EXCLI Journal 2005;4:91-104 – ISSN 1611-2156
Received: 22. September 2005, accepted 4. October 2005, published: 7. October 2005

Original article:

Construction of chimeric antibody binding green fluorescent protein for clinical application

Virapong Prachayasittikul*, Chartchalerm Isarankura-Na-Ayudhya, Yaneenart Suwanwong, Srisurang Tantimavanich

Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, 2 Prannok Rd., Bangkok-Noi, Bangkok, Thailand 10700, Telephone: 662-419-7172, Fax: 662-412-4110, e-mail: mtvpr@mucc.mahidol.ac.th (*corresponding author)

ABSTRACT

A chimeric antibody-binding green fluorescent protein (ZZGFPuv) was successfully constructed and applied as a powerful tool for immunological diagnosis. A gene encoding two repetitive sequences of Z-domain, derivative of IgG-binding B domain of staphylococcal protein A, was fused in-frame to the N-terminus of *gfpuv* gene. The chimeric gene was subsequently transformed and expressed in various strains of *E. coli*. Expression of chimeric protein in *E. coli* strain HB101 resulted in a protein translocation from cytoplasm to periplasmic space and cultivation medium. The chimeric ZZGFPuv could be purified using either IgG Sepharose column or immobilized metal (Cu^{2+}) affinity chromatography. The purified protein migrated in non-denaturing SDS-PAGE as two major bands. A fluorescent band was located at 36 kDa while another band at 48 kDa exhibited non-fluorescence. The fluorescent band was isolated and assessed for IgG-binding via fluorescent emission. The lowest amount of IgG that could be detected by dot immunobinding assay was approximately 630 ng. Indirect immunofluorescent assay for a serological detection of leptospirosis was performed by using the chimeric ZZGFPuv as IgG detector. A strong fluorescent intensity as comparable to that of the fluorescein isothiocyanate (FITC) conjugated system was significantly detected. All these findings support a high feasibility to apply the chimeric Ab-binding GFP for clinical applications in the future.

Keywords: Fc binding, Z-domain, green fluorescent protein

INTRODUCTION

In a variety of immunoassays, secondary antibody is considerably accounted for the sensitivity of the testing system. It can provide a high specific binding and a magnification of detection signal. Tracing of signal can be done by chemical labeling of a secondary antibody with reporter molecules such as radioisotope, enzyme, fluorescein and chemiluminescent compounds (Russell et al., 2004). In such cases, enzyme is the most frequently used due to its stability and ease to handle. However, in many circumstances disadvantages arise by the multiple assay steps, the use of carcinogenic or toxic substrates and the interference of endogenous enzymes and substrate of the system. Alternatively, antibody can be labeled with the fluorescent compounds and extensively be applied in the immunofluorescent assay. Tagging of fluorescence may simply be performed by chemical conjugation with fluorescent compounds while intensity variation between batches is generally occurred. Photobleaching during assay observation is also another problematic factor. Therefore,

development of a versatile stable reagent that can be utilized as universal secondary antibodies is utmost necessary to maximize the immunoassay testing. Construction of a chimeric protein providing a stable fluorescence emission activity along with a specific binding to immunoglobulin becomes a particular aim of our study.

Green fluorescent protein (GFP) is an autofluorescent protein isolated from the Pacific Northwest jellyfish, *Aequorea victoria* (Shimomura et al., 1962). It is a monomeric protein composed of 238 amino acids with molecular mass of 29 kDa. It can generate a green light emission (λ_{max} = 509 nm) upon illumination with long-wave UV light source (Morise et al., 1974). Neither additional substrates nor cofactors except molecular oxygen is required (Heim et al., 1994). GFP is a very stable molecule in which the fluorescence remains at high temperature (Tm at 78C), extreme pH (2-11), high molar denaturant (6 M guanidinium hydrochloride; GdnHCl), and prolonged incubation with many kinds of protease (Ward et al., 1982). The fluorescence is formed by autocatalytic cyclization of hexapeptide (residues 64-69) within the chromophore. This fluorescence can readily be measured using common modes of detection. Therefore, the GFP is widely used as a reporter molecule for gene expression (Chalfie et al., 1994), protein localization (Feilmeier et al., 2000; Phillips 2001), protein transportation (Kaether et al., 1995), protein binding events (Hernandez et al., 1998), and protein fusions (Strathdee et al., 2000). In this study, GFPuv is aimed to use as a reporter molecule owing to the special characteristics as follows: i) high efficiency of protein folding at 37C; ii) high solubility; iii) high intracellular protein diffusibility; iv) strong fluorescent intensity (18 times brighter than wild type GFP) (Crameri et al., 1996).

For the antibody-binding motif, much attention has been focused on the staphylococcal protein A (SpA) (Nilsson et al., 1987). It consists of five highly homologous immunoglobulin (Ig) binding domains in tandem (designated as domain E, D, A, B and C) proceeding from N to C termini. Each domain rearranges as a three-helix bundle consisting of 57-60 residues that can independently bind Fc portion of Ig. It has been well established that the SpA is relatively stable. It is partially but reversibly denatured upon exposure to 6M GdnHCl. Furthermore, it is partly intact even after heating at 80C. Supportive evidence from the circular dichroism (CD) shows that the secondary structure conformation remains intact over a pH range of 0.99-11.8 (Sjoholm 1975). Coupling of the SpA to certain enzyme has been applied as an enzyme-conjugated secondary antibody (Falini et al., 1980; Tuan et al., 1986). Conjugation of the SpA with other fluorescent and bioluminescent proteins at the genetic level further expands its application in immunoassay and immunological diagnosis (Aoki et al., 1996; Aoki et al., 2000; Kobatake et al, 1993). Furthermore, it has also been reported that secretion of protein A throughout the cell membrane effectively occurs due to the presence of SpA signal sequence. This opens up a feasibility to use such a vector containing the SpA signal sequence as a tool for secretion of target protein.

