Protein Expression and Purification 77 (2011) 124–130

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

A chimera of green fluorescent protein with single chain variable fragment antibody against ginsenosides for fluorescence-linked immunosorbent assay

Seiichi Sakamoto, Yusuke Tanizaki, Benyakan Pongkitwitoon, Hiroyuki Tanaka *, Satoshi Morimoto

Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 29 December 2010
and in revised form 21 January 2011
Available online 26 January 2011

Keywords:
Enzyme-linked immunosorbent assay
Fluobody
Fluorescence-linked immunosorbent assay
Ginsenosides
Green fluorescent protein
Single-chain variable fragment

ABSTRACT

A chimera of green fluorescent protein extracted from *Aequorea coerulescens* (AcGFP), a mutant that has been codon optimized for mammalian expression, with single-chain variable fragment (scFv) antibody against ginsenoside Re (GRe-scFv), named fluobody, has been successfully expressed in *Escherichia coli* (*E. coli*) to develop simple, speedy, and sensitive fluorescence-linked immunosorbent assay (FLISA). Two chimera proteins were constructed to contain GRe-scFv at the C-terminus of AcGFP (C-fluobody) and at the N-terminus of AcGFP (N-fluobody). These fluobodies were then purified by ion metal affinity chromatography and refolded by stepwise dialysis. The characterization of both fluobodies revealed that C-fluobody was found to be appropriate probe for FLISA as compare with N-fluobody. Furthermore, improvement of limit of detection (LOD) was observed in FLISA using C-fluobody (10 ng/mL) due to its strong fluorescence intensity of AcGFP compared with conventional enzyme-linked immunosorbent assay (ELISA) using parental monoclonal antibody against ginsenoside Re (G-Re), MAb-4G10 (100 ng/mL). Since some steps required in ELISA can be avoided in this present FLISA, speedy and sensitive immunoassay also could be performed using fluobody instead of monoclonal antibody and scFv.

Introduction

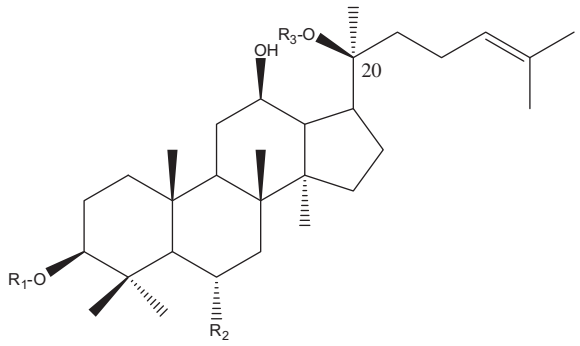
Ginsenosides, which possess a dammarane skeleton in their molecules, are well known as major bioactive compounds mainly produced in *Panax ginseng* (*P. ginseng*). So far, more than 30 ginsenosides have been identified from various ginsengs [1]. These ginsenosides mainly are classified into protopanaxatriol (Ginsenoside-Re, and -Rg1) or protopanaxadiol (Ginsenoside-Rb1, -Rc, and -Rd) as shown in Fig. 1. Recently, the demand of *P. ginseng* and its related species such as *P. japonicas*, *P. quinquefolium*, and *P. notoginseng* has been dramatically increased worldwide as an ingredient of dietary health supplements and additive in foods and beverages due to their pharmacological activities such as tonic, immunomodulatory, anti-mutagenic and anti-ageing activities [2,3]. Clinical studies have also demonstrated that ginseng may improve psychological function, conditions associated with diabetes, immune function [4.5]. Since the variety of total and individual ginsenoside concentration in the market products has been critical issue from the point of view of quality control, a sensitive, speedy, and simple method for standardizing ginseng samples is required.

In our previous study, we prepared monoclonal antibody (MAb) against G-Re (MAb-4G10) [6] and scFv against G-Re (GRe-scFv) [7] to develop an enzyme-linked immunosorbent assay for the determination of various ginsenosides. The developed ELISA using MAb-4G10 and GRe-scFv showed potential as accurate and reliable assay for the assessing the concentration of total ginsenosides in plant samples. However, even more simple, speedy, and sensitive immunoassay is required to deal with a large number of plant samples serially.

