

One-Step Purification of Chimeric Green Fluorescent Protein Providing Metal-Binding Avidity and Protease Recognition Sequence

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Various classical separation procedures basing on the physico-chemical properties of protein such as charge, size and hydrophobicity are employed for purification of recombinant proteins. These procedures are often time-consuming and laborious. Fusing of an amino acid sequence as affinity tag, therefore, has been extensively aimed for the simple and rapid approaches for purification (for recent review see Ref. 1). These include using the glutathione *S*-transferase and the maltose-binding protein as fusion partners. Protein A is also a popular fusion choice for purification of recombinant via immunoglobulin columns. Binding of biotin tag to avidin-agarose has been reported. Histidine tag has also been applied for purification via Immobilized Metal Affinity Chromatography (IMAC) for decade.²⁻⁴ The histidine tagging and IMAC have become a routine for easy first-time isolation

SUMMARY Gene fusion technique was successfully applied as a potential approach to create a metal-binding site to assist one-step purification of green fluorescent protein (GFP). The chimeric GFP carrying hexapolyhistidine (H6GFPuv) was purified to homogeneous protein via the Immobilized Metal Affinity Chromatography charged with zinc ions. Removal of metal tagger could readily be performed by using enterokinase enzyme. Engineering of the hexahistidine and enterokinase cleavage sites (DDDDK) onto the chimeric protein did not significantly affect the fluorescent property and the binding avidity to *Burkholderia pseudomallei* protease of a chimeric protease-binding GFP (H6PBGFuv). This concludes that engineering of repetitive histidine regions onto interested target protein along with the enterokinase cleavage sites will ease the complication of protein purification.

of newly expressed proteins. In most cases, the histidine tags neither affect protein folding nor interfere significantly with the biological functionality. Furthermore, the N-terminal tagging is more frequently used because several efficient endoproteases are available for precise cleavage of the tag after purification.⁵ Purification of protein with the histidine tag system provides more advantages than the other taggers since it can be performed even under denaturing conditions e.g. 8 M urea or 6 M guanidine hydrochloride.^{6,7} This is often necessary when recombinant proteins are highly expressed in *E. coli* in the form of inclusion bodies. Elution of protein can readily be done under mild conditions e.g. using metal-chelating agent (EDTA) or competitor (histidine or imidazole). Moreover, the high protein loading, simple regeneration and low cost are decisive when developing large-scale purification.

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cation procedures for industrial applications.²

Green fluorescent protein (GFP) is an autoilluminating protein isolated from the jellyfish, *Aequorea victoria*. Many researchers have focused their attention on applying the GFP as a fusion partner to monitor gene expression and protein localization in both prokaryotic and eukaryotic cells.^{8,9} In addition, the GFP has also been used for investigation of protein-protein interaction.¹⁰ Because of the vast varieties of application, an urgent need for improving of purification procedures have extensively been explored. These include using a wide variety of chromatography, e.g. ion exchange chromatography and gel filtration,³ purification via the organic extraction,^{11,12} and IMAC.^{3,4,13} For such certain conditions, to get the pure GFP from a complex mixture of biological materials can often be a difficult and time-consuming process. Therefore, it remains important to attain an approach with simple and effective for a particular recombinant protein. Furthermore, the purified protein should readily be used for further study without any other post-purification treatment.

Herein, the GFP has been expressed as a function protein together with a protease site including a stretch of six histidine residues integrated into the recombinant protein to facilitate purification by binding to a transition metal ion. Removal of the tagger can readily be observed upon addition of enterokinase enzyme. Furthermore, we also demonstrate that neither the histidine residues nor the enterokinase cleavage site significantly affect the fluorescence property or binding avidity for protease.

MATERIALS AND METHODS

Bacterial strain and plasmid

Escherichia coli TG1 (lac-pro), SupE, thi1, hsd D5/F' tra D36, pro A⁺ B⁺, lacI, lacZ, M15; (ung⁺, dut⁺) was used as host. Plasmid pGFPuv was obtained from Clontech Laboratories, USA.

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase and molecular weight marker (λ Hind III) were obtained from New England Biolabs, USA. Chelating Sepharose fast flow gel was purchased from Pharmacia Biotech, Sweden.

Chimeric genes construction

Cloning procedures were performed according to standard protocol as described by Maniatis *et al.*¹⁴ Purification of DNA from agarose gel was done via Qiaquick DNA purification system (Qiagen, USA). The oligonucleotides were synthesized by the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand.