To miniaturize the antibody-binding motif, a Fc-specific binding motif derived from the SpA B-domain (Z-domain) has been created and applied for protein conjugation (Nilsson et al., 1987). It has been stated that the Z domain shows a number of binding sites on IgG similar to SpA while providing significantly fewer sites on IgA, IgM and Fab (Ljungberg et al., 1993). This indicates a high specificity of the Z-domain to Fc portion of IgG. Owing to the small size and high stability, the Z-domain has successfully been fused to the proinsulin and insulin-like growth factor to facilitate protein purification (Nilsson et al., 1991; Kang et al., 1994). Therefore, this study has conducted to engineer a chimeric gene encoding chimeric GFPuv carrying two repetitive sequences of Z-domain. Expression, purification and characterization of the fusion protein have

been performed. Feasibility of applying the chimeric antibody-binding GFP as a powerful tool for clinical application is as well evaluated.

MATERIALS AND METHODS

Plasmids and bacterial strains

Plasmid pEZZ18 (Amersham Biosciences, Stockholm, Sweden) harbouring gene coded for double motifs of Z-domain (Nilsson et al., 1987) and plasmid pHis6-GFPuv (Prachayasittikul et al., 2000) were used for chimeric gene construction. *E. coli* strain HB101 (F-, *thi*-1, *hsd*S20(r_B-, m_B-), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20(str^r), *syl*-5, *mtl*-1), strain BL21(DE3) F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^r, and strain TG1 (*sup*E, *hsd*Δ5, *thi*Δ(lac-proAB), F'[*tra*D36 *pro*AB+ *lac*I^q lacZΔM15] were used as hosts for transformation and gene expression.

Enzymes and chemicals

Restriction endonucleases and calf intestinal alkaline phosphatase were purchased from New England Biolabs (USA). T4 DNA ligase was obtained from Sibenzyme (Russia). All other chemicals were of commercially available.

Gene construction

To construct a chimeric gene encoding two consecutive Fc-binding domains and GFP, a fragment of GFP encoding gene was cleaved out from the pHis6-GFPuv using *Sac*I and subsequently inserted into the *Sac*I site of pEZZ18, yielding a plasmid of pSZZGFP. The standard protocols for ligation and $CaCl_2$ transformation (Sambrook et al., 2001) were used. The in-frame fusing of chimeric gene was verified by restriction endonuclease analysis.

Protein expression and purification

Crude extract preparation

E. coli carrying plasmid pSZZGFP were grown in LB medium (Tryptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L) supplemented with ampicillin 100 mg/L. The culture was incubated at 30C with shaking (150 rpm) for 24 hrs. Cells were harvested by spinning at 6,000 rpm for 5 min. Cells were resuspended in working buffer (TST buffer for IgG sepharose purification or phosphate buffer for IMAC purification, ingredients below) and further disrupted by sonic disintegration at output 5 for 10 cycles of 60 sec pulse-on and pulse-off. Cell debris was removed by centrifugation at 10,000 rpm for 15 min.

Protein purification via IgG-sepharose

The crude extract was applied directly to an equilibrated IgG-sepharose column (Amersham Biosciences, Stockholm, Sweden). Unbound protein was washed out with 10 column volumes of TST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) followed by 2 column volumes of 5 mM ammonium acetate, pH 5.0. Bound protein was then eluted with 0.5 M acetic acid, pH 3.4. Fractions were immediately neutralized by addition of 0.5 M Tris, pH 10.0.

Protein purification via Immobilized Metal Affinity Chromatography (IMAC)

Bulk protein was partially eliminated by heating of crude extract at 65C for 5 min. The denatured proteins were then precipitated by centrifugation at 10,000 rpm for 20 min. The clear supernatant was loaded onto the Metal Chelating Sepharose 6B column charged with $CuSO_4$. After washing with phosphate buffer (50 mM Na_2HPO_4, 0.3 M NaCl, pH 7.4), bound protein was eluted upon addition of elution buffer (50 mM Na_2HPO_4, 0.3 M NaCl, 20 mM EDTA, pH 7.0). Green fluorescent fractions were then collected for further analysis.

Cell fractionation

After separating cells from extracellular medium, the osmotic shock was performed according to Brewer and Sassenfeld (Brewer et al., 1990) to release periplasmic protein. One milliliter of packed cells was resuspended in 8.0 mL periplasting buffer

(20% w/v sucrose, 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.15% Triton X-100). After leaving at room temperature for 5 min, cells were collected by spinning at 10,000 rpm. Pellet was resuspended in 4.0 mL cold water and swirled gently in ice bath for 10 min. Supernatant containing solubilized protein was collected by centrifugation at 6,000 rpm for 3 min at 4C. The spheroplasts were further sonicated and centrifuged at 10,000 rpm for 10 min at 4C to separate the cytoplasmic and debris fraction.

Protein characterization

SDS-PAGE and immunoblotting

Proteins separation was performed on 0.1% SDS-12% polyacrylamide gel without heat denaturation. Discontinuous buffer system was used as described (Laemmli, 1970). For immunoblotting, separated proteins were electrotransferred to nitrocellulose membrane using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, USA). The membrane was subsequently blocked with 3% BSA and then probed with human immunoglobulin (50 µg/mL). TBS (50 mM Tris, 200 mM NaCl, pH 7.2) was used as washing and diluting solution. After 3 times wash, the membrane was incubated with 1:2000 HRP-rabbit anti-human immunoglobulin (DakoCytomation, Glostrup, Denmark). Specific bound antibody was then visualized by incubating with 0.05% 3, 3'-diaminobenzidine tetrahydrochloride or DAB (Zymed, USA) and 0.01% hydrogen peroxide in TBS.

Dot-immunobinding assay

Proteins were spotted onto nitrocellulose membrane and dried at room temperature. The immunodetection was performed as described earlier.

Fluorescence measurement

Fluorescence was assayed by using FLx 800 microplate fluorescence reader (BIO-TEK, USA). Fluorescent emission of GFPuv at 508 nm was measured upon excitation at 395 nm.