Fluorescent labeled antibody has been widely used and conjugation between fluorescent labels and antibodies has been conventionally accomplished using chemical conjugation of organic fluorophores [8]. However, in these conjugation methods, it is difficult to control fluorophores number bound and there is a possibility that conjugation of fluorophores to paratope may occur, resulting in partial or complete inactivation of original antibody. To overcome these disadvantages, we designed a chimera of AcGFP with GRe-scFv, and expressed it in *Escherichia coli* to investigate its potential use in the immunoassay. In the format using conjugation of AcGFP with GRe-scFv, resultant protein is always expressed in a one-to-one ratio between the fluorochrome and scFv. So far, fluorescence-linked immunosorbent assay (FLISA) targeting a small molecules (hapten) has been developed for detecting two kinds of herbicide, picloram [9] and s-triazine [10], and bioactive naphthoquinone, plumbagin described in our group [11]. In our previous study, we demonstrated interesting characteristics of fluobody fusing scFv at the C-terminus of GFP called C-fluobody and showed its potential use in FLISA.

* Corresponding author. Fax: +81 (0) 92 6426582.
E-mail address: htanaka@phar.kyushu-u.ac.jp (H. Tanaka).

1046-5928/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2011.01.010



Ginsenoside	R_1	R_2	R_3
Protopanaxatriol			
Re	H	Rha^{1}-^{2}Glc-O-	Glc-
Rg_1	H	Glc-O-	Glc-
Protopanaxadiol			
Rb_1	Glc^{1}-^{2}Glc-	H	$Gluc^{1}$-^{6}Glc-
Rc	Glc^{1}-^{2}Glc-	H	$Ara(f)^{1}$-^{6}Glc-
Rd	Glc^{1}-^{2}Glc-	H	Glc-

Fig. 1. Structure of major ginsenosides in ginseng.

In the conventional ELISA using MAb-4G10 or GRe-scFv, the following steps that required almost 4.5 h are necessary: (i) fixation of coated antigen; (ii) blocking step to prevent plate from adsorbing non-specific protein; (iii) primary antibody reaction; (iv) secondary antibody reaction; (v) enzyme-substrate reaction. On the other hand, in the FLISA using a chimera of AcGFP with GRe-scFv described in present study, time- and cost-consuming secondary antibody reaction and following enzyme-substrate reaction can be avoided, making it possible to complete the assay within 3 h.

In this study, we successfully constructed two chimera proteins of AcGFP fused at the C-terminus of GRe-scFv (C-fluobody) and N-terminus of GRe-scFv (N-fluobody) with a flexible peptide linker $(Gly_4Ser)_2$ between the two domains and expressed them in *E. coli* to make sure that the format of C-fluobody is superior to that of N-fluobody as a probe for FLISA. Expression, purification, and characterization of fluobodies that can be generally used to develop simple, speedy, and sensitive FLISA are demonstrated in this paper.

Materials and methods

Chemicals and immunochemicals

Ginsenoside Re (G-Re), ginsenoside Rb1 (G-Rb1), ginsenoside Rc (G-Rc), ginsenoside Rg1 (G-Rg1), and ginsenoside Rd (G-Rd) were purchased from Wako Pure Chemical (Osaka, Japan). Human serum albumin (HSA) was purchased from Sigma–Aldrich (Steinheim, Germany). Horseradish peroxidase (HRP)-labeled anti-T7-tag conjugates was obtained from Invitrogen (CA, USA). DNA polymerase and DNA restriction enzymes were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of an analytical grade.

Table 1
Primers used for construction of the C-fluobody and N-fluobody.