To construct a chimeric gene encoding GFP in combination with hexapolyhistidine and enterokinase cleavage site, two synthetic oligonucleotides I (5'-AGCTTAC-ACCACCACCATCACCATGCG-AGCTCTGATGACGATGATAAA-GCCTGCA-3' and 5'-GGCTTTA-TCATCGTCATCAGAGCTCGCA-TGGTGATGGTGGTGGTGTA-3') were heated at 95°C and slowly cooled down to allow hybridization. The oligonucleotides were subsequently treated with T4 polynucleotide kinase enzyme in order to

add a phosphate group at the 5'-terminal end. These oligonucleotides were further inserted between the Hind III and Pst I sites of pGFPuv, generating plasmid pH6GFPuv.

To further generate a chimeric GFP having a protease-binding region, the H6GFPuv was cleaved with *Xba* I and *Kpn* I and the synthetic oligonucleotides II (5'-CTAGCTTGTTCCTTCATGC-CGCACACTTTCTGTTCTAGAG-CATCGATGGTAC-3' and 5'-CAT-CGATGCTCTAGAACAGAAAG-TGTGCGGCATGAAGAAACAA-G-3') encoding one region of protease-binding peptide¹⁵ were inserted between these sites, yielding plasmid pH6PBGFuv.

All these chimeric genes were subsequently transformed into *E. coli* strain TG1. Transformants that possessed greenish fluorescence were selected and the inframe-fusing of chimeric gene was checked via restriction endonucleases analysis.

Crude chimeric protein preparation

Both of the native and chimeric green fluorescent proteins were isolated from cultures of *E. coli* TG1 carrying plasmids pGFPuv, pH6GFPuv or pH6PBGFuv. Cultures were initiated in LB broth supplemented with 100 mg/l ampicillin at 37°C with shaking (150 rpm). Expression of the genes was induced by addition of 1 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) at OD₅₅₀ = 0.1. Late log phase cultures were spun at 5,000 x g for 5 minutes and the cell pellets were resuspended in 50 mM sodium phosphate buffer, pH 7.4 containing 0.3 M NaCl. The cell suspensions

were sonic disintegrated and debris was removed by centrifugation (8,000 x g, 5 minutes). The derived homogenates of native GFPuv was then heated to 65°C for 15 minutes in order to precipitate other host proteins¹⁶ while none of the chimeric H6GFPuv and H6PBGFuv was performed. Precipitate was removed by 5 minutes centrifugation at 8,000 x g and the supernatant was attained as crude chimeric protein preparation prior to further purification.

Purification of chimeric proteins via Immobilized Metal Affinity Chromatography (IMAC)

Crude protein was loaded on a metal chelating Sepharose 6B column (0.7 x 8 cm) charged with 20 mM of the desired metal ions. The column was washed with at least 5 column volumes of distilled water and equilibrated with 50 mM phosphate buffer, pH 7.4. Crude extract was loaded at a flow rate of 0.58 ml/minute. Unbound material was removed by washing with 300 ml phosphate buffer. Bound protein was eluted by using 20 mM EDTA in phosphate buffer. Fractions were collected and those possessed greenish fluorescence were pooled and dialyzed in two liters of phosphate buffer for five times. Fractions were stored at -20°C until use. To the procedure herein, copper ion was charged to the affinity column for purification of native GFP while zinc ion was used for all chimeric proteins purification.

Removal of the hexahistidine by enterokinase cleavage

Enterokinase (New England Biolabs, Beverly, MA, USA; EK) specifically recognized an Asp-Asp-Asp-Asp-Lys sequence (DDDDK)

and cleaved the peptide bond after the lysine residue.¹⁷ Removal of the hexahistidine after protein purification was performed by the enterokinase cleavage assay. The assay was performed according to the manufacturer's instruction. Briefly, the chimeric protein was incubated with enterokinase enzyme at 25°C for 16 hours. The protein mixture was then loaded onto the IMAC-Zn²⁺ column as previously described.

Binding of the chimeric protease-binding GFP to *B. pseudomallei* protease

Binding of the chimeric H6PBGFuv to *B. pseudomallei* protease was investigated by fluorescence gel retardation assay. Purified chimeric H6PBGFuv was incubated at 25°C with partial purified protease for 15 minutes. Samples were mixed with loading buffer (4x) prior applying to the gel. The mixtures were loaded onto a SDS discontinuous polyacrylamide gel. The gel was subjected to electrophoresis at constant voltage of 150 V. After electrophoresis, the fluorescence was immediately observed under UV irradiation. Binding interaction between the chimeric H6PBGFuv and protease was evidenced by decreasing of the protein mobility due to the complex formation.