Application of chimeric protein in indirect fluorescent assay

Glass slides coated with 0.5 McFarland cell suspension of *Leptospira enterogans* were probed with 1:10 serum from leptospirosis patient. Two times washing with PBS were done and the slides were incubated with various amounts of ZZGFPuv or FITC-conjugated rabbit anti-human immunoglobulin (DakoCytomation, Glostrup, Denmark). After wash off excess protein or antibodies, fluorescent signal was detected under fluorescent microscope using FITC filter.

RESULTS

Construction of chimeric ZZGFPuv gene

A plasmid pSZZGFP encoding a chimeric GFP carrying IgG-binding domain (Z-domain) was successfully constructed. Verification of the in-frame fusing was performed by restriction endonuclease analysis. An increase in size of the pSZZGFP up to 5.3 kb was observed upon digestion with *Eco*RI. Addition of *Sac*I resulted in the presence of two corresponding bands of 4.6 and 0.7 kb for the pEZZ18 and *gfp* gene, respectively (data not shown). A plausible conformation of the chimeric ZZGFPuv was illustrated in Fig. 1**.**

Protein purification and characterization

E. coli strain HB101 was selected as a host of choice for chimeric protein purification. Since the chimeric protein carried the Fc-binding domain, therefore, it could easily be purified using IgG immobilized affinity chromatography. However, it is worth to note that the protein migrated as 2 major bands at approximately 48 and 36 kDa on the non-denaturating SDS-PAGE. These two bands possessed IgG binding capability determined by immunoblotting. The upper band was supposed to be the unfolding state of complete fusion protein with the calculated size of 48 kDa. While the lower band rearranged as the compact form in which a strong fluorescent emission could be detected (Fig. 2).

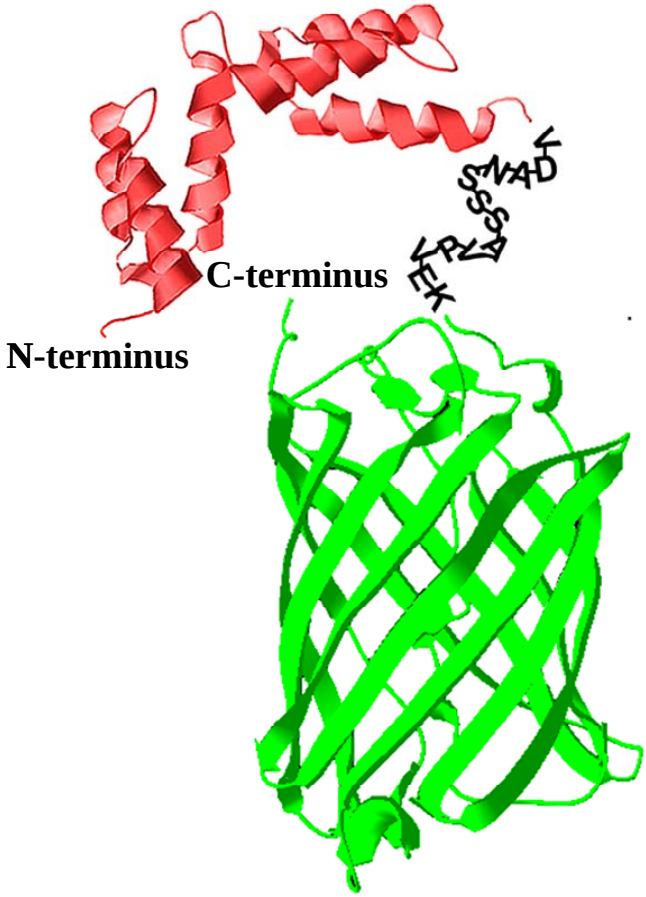

Figure 1: Schematic representation of the chimeric green fluorescent protein carrying two consecutive regions of Fc-binding domain (ZZGFPuv).

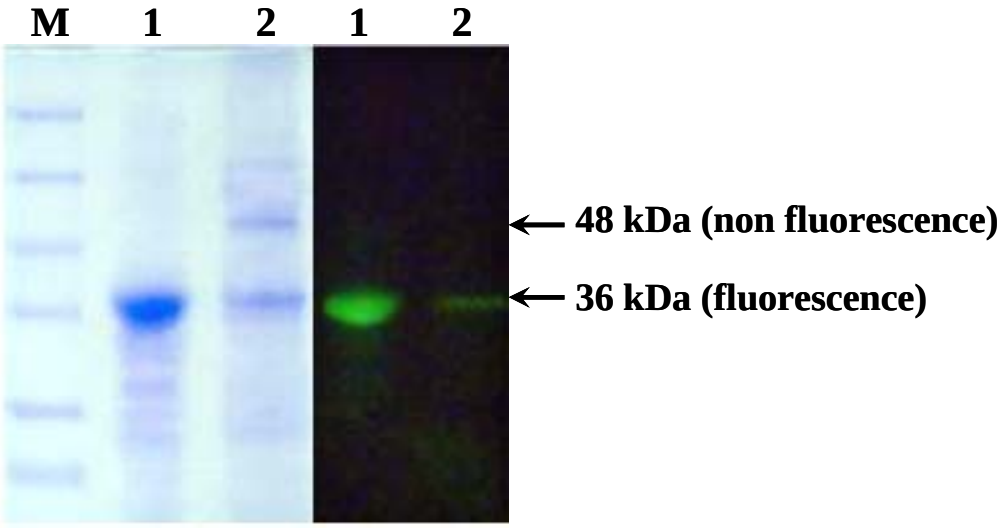

Figure 2: SDS-PAGE analysis of purified ZZGFPuv. Two identical gels were demonstrated. Gel on the left side was Coomassie brilliant blue stained and on the right side was observed under UV illumination. Lane 1 represents purified protein isolated from SDS-PAGE gel, lane 2 represents purified protein obtained from IMAC-Cu(II) and M indicates molecular weight marker.

