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Recombinant *Gaussia* luciferase with a reactive cysteine residue for chemical conjugation: Expression, purification and its application for bioluminescent immunoassays

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ABSTRACT

The mutated recombinant *Gaussia* luciferase (hgGLase) having the hinge sequence with a reactive cysteine residue at the carboxyl terminal region was purified from *Escherichia coli* cells by nickel-chelate affinity chromatography and hydrophobic chromatography. The biotinylated hgGLase (Biotin-hgGLase) was prepared by chemical conjugation with a maleimide activated biotin and apply to bioluminescent immunoassay. In the streptavidin and biotin complex system using Biotin-hgGLase, the measurable range of α-fetoprotein as a model analyte was 0.02–100 ng/ml with the coefficient of variation between 2.5% and 5.2%. The sensitivity of Biotin-hgGLase was similar to that by using the detection system of aequorin, alkaline phosophatase and horseradish peroxidase as a label enzyme.

1. Introduction

The marine copepod *Gaussia princeps* secretes a luciferase that catalyzes the oxidation of coelenterazine to emit light (λ_{max} = ~490 nm), according to the following reaction scheme [1–3]:

$$\text{Coelenterazine} + O_2 \xrightarrow{\textit{Gaussia}\text{Luciferase}} \text{Coelenteramide} + CO_2 + h\nu$$

Gaussia luciferase (GLase) dose not require any cofactors in the luminescence reaction and is a potential candidate for a reporter protein in various assay systems.

The cDNA of *Gaussia* luciferase (*GLuc*) has been isolated [3] and the deduced amino acid sequence of *Gaussia* luciferase (GLase) from *GLuc* shows 185 amino acid residues (19.9 kDa protein) including a signal peptide sequence for secretion. In the primary structure, GLase has 10 cysteine residues and consists of two repeat sequences with 71 amino acid residues containing four conserved cysteine residues [4]. The active GLase was expressed both in *Escherichia coli* [4–8] and mammalian cells [6]. The recombinant protein was also prepared using *in vitro* translation system and purified [9]. In addition, the result of the independent expression of two repeat sequences in GLase suggested that two functional domains for the luminescence reaction are present [4]. However, the protein structure of GLase including the positions of the disulfide bonds was not determined.

Recently, the codon-optimized GLase gene has been applied to *in vivo* imaging including the cultured cells [6,10] and mice [6]. However, the protein application of recombinant GLase was only reported in DNA hybridization assays [5]. The fusion protein of GLase with a biotin-acceptor peptide and a biotin protein ligase (birA) was expressed in *E. coli* cells and the biotinylated GLase was purified by affinity chromatography using a monomeric avidin resin. The complex of *in vivo* biotinylated GLase with streptavidin was used for detecting target DNA [5].

As previously reported, we have expressed GLase in *E. coli* cells using the cold induction system and the purified GLase was characterized [4]. The luminescence activity of GLase was significantly lost by treatment with reducing reagents such as dithiothreitol and 2-mercaptoethanol, suggesting that the disulfide bonds are essential for full activity of GLase [4]. Further, the chemical biotinylation of the amino group in GLase with succinimidyl 6-(biotinamido) hexanoate caused the loss of luminescence activity, indicating that the modification of lysine residue(s) might effect on the luminescence reaction of GLase (data not shown). On the other hand, the chemical modification of GLase by the maleimide reagents such as *N*-ethylmaleimide (0.1 mM) and iodoacetamide (0.1 mM) gave no significant loss of luminescence activity (data not shown). In

Abbreviations: GLase, *Gaussia* luciferase; *GLuc*, *Gaussia* luciferase cDNA; hgGLase, *Gaussia* luciferase with the hinge sequence and a reactive cysteine residue; Biotin-hgGLase, biotinylated hgGLase; AFP, α-Fetoprotein; CV, coefficient of variation; SD, standard deviation; PBS, 10 mM phosphate buffer (pH 7.4) containing 137 mM NaCl and 2.7 mM KCl; PBST-E, PBS containing 0.01% Tween 20 and 10 mM EDTA; TBS, 20 mM Tris–HCl (pH 7.6) containing 150 mM NaCl; TBST-E, TBS containing 0.01% Tween 20 and 10 mM EDTA; PBST-EB, PBST-E containing 0.4% Block Ace.