Analytical assays

The fluorescence assay of chimeric GFPs was performed by measuring fluorescence intensity using a fluorometer (FLx 800, BIOTEK instrument, USA) at an excitation wavelength of 395 nm and emission at 508 nm. SDS-PAGE was performed on a 12% polyacrylamide gel in a Tris-glycine, pH 8.3

discontinuous buffer system as described by Laemmli.¹⁸ Protein concentration was quantitated by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin was used as standard.

RESULTS

Construction and expression of chimeric genes encoding GFP having hexahistidine and enterokinase cleavage site

Gene fusion technique was applied for construction of a chimeric gene coding for GFP carrying hexahistidine and enterokinase cleavage site. A plasmid pGFPuv was cleaved by *Hind* III and *Pst* I. Synthetic oligonucleotides I was inserted into the *Hind* III and *Pst* I sites of the pGFPuv, generating a chimeric gene encoding hexahistidine-enterokinase-green fluorescent protein as shown in Fig. 1. The resulting plasmid, pH6GFPuv, was subsequently transformed into *E. coli* strain TG1. Strong greenish fluorescence could then be observed under UV illumination from cells grown on agar plates. Spectroscopic monitoring of cell density (OD₆₀₀) throughout cultivation exhibited non-significant difference in growth rate between cells expressing chimeric metal-binding GFPs and the cells expressing native GFP or host cells. This indicated that addition of metal-binding region possessing non-significance metabolic burden on the cells.

Purification of chimeric H6GFPuv via immobilized metal affinity chromatography

The H6GFPuv was simply purified by one-step affinity chromatography using the IMAC charged with zinc ions. While the native