In an attempt to isolate the fluorescent-active form, the chimeric protein was further purified by means of immobilized metal ion affinity chromatography charged with copper ions. However, the result remained the same as obtained from IgG sepharose column (Fig. 2; lane 2). Finally, separation of the active form was carried out by cutting of the fluorescent band from the polyacrylamide gel. The gel fragment was then placed in a dialysis bag filled with Tris-HCl, pH 8.0 and the electrical current was applied to elute protein out of the gel. The result of the purification of ZZGFPuv was summarized in Table 1. The purified protein showed a great purity (Fig. 2; lane 1) with the purification fold of approximately 20 times. The purified ZZGFPuv possessed a specific fluorescent intensity of 4.8 x 10^5 RFU/mg. This fluorescence was approximately 2 times less than the native GFPuv.

Table 1 Purification of ZZGFPuv from crude extract of *E. coli* strain HB101/pSZZGFP

Purification step	Vol. (mL)	Protein		Total Fluorescence (RFU x 10^5)	Specific Fluorescence (RFU x 10^5/mg)	Purification fold	Recovery (%)
		Total (mg)	Conc. (mg/mL)				
Crude extract	200	1240	6.2	291.0	0.2	1.0	100
Heating at 65C	180	378	2.1	180.8	0.5	2.5	62.1
IMAC-Cu	70	147	2.1	149.1	1.0	5.0	51.2
SDS-PAGE	25	10.5	0.42	50.5	4.8	24.0	17.3

IgG-binding activity of the chimeric protein

To evaluate the IgG-binding activity, the ZZGFPuv was applied as a fluorescent-labeled antibodies-specific ligand for fluoroimmunoassay. The detection limit of fluorescent signal on nitrocellulose membrane was approximately 250 ng protein/spot (Fig. 3A). The sensitivity of immunoglobulin-binding assay was at 630 ng Ig/spot observed under UV transilluminator (Fig. 3B).

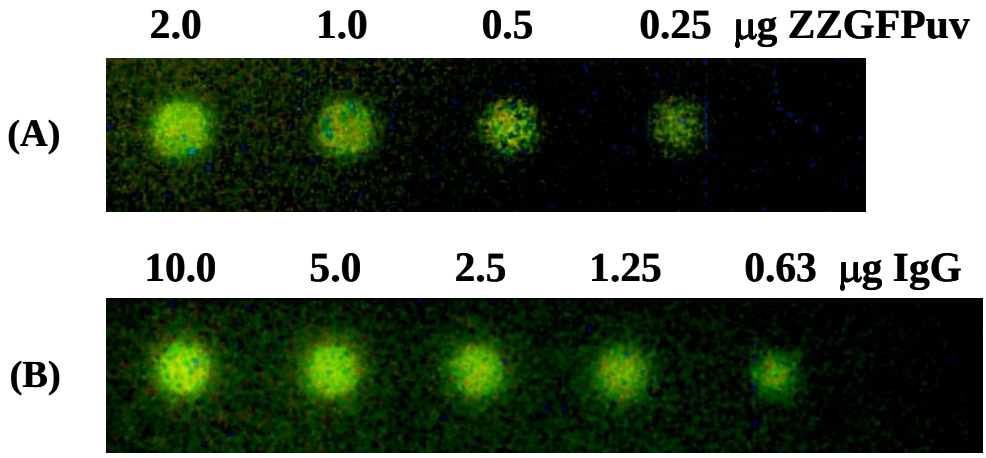

Figure 3: (A) Various amounts of ZZGFPuv (0.25-2.0 μg) were spotted on nitrocellulose membrane and (B) a series of human IgG (0.63-10 μg) spotted on nitrocellulose membrane were probed with ZZGFPuv. The fluorescent signals excited by UV light were observed and captured (BIO-RAD Digi Doc).

Detection of Leptospira antibodies

A feasibility of using the chimeric ZZGFPuv as a secondary labeling antibody was evaluated. Detection of Ab against *Leptospira* in patient serum was performed. The slides coated with *L. enterogans* were incubated with 3 different concentrations of ZZGFPuv (Figs. 4A-C). The fluorescent signal of the chimeric GFP was observed under fluorescent microscope as compared to the FITC-system. Non-specific binding was applied as a control by incubating the slides with normal serum. Our findings indicated that increasing of fluorescent intensity corresponded to the protein concentration. From this experiment, the minimum amount of 300 ng of the ZZGFPuv provided a great discrimination over the background interference for detection of *Leptospira* antibody using indirect immunofluorescent assay**.**