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addition, when the chemical conjugation of GLase with fluorescein-5-maleimide was performed, GLase isolated from the reaction mixture did not show fluorescence intensity (data not shown). From our preliminary results, we concluded that an active GLase has no cysteine residues for the chemical conjugation and plan to introduce a new reactive cysteine residue into GLase for the chemical conjugation with the maleimide-activated ligands.

In this report, we describe the purification of recombinant GLase with a reactive cysteine residue in the hinge sequence at the C-terminus (hereafter call hgGLase) and its application to an immunoassay.

2. Materials and methods

2.1. Construction of the expression vector for hgGLase in E. coli cells

The expression vector, pCold-hgGL (Fig. 1A), consisting of the coding region of GLase (18–185 a.a.) and the hinge sequence with a new reactive cysteine residue (SLSTPPPPSPSPSTPPC, new cysteine residue underlined) at the C-terminal region was constructed as follows. The synthetic oligonucleotide set of hinge Linker-F (5′ G agc tta tcc acc ccg ccg acc ccg tcc ccg tcc acc ccg ccg TGc CTC GAG TCT AGA G 3′) and hinge Linker-R (5′ TC GAC TCT AGA CTC GAG gCA cgg cgg ggt gga cgg gga cgg ggt cgg cgg ggt gga taa gct CTG CA 3′, new cysteine residue underlined) for the hinge sequence with a new cysteine residue was inserted into pPICZα-Linker vector derived from pPICZα A vector (Invitrogen, Carlsbad, CA) to give pPICZα-hgLinker (SFig. 1). The DNS fragment of GLase obtained by PCR procedure with a primer set of GL6-N/EcoRI (5′ gcc GAA TTC AAG CCC ACC GAG AAC AAC GAA 3′, *Eco*RI site underlined) and GL26C-TAA/PstI (5′ ggc CTG CAG GTC ACC ACC GGC CCC CTT GAT 3′, *Pst*I site underlined) was inserted into the *Eco*RI/*Pst*I sites of pPICZα-hgLinker to give pPICZα-hgGL (SFig. 2). The *Asp*718-*Xba*I fragment of pPICZα-hgGL containing GLase and the hinge sequence with a new cysteine residue was inserted into the *Asp*718/*Xba*I sites of pColdII [11] (Takara-Bio., Kyoto, Japan) to give pCold-hgGL (Fig. 1A). The expressed GLase had a histidine-tagged sequence at the N-terminus for nickel-chelate affinity chromatography and a hinge sequence with a new reactive cysteine residue at the C-terminal region (Fig. 1B, SFig. 3). The *E. coli* BL21 strain was used as a host strain (Novagen, Madison, WI).