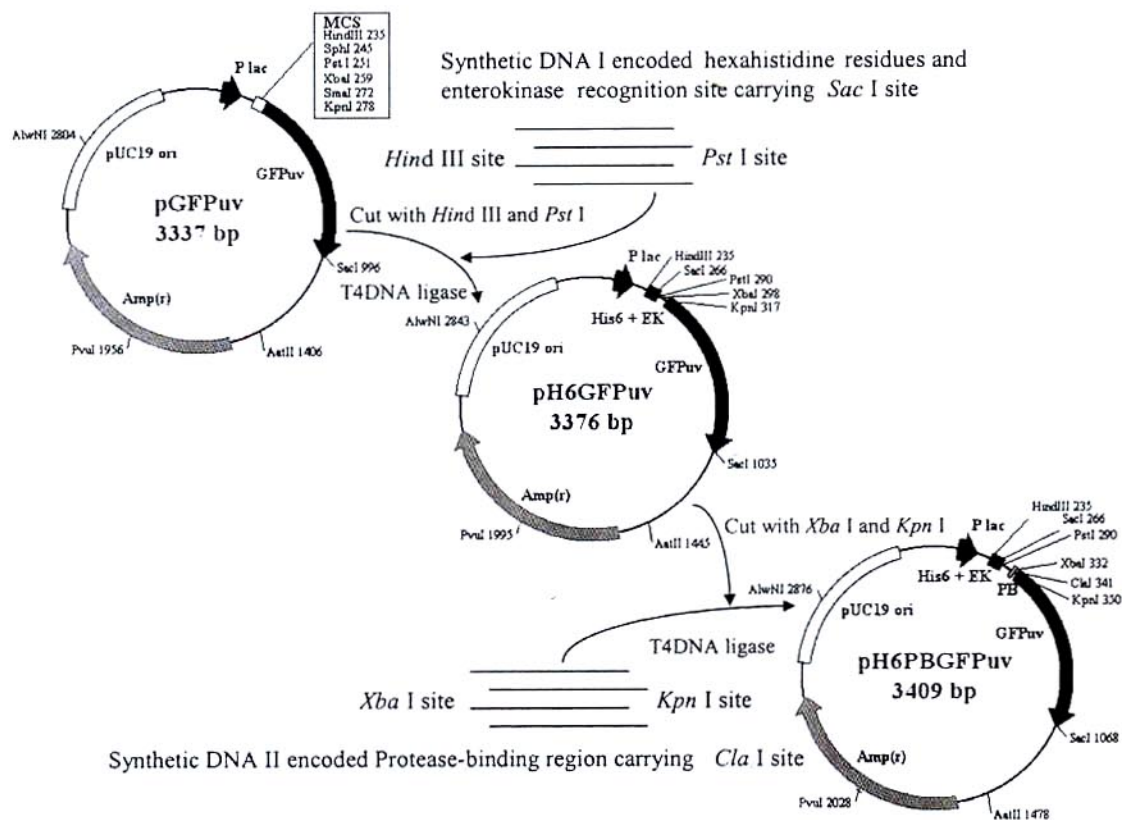


Fig. 1 Construction of chimeric genes encoding chimeric green fluorescent protein having hexapolyhistidine, enterokinase cleavage site and one region of protease-binding peptide.

GFPuv, which did not possess the hexahistidine site, was purified by multiple steps of heat treatment (65°C for 15 minutes) following by the IMAC charged with copper ions (see Table 1 for detail). The specific activity for fluorescence emission of the H6GFPuv (435×10^9 RFU/mg) was 3 folds higher than that of the GFPuv (173×10^9 RFU/mg). As shown in Fig. 2A, the GFPuv and the H6GFPuv located at *Mr* 30 and 33.7 kDa, respectively. The purified chimeric H6GFPuv showed the same excitation and emission maxima as the native protein (Fig. 2B). All these findings indicated that the polyhistidine could easily be inserted onto the protein of interest to assist protein purification.

Removal of hexahistidine fusion partner by enterokinase enzyme

To investigate whether the hexahistidine could be removed after protein purification, the chimeric H6GFPuv was incubated with enterokinase enzyme at room temperature for 16 hours. The enterokinase cleaved specifically at the recognition sequence (DDDDK). As shown in Fig. 3, the binding capability to zinc ions of the chimeric H6GFPuv was lost and the decreasing of molecular size was observed upon addition of the enzyme (lane 5). This finding was again supported by an evidence that almost all of the proteins were retained in the wash fraction (lanes 6-

8). Meanwhile, omitting of the enterokinase addition resulted in a complete binding to the IMAC-gel and recovery of the protein was done by elution with chelating agent (lanes 1-4).

Effect of the fusion partner on the protein function

Eventhough the enterokinase cleavage site and the hexahistidine tag were relatively small, their interaction with the partner fusion protein still could not be excluded. Therefore, the chimeric pH6GFPuv was further applied for further construction (Fig. 1). The synthetic oligonucleotides coding for protease-binding peptide (Cys-Phe-Phe-

Table 1 Purification of chimeric green fluorescent proteins

Protein	Purification step	Total protein (mg)	Total fluorescence (RFU)	Specific activity (RFU/mg)	Purification fold
GFPuv	Crude extract	7.2	95.85×10^9	13.3×10^9	-
	Heat treatment ^c	1.61	93.01×10^9	57.8×10^9	4.35
	IMAC-Cu	1.59	275×10^9	173×10^9	13
H ₆ GFPuv ^a	Crude extract	18.69	910×10^9	48.72×10^9	-
	IMAC-Zn	2.53	1101×10^9	435×10^9	8.93
H ₆ PBGFPuv ^b	Crude extract	11.63	14.5×10^9	1.25×10^9	-
	IMAC-Zn	1.498	17.1×10^9	11.4×10^9	9.12

^cthe crude protein extract was heated to 65°C for 15 minutes.

^aH₆GFPuv denoted chimeric green fluorescent protein having hexapolyhistidine and enterokinase cleavage site.

^bH₆PBGFPuv denoted chimeric green fluorescent protein having hexapolyhistidine, enterokinase cleavage site and one region of protease-binding peptide.