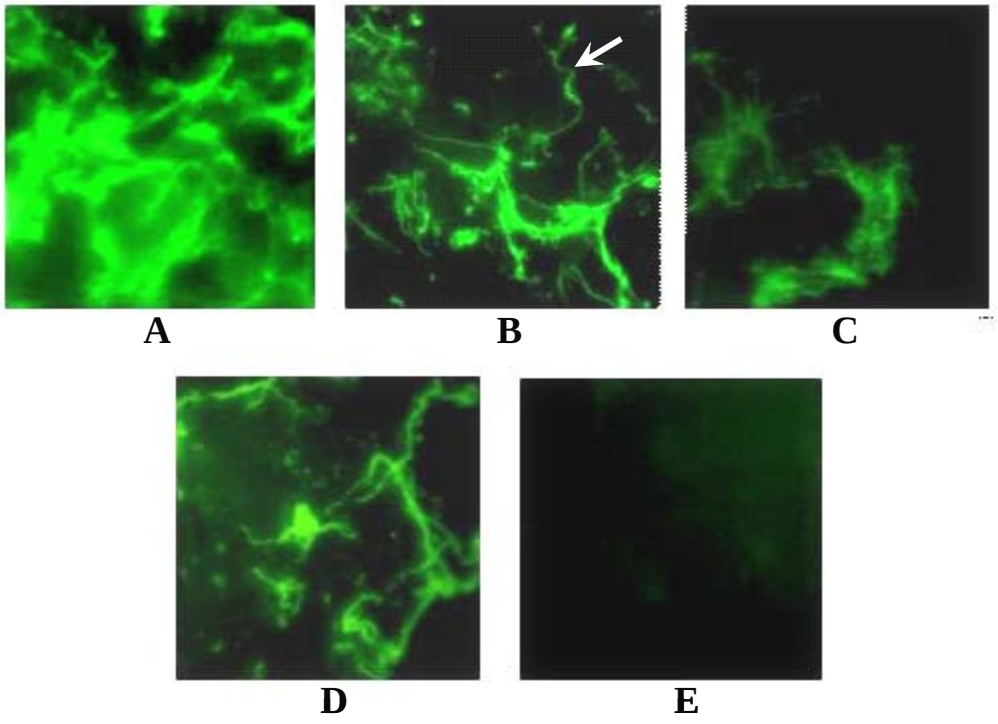

Figure 4: Detection of antibodies against *leptospira* in patient serum by IFA using ZZGFPuv as a detection probe. Various amounts of ZZGFPuv were used; (A) 1200 ng, (B) 300 ng and (C) 75 ng. FITC system was also used for comparison (D). Non-specific binding was monitored by incubating *leptospira*-coated slide with normal serum before probing with chimeric protein (E)

Strain-specific for chimeric protein expression

To further investigate whether expression of the chimeric ZZGFPuv relied on specific host, two more strains of *E. coli* including TG1 and BL21 were selected for comparison. Expression of the chimeric protein in different hosts was monitored by measuring the fluorescent intensity of engineered cells. As shown in Fig. 5, the fluorescent emission was in the order of HB101>TG1>BL21. This indicated that expression of the chimeric ZZGFPuv was in a strain-dependent fashion.

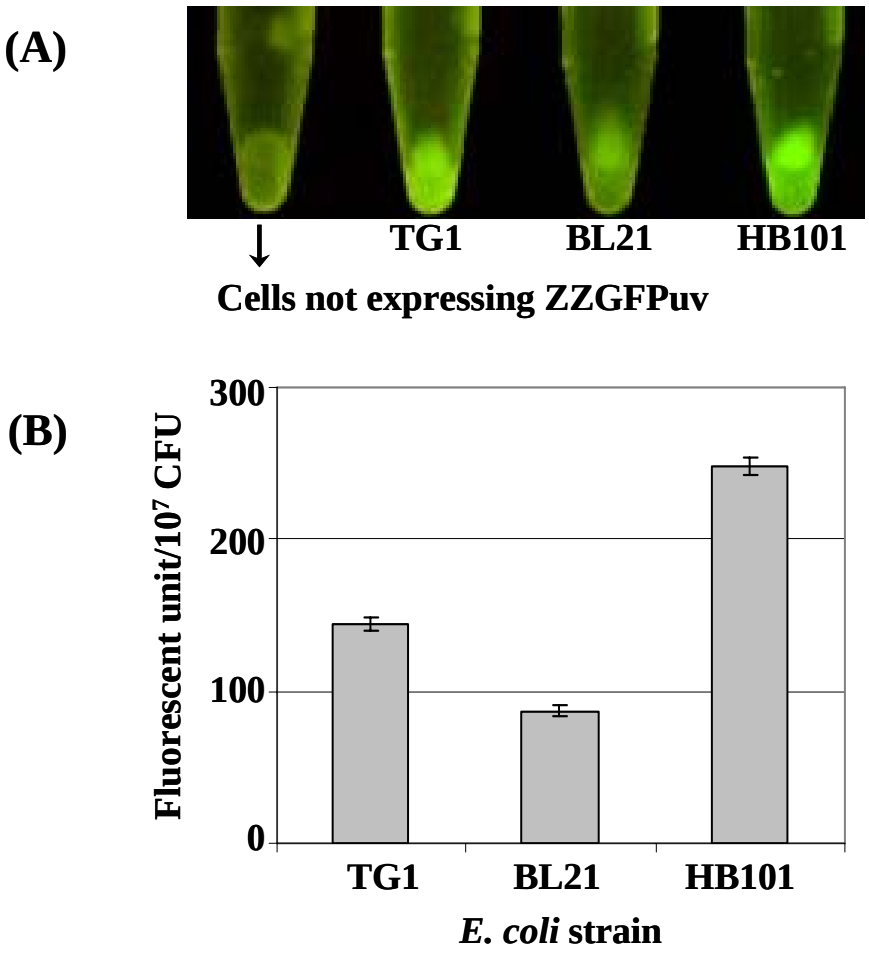

Figure 5: (A) Suspension of cells expressing ZZGFPuv was observed under UV irradiation. Fluorescent emissions of chimeric ZZGFPuv expressed in 3 strains of *E. coli* (TG1, BL21, and HB101) were compared. (B) The fluorescent intensities of cells expressing ZZGFPuv were quantified by measuring fluorescent emission light (508 nm) upon excitation with UV light (395 nm) using microplate fluorescence reader. The values are mean values of triplicate measurements and error bars indicate standard deviation.

Translocation of chimeric ZZGFPuv in E. coli

Further question arose whether the presence of SpA signal sequence facilitated protein transportation across the bacterial membrane. A chimeric ZZGFPuv was expressed and secretion of the protein to the medium was investigated. Each of culture medium and crude extract was individually loaded onto the IgG Sepharose column. Eluate fractions were collected and subjected to dot-immunobinding assay. As represented in Fig. 6, the majority of chimeric protein was found in the crude extract. This indicated the ineffectiveness of protein transportation to the medium.

