrease when stored (for at least 1 month at 4°C); and (v) purification of PA-GFP is not required for blotting: therefore, the crude cell extract of the strain harboring pPAGFP127 can be applied directly without purification.

In this study, the second antibody against rabbit IgG was not combined in the PA-GFP detection and only a polyclonal antibody was examined. If a second antibody is used, the sensitivity should increase, which will also allow the detection of monoclonal antibodies.

Acknowledgements: We thank Dr. M. Chalfie for providing the plasmid TU#65, and Messrs. M. Kaneta and H. Onagi for technical assistance.

References

[1] Morise, H., Shimomura, O., Johnson, F.H. and Winant, J. (1974) Biochemistry 13, 2656–2662.
[2] Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cormier, M.J. (1992) Gene 111, 229–233.
[3] Prendergast, F.G. and Mann, K.G. (1978) Biochemistry 17, 3448–3453.
[4] Shimomura, O. (1979) FEBS Lett. 104, 220–222.
[5] Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G. and Ward, W.W. (1993) Biochemistry 32, 1212–1218.
[6] Bokman, S.H. and Ward, W.W. (1981) Biochem. Biophys. Res. Commun. 101, 1372–1380.
[7] Ward, W.W. and Bokman, S.H. (1982) Biochemistry 21, 4535–4540.
[8] Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) Science 263, 802–805.
[9] Barthmaier, P. and Fyrberg, E. (1995) Dev. Biol. 169, 770–774.
[10] Baulcombe, D.C., Chapman, S. and Cruz, S.S. (1995) Plant J. 7, 1045–1053.
[11] Hu, W. and Cheng, C.-L. (1995) FEBS Lett. 369, 331–334.
[12] Ikawa, M., Kominami, K., Yoshimura, Y., Tanaka, K., Nishimune, Y. and Okabe, M. (1995) Dev. Growth Differ. 37, 455–459.
[13] Wang, S. and Hazelrigg, T. (1994) Nature 369, 400–403.
[14] Olson, K.R., McIntosh, J.R. and Olmsted, J.B. (1995) J. Cell Biol. 130, 639–650.
[15] Zabeau, M. and Stanley, K.K. (1982) EMBO J. 1, 1217–1224.
[16] Nilsson, B., Abrahmsén, L. and Uhlén, M. (1985) EMBO J. 4, 1075–1080.
[17] Aoki, T., Kimura, M., Keneta, M., Kazama, H., Morikawa, J. and Watabe, H. (1993) Tumor Biol. 14, 261–270.
[18] Mandel, M. and Higa, A. (1970) J. Mol. Biol. 53, 159–162.
[19] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[22] Inouye, S. and Tsuji, F.I. (1994) FEBS Lett. 351, 211–214.
[23] Lim, C.R., Kimata, Y., Oka, M., Nomaguchi, K. and Kohno, K. (1995) J. Biochem. (Tokyo) 118, 13–17.