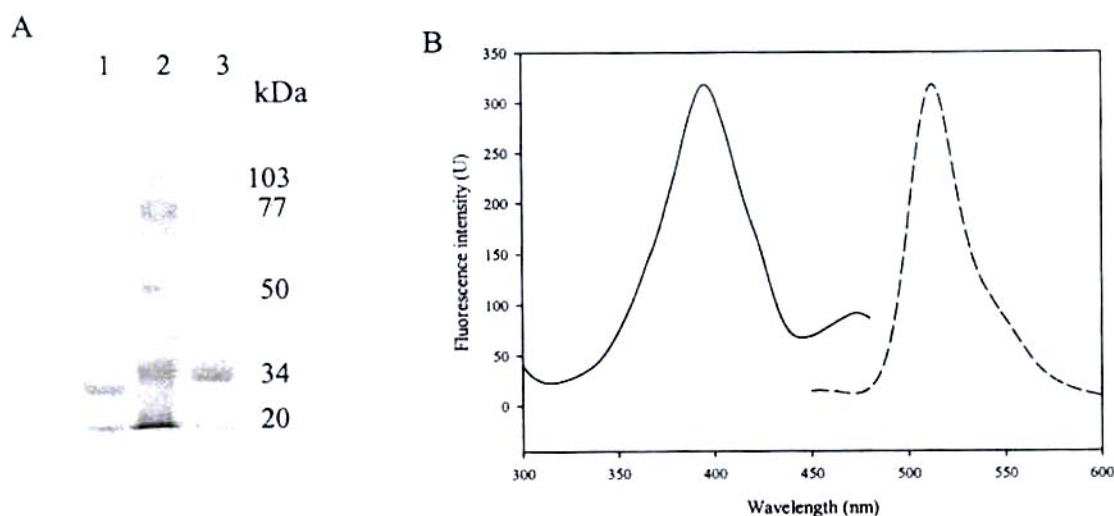


Fig. 2 A) SDS-PAGE of chimeric green fluorescent protein having hexapolyhistidine and enterokinase cleavage site (H₆GFPuv). Lane 1: native GFP, lane 2: standard protein markers and lane 3: H₆GFPuv. B) Excitation (solid line) and emission (broken line) spectra of the chimeric H₆GFPuv.

Met-Pro-His-Thr-Phe-Cys), which was previously derived from phage display technology, was used for construction of a chimeric gene encoding GFPuv providing protease binding avidity. The pH₆GFPuv was cleaved by *Xba* I and *Kpn* I then the synthetic oligonucleotide II was inserted, yielding a chimeric gene en-

coding GFP providing a protease-binding region (H₆PBGFPuv). The H₆PBGFPuv was subsequently purified via a similar manner (Table 1). Fluorescent activity of the purified chimeric H₆PBGFPuv was detected by native-polyacrylamide gel electrophoresis (PAGE) observed under UV light as shown in Fig. 4A. To test

the protease-binding avidity of the chimeric H₆PBGFPuv, the fluorescence gel retardation was applied. As represented in Fig. 4B, binding of the H₆PBGFPuv to protease from *B. pseudomallei* led to the mobility shift of the fluorescent protein (lane 3) as compared to the control protein (lane 1). In addition,

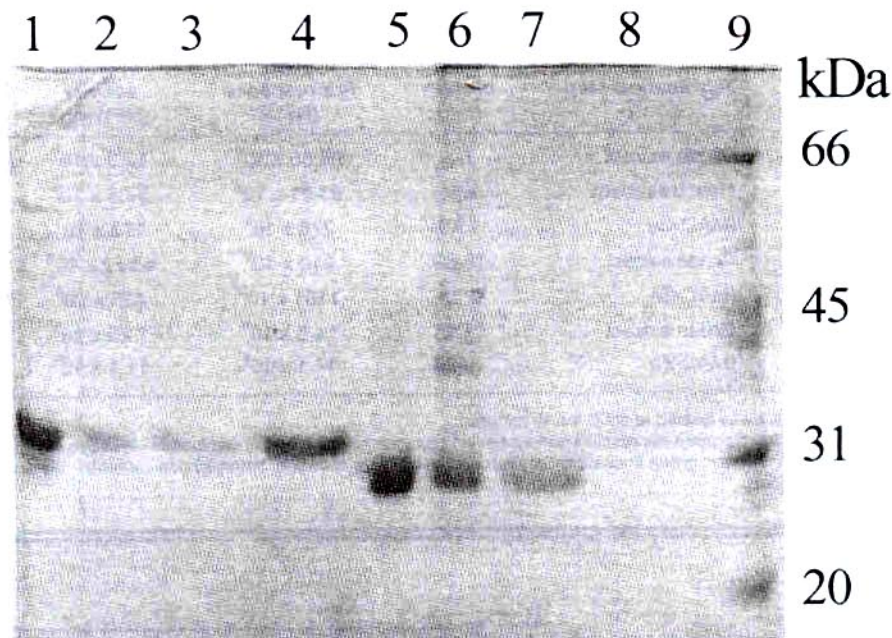


Fig. 3 Removal of hexapolyhistidine via enterokinase (EK) cleavage. Lane 1: purified H6GFPuv, lane 2: H6GFPuv (fraction after loaded onto IMAC-Zn²⁺), lane 3: H6GFPuv (wash fraction), lane 4: H6GFPuv (eluate fraction), lane 5: H6GFPuv incubated with EK for 16 hours, lane 6: H6GFPuv + EK (fraction after loaded onto IMAC-Zn²⁺), lane 7: H6GFPuv + EK (wash fraction), lane 8: H6GFPuv + EK (eluate fraction) and lane 9: standard protein markers.