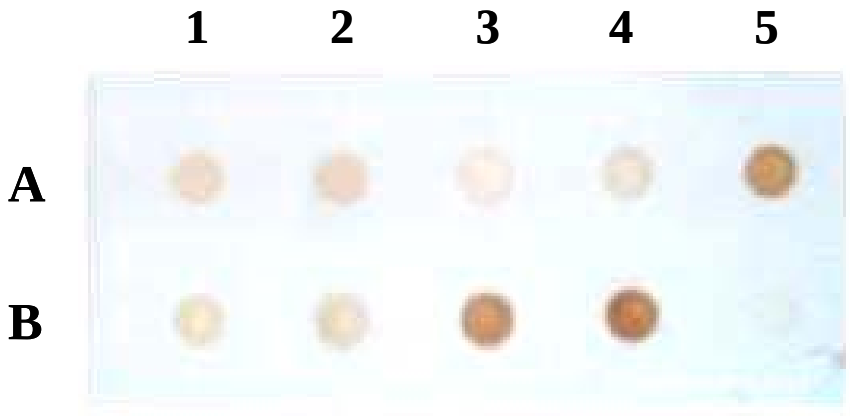

Figure 6: Dot-immunobinding assay of cultured medium (lane 1), eluate fraction from IgG-sepharose column of culture medium (lane 2), crude extract (lane 3), and eluate fraction from IgG-sepharose column of crude extract (lane 4). Panel A represents fractions from cell not expressing ZZGFPuv whereas panel B represents fraction from ZZGFPuv expressing cells. SpA 100 ng was blotted as a control (5A).

To enhance the efficiency of transportation, cultivation of the engineered cells was controlled by the heat induction process. *E. coli* HB101 carrying pSZZGFP was grown in the medium at 30C for 24 hrs. After that, one portion was kept constant at 30C while the temperature of another portion was raised up to 42C to induce protein secretion and continued incubating for 3 hrs (Abrahmsen et al., 1986; Moks et al., 1987). Protein obtained from each compartment (extracellular, periplasm, cytoplasm and cell debris) was analyzed by both of SDS-PAGE and immunoblotting. As shown in Fig. 7 and Table 2, approximately 60 % of ZZGFPuv was secreted into the medium upon induction with the temperature shift while less than 10% was secreted under the normal growth condition.

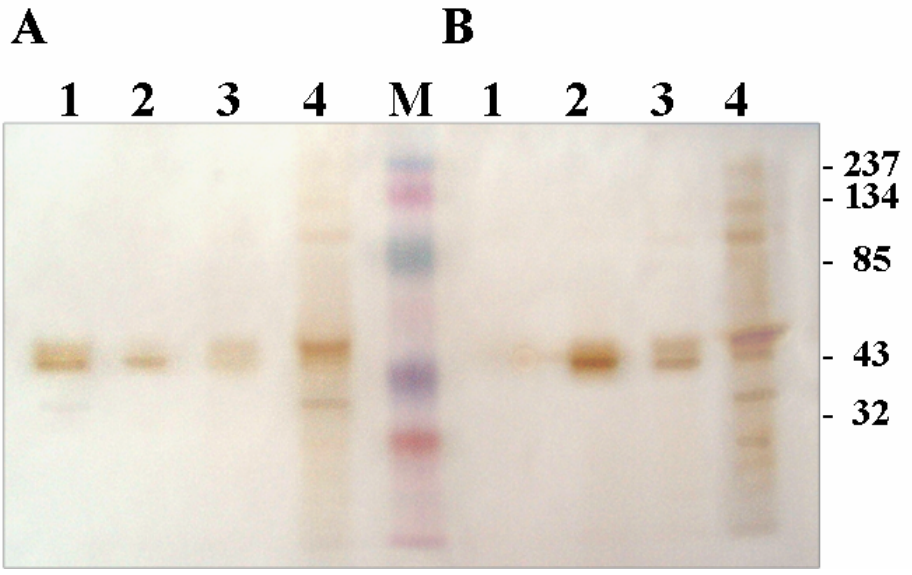

Figure 7: Immunoblotting of proteins from each fraction of HB101/pSZZGFP. Panel A and B represent fractions from HB101/pSZZGFP cultured in heat-induced and normal condition, respectively. Lane 1 extracellular medium, lane 2 periplasm, lane 3 cytoplasm and lane 4 cell debris. M indicates molecular weight marker shown in kDa.

Table 2 Protein and fluorescent determination of each compartment of *E. coli* strain HB101/pSZZGFP cultured in normal and heat-induction condition

Fraction	Volume (mL)	Protein		Total fluorescence (RFU x 10^5)	Specific fluorescence (RFU x 10^5/mg protein)	% yield
		Total (mg)	Conc. (mg/mL)			
Heat shock culture (42C)						
Medium	4.2	14.1	3.36	3.46	0.25	59.1
Periplasm	3.8	2.2	0.57	0.30	0.14	5.1
Cytoplasm	8	19	2.38	0.75	0.04	12.8
Cell debris	1	2.8	2.76	1.35	0.48	23
Normal culture (30C)						
Medium	4.3	4.4	1.03	1.37	0.31	9.1
Periplasm	4.5	6.4	1.42	3.26	0.51	21.6
Cytoplasm	11	28.5	2.59	3.71	0.13	24.5
Cell debris	2	3.5	1.75	6.78	1.94	44.8

DISCUSSION

Feasibility of using chimeric antibody binding GFP for clinical application

A potential approach of using a chimeric antibody-binding green fluorescent protein (ZZGFPuv) as a tool for immunological diagnosis has been explored. The Fc-binding portion (Z-domain) has successfully been fused to the N-terminus of the green fluorescent protein at the genetic level (Fig. 1). The chimeric protein possessed dual characteristics of both antibody binding and autofluorescent emission has been effectively applied for detection of antibodies against *Leptospira* in patient serum. A strong fluorescent signal as comparable to that of the FITC system has been visualized under fluorescent microscope (Fig. 4). Advantages of using the same set of excitation/emission filter and the minimized photobleaching of the GFP compared to the FITC enlarge the scope of utilization (Griep et al., 1999). Furthermore, detection of fluorescence in real-time expands its applicability for single-step immunoblotting. The complete reaction can be finished within an hour, which is approximately 3 times faster than that of the conventional HRP/DAB detection system (Aoki et al., 2000). These findings open up a high feasibility to apply the chimeric antibody-binding GFP for clinical applications in the future.