Primer name	Sequence
PC1	5′-CGCGGATCCGTGAGCAAGG-3′
PC2	5′-GCTGCCACCTCCACCGCTACCGCCGCCTCCCTTGTACA-3′
PC3	5′-GGTGGAGGTGGCAGCCAGGTTCAGCTGCAGCAG-3′
PC4	5′-AGCTTTGTCGACCTAACGTTTTATTTCCAACTT-3′
PN1	5′-CGCGGATCCCAGGTTCAGCTGCAGCAGTCTGGA-3′
PN2	5′-GCTGCCACCTCCACCGCTACCGCCGCCTCCACGTTTTATTC-3′
PN3	5-GGTGGAGGTGGCAGCGTGAGCAAGGGC-3′
PN4	5′-AGCTTTGTCGACCTACTTGTACAGCTCATCCAT-3′

Preparation of G-Re-human serum albumin conjugates

Synthesis of G-Re-human serum albumin (GRe–HSA) conjugates was carried out by periodate oxidation method [12]. G-Re (3 mg) in 0.4 mL dimethyl sulfoxide (DMSO) was added dropwise to 0.6 mL of a solution containing $NaIO_4$ (3 mg) and then stirred at room temperature for 1 h. After that, HSA (8 mg) in 1 mL carbonate buffer (50 mM, pH 9.6) was added and stirred for 5 h. The reaction mixture was dialyzed against five changes of distilled water and then lyophilized to yield 6.7 mg of GRe–HSA conjugates, which was used as a coating antigen in both ELISA and FLISA.

Construction of expression vector for fluobody

A pET28a expression vector (Novagen) encoding GRe-scFv and a pAcGFP1-N1 vector (Clontech) encoding AcGFP were used as templates for constructing a fluobody expression vector by means of splicing by overlapped extension PCR (SOE-PCR) [13]. Eight primers (Table 1) for PCR were designed based on the GRe-scFv and AcGFP sequences to construct two kinds of chimera fused with GRe-scFv at the C-terminus of AcGFP (C-fluobody) or the N terminus of AcGFP (N-fluobody). PC and PN indicate the primers used to construct the C-fluobody and N-fluobody, respectively.

First, the AcGFP domains were amplified from the pAcGFP1-N1 vector by PCR using the primers set of PC1 and PC2 containing *Bam*H I restriction enzyme site and linker sequence for AcGFP domains of C-fluobody, and the set of PN3 and PN4 containing linker sequence and *Sal* I restriction enzyme site for AcGFP domains of N-fluobody with the PCR conditions as follow: 30 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 5 s), and extension (72 °C, 1 min) with PrimeStar HS DNA polymerase (Takara, Kyoto, Japan).

Subsequently, GRe-scFv domains were amplified from the pET28a expression vector by PCR using the primers set of PC3 and PC4 containing linker sequence and *Sal* I restriction enzyme site for GRe-scFv domains of C-fluobody, and the set of PN1 and PN2 containing *Bam*H I restriction enzyme site and linker sequence for GRe-scFv domains of N-fluobody with the PCR conditions as follow: 30 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 5 s), and extension (72 °C, 1 min) with PrimeStar HS DNA polymerase (Takara).

The AcGFP domains amplified using PC1 and PC2 were then joined to the GRe-scFv domains amplified using PC3 and PC4 by SOE-PCR using PC1 and PC4 to construct the C-fluobody in a AcGFP/GRe-scFv format. On the other hand, the AcGFP domains amplified using PN3 and PN4 were joined to the GRe-scFv domains amplified using PN1 and PN2 by SOE-PCR using PN1 and PN4 to construct the N-fluobody in a GRe-scFv/AcGFP format. The PCR conditions for amplification of C-fluobody and N-fluobody were as follow: 30 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 5 s), and extension (72 °C, 3 min) with PrimeStar HS DNA polymerase (Takara).

The amplified genes encoding the C-fluobody and N-fluobody were then purified, digested with *Bam*H I and *Sal* I, and ligated downstream of the His6 and T7-tags of the pET28a expression vector (Novagen) to generate the pET28a/C-fluobody and pET28a/N-fluobody plasmids.

Expression and purification of recombinant fluobodies

Resultant plasmids were then transformed into the *E. coli* BL21 (DE3) strain (Novagen) for the expression and purification of the