the H6PBGFuv lost its fluorescence upon thermal-induced denaturation (lane 2) while heating effect on the protease led to unfolding of protein, which was unsuitable for binding interaction between these two proteins as represented in lane 4. All these findings indicated that neither the hexahistidine nor the enterokinase cleavage site affected both the fluorescent emission and the binding avidity to protease of the chimeric protease-binding GFPuv.

DISCUSSION

Our findings indicate that metal-binding tag, the N-terminal hexahistidine, makes GFPuv amenable to be simply purified by the

IMAC-Zn²⁺ in single step. The purity of the chimeric GFPs is relatively high as evidenced by a single protein band on the SDS-PAGE and in comparable with the GFP purification via the organic extraction.¹¹ Such advantage is taken from the fact that histidine is not an abundant amino acid in most proteins and hardly found in a repetitive arrangement on the protein sequence. It accounts for only 2.1% of total amino acids in globular proteins and only few are located on the surface.¹⁹ Molecular engineering of the hexahistidine to the GFPuv can then be applied to create a very strong metal-binding domain to allow a simplified purification. This domain contains electron donor groups on the imidazole ring, which readily form

coordination bonds with the immobilized transition metal ions including Cu²⁺, Ni²⁺, Zn²⁺ and Co²⁺. Herein, zinc has been chosen as linker due to having low adsorptivity to the native GFPuv as compared to copper and nickel ions.^{3,20} In addition, replacement of copper by zinc has prevented the protein damage caused by metal-catalyzed oxidation reactions.²¹

In the case of native GFPuv, two steps of purification; heat treatment (at 65°C for 15 minutes) and IMAC-Cu²⁺ are needed. This is based on the relatively high thermostability ($T_m = 78^\circ\text{C}$) and the presence of rarely surface exposed histidine residues (e.g. His25, His139, His77/-His81 and His231) on the native GFP

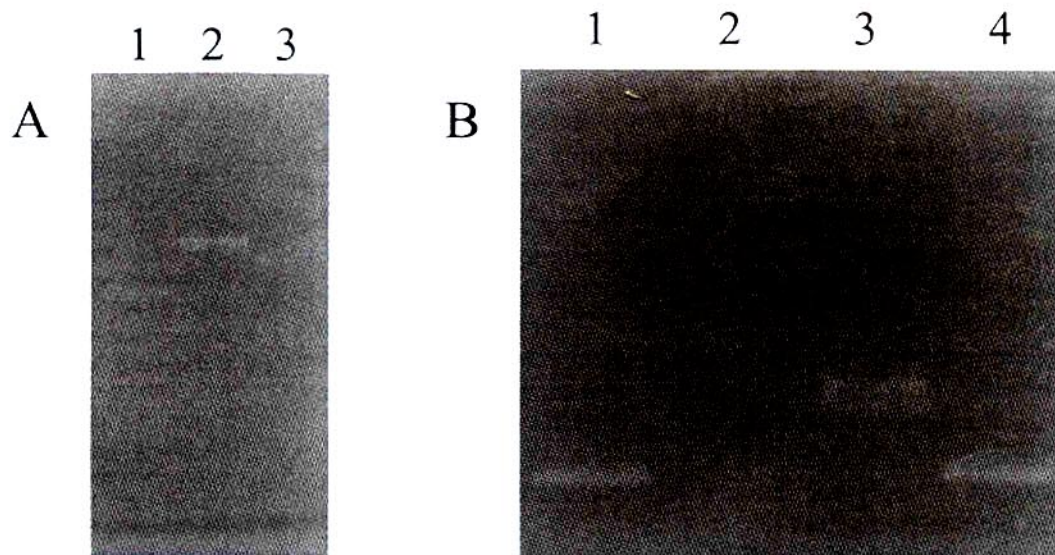


Fig. 4 A) Fluorescence property of chimeric green fluorescent proteins. Lane 1: native GFP, lane 2: H6GFPuv, and lane 3: H6PBGFPuv. B) Binding avidity to protease of the chimeric green fluorescent protein having protease-binding region as determined by fluorescence gel retardation assay. Lane 1: purified H6PBGFPuv, lane 2: H6PBGFPuv (heated at 95°C) incubated with protease, lane 3: H6PBGFPuv incubated with protease and lane 4: H6PBGFPuv incubated with protease (heated at 95°C).