Characteristics of chimeric Ab-binding GFP

Based on the previous studies, it is well established that the IgG binding capability of the staphylococcal protein A has not been changed dramatically upon fusion to the bioluminescent or fluorescent molecule (Aoki et al., 1996; Kobatake et al., 1993). Meanwhile, little is known on the other Ig-binding protein derivatives. In this study, we report for the first time the binding affinity to IgG of the Fc-binding monodomain (Z-domain) derived from the B-domain of staphylococcal protein A. Two consecutive sequences of Z-domain possess a strong binding interaction to immobilized IgG while maintaining a strong fluorescent emission (Fig. 3B). This high binding affinity and specificity to IgG as comparable to the SpA native domain has been reported (Jansson et al., 1998). However, the relative smaller size and high protein solubility are also being counted as its advantages. Furthermore, the stability against hydroxylamine and cyanogen bromide due to the substitution of Ala1 → Val and Gly29 → Ala makes it more reliable than the B-domain.

The presence of active form (fluorescent band; migrated at 36 kDa) and inactive form (non-fluorescent band; migrated at 48 kDa)

of chimeric ZZGFPuv have been observed on the non-denaturation SDS-PAGE (Fig. 2). While, a complete denaturation upon heat treatment (95C, 5 min) reveals a single band at 48 kDa. These findings indicate that the 36 kDa band exhibits a compact conformation of folded state while the 48 kDa band represents an unfolded state of ZZGFPuv. Similar evidences have also been reported demonstrating that some portions of chimeric protein A-GFP and protein G-EGFP expressed in *E. coli* remain in inactive state (Aoki et al., 1996; Arai et al., 1998). It is worth to mention that active form of antibody-binding GFPs (ZZGFPuv, protein A-GFP and protein G-EGFP) moves faster than the inactive form in non-denaturing gel. Contrarily, the opposite mobility pattern has been found in the wild-type GFP (Inouye et al., 1994). A plausible explanation is that incorporation of a certain amount of sodium dodecyl sulphate (SDS) to the antibody-binding portion facilitates a larger extent on protein mobility than the GFP molecule. However, effects of isoelectric point and hydrophilicity between these two molecules have to be taken into consideration (Aoki et al., 2002; Aoki et al., 2003).

Expression and localization of chimeric Ab-binding GFP in E. coli

Based on our findings, bacterial host strain as well as the presence of signal peptide is a major factor affecting the expression and localization of chimeric ZZGFPuv. *E. coli* strain HB101 has experimentally been proven to be a suitable host for high level expression and secretion of chimeric ZZGFPuv throughout the cellular membrane (Figs. 5 and 7). A gene encoding ZZGFPuv has been placed downstream of the SpA signal sequence and the gene expression has been regulated by the SpA promoter, in which the secretion of heterologous protein can successfully be potentiated in *E. coli*. The presence of SpA signal sequence facilitates protein translocation from cytoplasm to extracellular via the Sec pathway. For circumstances, the insulin-like growth factor I (IGF-I) and proinsulin with complete function have successfully been secreted by this system (Kang et al., 1994; Loewenadler et al., 1987). Since the GFP has a unique structure and relatively larger than the IGF-I and proinsulin, we doubt whether a mature GFP can be secreted throughout the cell membrane by the same system. From our findings, the ZZGFPuv has successfully been secreted to the periplasm and the medium. However, notification has to be made that induction of temperature up to 42C in the cultivation process facilitates the protein transportation up to 60%. Supportive evidences on the production of protein content (up to 20 folds) and the enhancement of secreted product upon temperature shift after stationary phase have also been reported (Abrahmsen et al., 1986). Our studies lend support the notion that expression and secretion of the GFP can be obtained via the Sec-dependent protein translocation. Feilmeier et al. (2000) have previously demonstrated that the GFP can be exported to the periplasmic space by fusing with the maltose-binding protein carrying a typical Sec-dependent signal peptide. However, the GFP hybrid protein in periplasm exhibits a complete non-fluorescent indicating the improper folding. In contrast, Casey et al have demonstrated the successfulness of periplasmic expression of fluorescent GFP upon fusion with the scFv and PelB signal sequence (Casey et al., 2000). In our case, some portion of the ZZGFPuv (approximately 40%) remains located inside the cells (Table 2). The presence of protein residual in cell debris may be attributable to the binding interaction between the hydrophobic region of the signal sequence and lipid membrane. This phenomenon has been evidenced by the incorporation of chimeric cadmium-binding GFP to the inner membrane of *E. coli* (Prachayasittikul et al., 2004).

Future perspectives

Future perspectives for the development of a versatile reagent for clinical application are now taken as the ongoing research in our lab. For instances, genetic re-manipulations of the chimeric Ab-binding GFP are needed to be performed in order to attain a high level

protein expression. These include construction of the chimeric gene encoding chimeric GFP carrying the repetitive sequences of Z-domain on the C-terminus in which the high level fluorescent emission can be obtained. Expression of cytoplasmic chimeric Ab-binding GFP without the signal peptide is performed. Since the presence of signal peptide e.g. CAT and PelB has been reported to reduce the gene expression while production of the protein A and protein G fused to GFP intracellularly yield the protein content up to 30 mg/L (Griep et al., 1999; Arai et al., 1998; Casey et al., 2000; Fujino et al., 2002).

Acknowledgements

Y.S. is a postgraduate student supported by the collaborative Ph.D. fellowship from Royal Thai Government. We thank to Dr. Wimol Petkanchanapong for providing serum samples.