2.2. Expression and purification of hgGLase from E. coli cells

The seed culture of *E. coli* cells possessing the expression vector pCold-hgGL was grown in 10 ml of Luria-Bertani broth containing ampicillin (50 μg/ml) at 37 °C for 18 h and was transferred into 400 ml of LB broth in a 3L flask. After incubating for 3 h at 37 °C, the culture medium was cooled in an ice-water bath for over 30 min and IPTG was added to the cultured medium at the final concentration of 0.2 mM and further incubated at 15 °C for 17 h. The cells harvested by centrifugation at 5,000*g* for 5 min from 2 L of the cultured medium were suspended in 200 ml of 50 mM Tris–HCl (pH 7.6) and disrupted by sonication using a Branson model 250 sonifier (Danbury, CT) for 12 min (3 min × 4) in an ice-water bath. After centrifugation at 12,000*g* for 20 min, the soluble fraction (200 ml) was applied on a nickel-chelate column (2.5 × 6 cm, GE Healthcare, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl (pH 7.6) and washed the column with 300 ml of 50 mM Tris–HCl (pH 7.6) at room temperature. The adsorbed proteins were eluted with 0.1 M imidazole in 50 mM Tris–HCl (pH 7.6). The fractions with luminescence activity were combined, adjusted to 1.2 M $(NH_4)_2SO_4$ and applied on a Butyl-Sepharose column (2.5 × 5.5 cm, GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 7.6)-2 mM EDTA ("Buffer A") containing 1.2 M $(NH_4)_2SO_4$. After washing with 110 ml of 1.2 M $(NH_4)_2SO_4$ in Buffer A, hgGLase was eluted with 0.4 M $(NH_4)_2SO_4$ in Buffer A.

2.3. Protein analysis

Protein concentration was determined by the dye-binding method of Bradford [12] using a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce; Rockford, IL). SDS–PAGE analysis was carried out under reducing conditions using a 12% separation gel (TEFCO, Tokyo, Japan) [13]. Electrophoresis was run at the current of 25 mA for

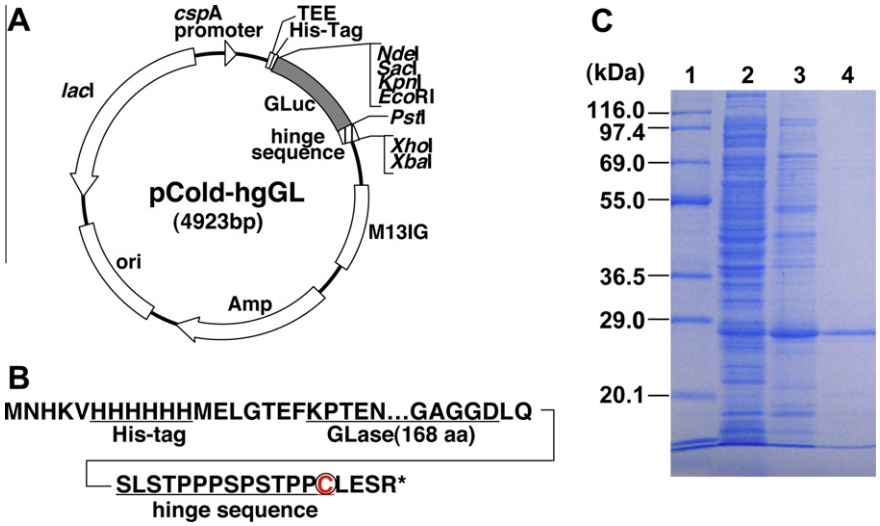

Fig. 1. Expression vector for *Gaussia* luciferase with the hinge sequence containing a reactive cysteine residue (hgGLase) in *E. coli* cells. (A) Plasmid map of pCold-hgGL expression vector. *GLuc*, *Gaussia* luciferase gene; *cps*A, the promoter of cold shock protein A; TEE, translational enhancing element; His-Tag, six histidine-tagged sequence. (B) Amino acid sequence of the N- and C-terminal regions of hgGLase. GLase, *Gaussia* luciferase (from 18 to 185 a.a.); Open circle at the hinge sequence is a new cysteine residue. (C) SDS–PAGE analysis of proteins in various steps of hgGLase purification under reducing conditions. Lane 1, molecular weight markers (TEFCO): β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69,0 kDa), glutamic dehydrogenase (55.0 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29.0 kDa) and trypsin inhibitor (20.1 kDa); lane 2, crude extracts of bacterial cells (18.5 μg protein); lane 3, eluted fraction from a Ni-chelate column (7.4 μg protein); lane 4, eluted fraction from a Butyl-Sepharose column (1.1 μg protein).