molecule.^{20,22} Our finding is agreed with the previous report that the native GFPuv has been purified up to 3.3 folds from crude extract via only one step of IMAC-Cu²⁺. When the purification product has further been subjected to dialysis then loaded onto the IMAC-Ni²⁺ column the purification factor has been increased up to ten folds.²⁰ Although, the purification factor of native GFP seems to be higher than that of the chimeric GFP (Table 1). However, it is worth noting that the increasing of purification factor from 4.35 to 13 was achieved. A plausible explanation for the increase of purification factor may be due in part to the fluorescence recovery of GFP molecules upon renaturation process during purification via IMAC-Cu²⁺ and dialysis.^{23,24}

Introduction of the histidine tag onto the green fluorescent protein can be designed as a permanent or a temporary purification tag. Permanent incorporation of the N- or C-terminal fusion can be performed if the tagger does not alter the structure or the biological activity of the chimeric protein. While a site-specific cleavage site for removal of the tagger by enzymatic or chemical modification is needed for the temporary tagging. The chemical cleavages (e.g. cyanogen bromide, formic acid or hydroxylamine) are usually hazardous for protein degradation and undesirable. Using of proteolytic enzymes such as coagulation factor Xa, thrombin, carboxypeptidase and enterokinase which recognize specific sequence are preferable.²⁵⁻²⁸ The enterokinase

has been reported as an ideal enzyme providing cleavage efficiencies ranging from 60% to 90% depending on the first amino acid residue downstream of the recognition sequence.²⁸ In our case, alanine is the first amino acid in which the highest efficiency of cleavage is achieved as compared to all the other amino acid residues. Therefore, removal of the histidine tag from the GFPuv is successfully obtained as has been indicated by the loss of metal-binding avidity (Fig. 3).

It has been shown in this study that the hexahistidine in combination with the enterokinase cleavage site do not have any effect or interference on the biological activities as well as the binding avid-

ity to other macromolecules. Fluorescent activity of the H6GFPuv was relatively higher than that of the native GFP while exhibited the same maximum excitation and emission wavelength (Table 1, Fig. 2B). This is in a good agreement with the previous report that extension of the fusion partners at the N-terminus did not interfere the barrel formation of tertiary structure of GFP.²² Although, further insertion of the protease-binding peptide onto the H6GFPuv resulted in a markedly decrease of the fluorescent intensity (Table 1). However, this intensity was high enough to apply for investigation of binding interaction to protease from *B. pseudomallei* via fluorescence gel retardation assay (Fig. 4). From our findings, the chimeric H6PBGFuv provided strong binding avidity to the protease, which clearly indicates that the tagger exerts non-significant effect on binding interaction to other macromolecules. Therefore, the small affinity tags do not have to be removed after purification. This has also been reported for tagging of various proteins.²⁹⁻³¹ However, interference of the hexahistidine tagging has been notified in many cases for the protein application.³²⁻³⁴

In conclusion, this study explores a potential approach of applying the histidine residues for simple and effective one-step purification of chimeric green fluorescent protein via Immobilized Metal Affinity Chromatography (IMAC). Engineering of the histidine tag provides a number of advantages over other bioaffinity chromatography methods. For examples, the metal chelates are stable in the presence of a wide range of solvents and over a large temperature range. The sorbents are usually capable of high

metal ions loading, which permits high protein-binding capacities. In addition, mild elution conditions can be selected to prevent denaturation. The resin can also be recycled and regenerated by replenishing the supply of metal ions. Furthermore, the presence of histidine tag does not significantly affect the biological activity or binding avidity to other molecules of the protein of interest. Therefore, the chimeric protein can directly be applied for further investigation without any post-purification requirement.

ACKNOWLEDGEMENTS

The author would like to thank to the Faculty of Medical Technology, Mahidol University, for supporting partly of the expenses and utilities in this project. T.P. is particularly indebted to the Ministry of University Affairs for the graduate scholarship.

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