REFERENCES

Abrahmsen L, Moks T, Nilsson B, Uhlen M. Secretion of heterologous gene products to the culture medium of *Escherichia coli*. *Nucleic Acids Res* 1986;14:7487-500

Aoki T, Takahashi Y, Koch K, Leffert H, Watabe H. Construction of a fusion protein between protein A and green fluorescent protein and its application to western blotting. *FEBS Lett* 1996;384:193-7

Aoki T, Miyashita M, Fujino H, Watabe H. A flexible single-step detection of blotted antigen using a fusion protein between protein A and green fluorescent protein. *Biosci Biotechnol Biochem* 2000;64:1547-51

Aoki T, Tahara T, Fujino H, Watabe H. "GFP-display", an easy detection method for single amino acid changes in a target polypeptide: application to random mutagenesis. *Anal Biochem* 2002;300:103-6

Aoki T, Tahara T, Satoh K, Fujino H, Watabe H. General properties of GFP-display, an electrophoretic analysis for single amino acid changes in target polypeptides. *Anal Biochem* 2003;317:107-15

Arai R, Ueda H, Nagamune T. Construction of chimeric proteins between protein G and fluorescence-enhanced green fluorescent protein, and their application to immunoassays. *J Ferment Bioeng* 1998;86:440-5

Brewer SJ, Sassenfeld HM. Engineering proteins for purification. In: Angal S, editor. Protein purification applications - a practical approach. 1st ed. Oxford: Oxford University Press; 1990. p. 96

Casey JL, Coley AM, Tilley LM, Foley M. Green fluorescent antibodies: novel *in vitro* tools. *Protein Eng* 2000;13:445-52

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science* 1994;263:802-5

Crameri A, Whitehorn EA, Tate E, Stemmer WP. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat biotechnol* 1996;14:315-9

Falini B, Tabilio A, Zuccaccia M, Martelli MF. Protein A-peroxidase conjugates for two-state immunoenzyme staining of intracellular antigens in paraffin-embedded tissues. *J Immunol Methods* 1980;39:111-20

Feilmeier BJ, Iseminger G, Schroeder D, Webber H, Phillips GJ. Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. *J Bacteriol* 2000;182:4068-76

Fujino H, Aoki T, Watabe H. A highly sensitive assay for proteases using staphylococcal protein A fused with enhanced green fluorescent protein. *Biosci Biotechnol Biochem* 2002;66:1601-4

Griep RA, Twisk C, Wolf JM, Schots A. Fluorobodies: green fluorescent single-chain Fv fusion proteins. *J Immunol Methods* 1999;230:121-30

Heim R, Prasher DC, Tsien RY. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc Natl Acad Sci U S A* 1994;91:12501-4

Hernandez EC, Daunert S. Recombinant green fluorescent protein as a label in binding assays. *Anal Biochem* 1998;261:113-5

Inouye S, Tsuji FI. Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett* 1994;341:277-80

Jansson B, Uhlen M, Nygren PA. All individual domains of staphylococcal protein A show Fab binding. *FEMS Immunol Med Microbiol* 1998;20:69-78

Kaether C, Gerdes HH. Visualization of protein transport along the secretory pathway using green fluorescent protein. *FEBS Lett* 1995;369:267-71

Kang Y, Yoon JW. Effect of modification of connecting peptide of proinsulin on its export. *J Biotechnol* 1994;36:45-54

Kobatake E, Iwai T, Ikariyama Y, Aizawa M. Bioluminescent immunoassay with a protein A-luciferase fusion protein. *Anal Biochem* 1993;208:300-5

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5

Ljungberg UK, Jansson B, Niss U, Nilsson R, Sandberg BE, Nilsson B. The interaction between different domains of staphylococcal protein A and human polyclonal IgG, IgA, IgM and F(AB')2: separation of affinity from specificity. *Mol Immunol* 1993;30:1279-85

Lowenadler B, Jansson B, Paleus S, Holmgren E, Nilsson B, Moks T, Palm G, Josephson S, Philipson L, Uhlen M. A gene fusion system for generating antibodies against short peptides. *Gene* 1987;58:87-97

Moks T, Abrahamsen L, Österlöf B, Josephson C, Östling M, Enfors SO, Persson I, Nilsson B, Uhlen M. Large-scale affinity purification of human insulin-like growth factor I from culture medium of *Escherichia coli*. *Bio/Technology* 1987;5:379-82

Morise H, Shimomura O, Johnson FH, Winant J. Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry* 1974;13:2656-62

Nilsson B, Moks T, Jansson B, Abrahmsen L, Elmblad A, Holmgren E, Henrichson C, Jones TA, Uhlen M. A synthetic IgG-binding domain based on staphylococcal protein A. *Protein Eng* 1987;1:107-13

Nilsson B, Forsberg G, Hartmanis M. Expression and purification of recombinant insulin-like growth factors from *Escherichia coli*. *Methods Enzymol* 1991;198:3-16

Phillips GJ. Green fluorescent protein --a bright idea for the study of bacterial protein localization. *FEMS Microbiol Lett* 2001;204:9-18

Prachayasittikul V, Isarankura Na Ayudhya C, Mejare M, Bulow L. Construction of a chimeric histidine6-green fluorescent protein: role of metal on fluorescent characteristic. *Thammasat Int J Sc Tech* 2000;5:61-8

Prachayasitikul V, Isarankura Na Ayudhya C, Boonpangrak S, Galla HJ. Lipid-membrane affinity of chimeric metal-binding green fluorescent protein. *J Membrane Biol* 2004;200:47-56

Russell J, Colpitts T, Holets-MaCormack S, Spring T, Stroupe S. Defined protein conjugates as signaling agents in immunoassays. *Clin Chem* 2004;50:1921-9

Sambrook J, Russell DW. Molecular cloning. A laboratory manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001

Shimomura O, Johnson FH, Saiga Y. Extraction, purification, and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J Cell Comp Physiol* 1962;59:223-39

Sjoholm I. Protein A from *Staphylococcus aureus*. Spectropolarimetric and spectrophotometric studies. *Eur J Biochem* 1975;51:55-61

Strathdee CA, McLeod MR, Underhill TM. Dominant positive and negative selection using luciferase, green fluorescent protein and beta-galactosidase reporter gene fusions. *Biotechniques* 2000;28:210-2, 214

Tuan RS, Fitzpatrick DF. Alkaline phosphatase conjugated protein A as a sensitive reagent to immunoscreen an expression cDNA plasmid library: isolation of cDNA to the calcium-binding protein of the chick embryonic

chorioallantoic membrane. *Anal Biochem* 1986;159:329-35

Ward WW, Bokman SH. Reversible denaturation of *Aequorea* green-fluorescent protein: physical separation and characterization of the renatured protein. *Biochemistry* 1982;21:4535-40