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The effect of varying the peptide linker length in a single chain variable fragment antibody against wogonin glucuronide

Madan Kumar Paudel[a], Seiichi Sakamoto[a], Le Van Huy[b], Hiroyuki Tanaka[a,*], Tomofumi Miyamoto[b], Satoshi Morimoto[a]

[a] Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Natural Product Chemistry, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

Peptide linkers of three different lengths were constructed to join the variable regions of the heavy chain (VH) and the light chain (VL) in a single-chain variable fragment antibody (scFv) specific for wogonin glucuronide (Wgn) that has the structure VH-$(GGGGS)_n$–VL (n = 3, 5, or 7). The scFv antibodies, which were expressed in *Escherichia coli*, were derived from an anti-Wgn monoclonal antibody (315A). An indirect competitive enzyme-linked immunosorbent assay (icELISA) was used to evaluate their reactivity and sensitivity, which is also used for quantitative analysis of Wgn. Our results, showed that the reactivity and specificity of the three different scFvs were, in fact, similar. Subsequently, the scFv having a VH-$(GGGGS)_3$–VL linker which was slightly better that other two scFvs against Wgn, was applied to indirect competitive ELISA (icELISA) to analyze Scutellariae Radix (S. Radix). The utility of the icELISA was demonstrated for quality control and analysis of S. Radix in this report.

1. Introduction

Immunoassays are well known for their attractive immunochemical applications for analysis and quality control of multiple samples in a short period of time (Kido et al., 2008; Paudel et al., 2011, 2012, 2013). Recently, we raised a monoclonal antibody specific for wogonin 7-*O*-β-D-glucuronide (Wgn) in order to develop an indirect competitive enzyme-linked immunosorbent assay (icELISA) using anti-Wgn monoclonal antibody (MAb) for determining Wgn in our lab (Paudel et al., 2017). Wgn (Fig. 1) is a bioactive flavone present in the dried root of *Scutellaria baicalensis* Georgi (Li et al., 2004, 2011a,b), and shows a variety of pharmacological activities such as anti-allergic, anti-inflammatory, anti-human immunodeficiency virus[t], anti-cancer, anti-oxidant, and anti-viral properties. Due to these activities, Wgn is believed to be one of the indices of Scutellariae Radix which is the dried root of *S.baicalensis* and is frequently used as an ingredient of Kampo medicines.

Several analytical methods such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, gas chromatography with mass spectrometry (MS), HPLC with electrochemical detection, ultra-performance liquid chromatography (UPLC), HPLC-MS, and UPLC-electrospray ionization (ESI)-MS/MS have been applied to the study of Wgn, and for high-sensitivity quantitative analysis in Kampo medicines (Bhattaram et al., 2002; Li et al., 2011a,b; Kim et al., 2006; Chung et al., 2012; Zhang et al., 2005, 2006; Hou et al., 2011). Comparing with these analytical methods for Wgn, the icELISA using anti-Wgn MAb developed by us has advantages in eco-friendly and simultaneous multiple sample analysis. However, there are some disadvantages such as the risk for microbial contamination of hybidoma cell culture and use of costly MAbs. To overcome the disadvantages, recombinant antibodies are paid attention to now. Among the recombinant antibodies, single chain variable fragment (scFv) are well-known recombinant antibodies expressed in bacteria that are composed of a heavy chain variable region (VH) and a light chain variable region (VL) joined by a short flexible peptide $(GGGGS)_n$. They are very small in size comparison to full-length immunoglobulin G molecules. Furthermore, the recombinant antibodies could be genetically engineered with specific properties to meet the needs of particular therapeutic and analytical applications (Gu et al., 2010).

Up to date, linker length between VH and VL has been investigated using scFv against large molecule such as proteins (Atwell et al., 1999; Wang et al., 2008). The scFvs possessing the linker length of 5–12 amino acids formed bivalent dimer (diabody) and these possessing no linker (direct ligation) formed trivalent trimer (triabody). These results accounted for the limited flexibility due to short linker length. When the flexibility between VH and VL is strictly limited, VH is enable to

* Corresponding author.
E-mail address: htanaka@phar.kyushu-u.ac.jp (H. Tanaka).

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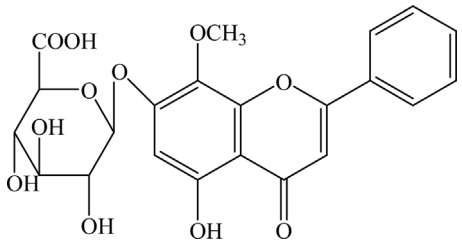

Fig. 1. Structural formula of wogonin 7-*O*-β-D-glucuronide (Wgn).

associate with its VL in the same scFv molecule. Instead, it binds to the VL of separate scFv molecule, resulting in formation of non-functional multimer scFvs (Holliger et al., 1993). Although the linker length of scFvs targeted to proteins has been studied, that targeted to small molecules has not been well elucidated except one report from our group (Yusakul et al., 2016), especially their effect on reactivity and sensitivity. In our previous studies, the scFvs having VH-$(GGGGS)_3$–VL against artemisinin, paeoniflorin and plunbagin were prepared and applied to icELISA (Paudel et al., 2012; Lu et al., 2006; Sakamoto et al., 2011). In this study, we prepared scFvs with three kinds of peptide linkers, VH-$(GGGGS)_n$–VL (n = 3, 5, or 7) against Wgn were constructed and evaluated them in terms of specificity, sensitivity and reliability and applied to icELISA as qualitative analysis of Wgn.

2. Materials and methods

2.1. Reagents and standards

Human serum albumin (HSA) was obtained from Sigma-Aldrich (Steinheim, Germany). Freund's complete and incomplete adjuvants were obtained from Difco (Detroit, USA). Peroxidase (POD)-labeled anti-mouse IgG (Fc) was purchased from MP Biomedicals (Santa Ana, CA, USA). POD-labeled anti-T7-Tag conjugate was purchased from Novagen (San Diego, CA, USA). Ex Taq DNA polymerase, Prime STAR HS DNA polymerase, and DNA reaction enzymes were acquired from Takara (Kyoto, Japan). Primers were obtained from Fasmac (Kanagawa, Japan). All other compounds and natural solvent were of analytical reagent grade.

2.2. Sample preparation

Samples of various S. Radix were obtained from Tochimototenkaido Co., Ltd. (Osaka, Japan), Takasago Yakugyo K.K. (Osaka, Japan), Daido Corp. (Osaka, Japan), and Yamada Corp. (Osaka, Japan). Kampo medicines were purchased from Tsumura and Co. (Tokyo, Japan).

Thirty milligrams of each S. Radix, including a sample cultivated in the herbal garden of Kyushu University were weighed, sliced, and ground into a fine powder. Each sample was extracted with 0.5 mL methanol using a sonicator for 10 min. The concentrate was subsequently centrifuged at 12,000 rpm for 15 min and transferred into a glass test tube. This extraction step was done three times totally and the supernatants were mixed to generate a sample solution. Subsequently, the solution was evaporated to yield the extract. The extract was dissolved in methanol (1 mL) and diluted for ELISA and HPLC analysis.

2.3. Preparation of wogonin glucuronide-human serum albumin conjugates (Wgn–HSA)

The Wgn was used as a hapten and conjugated to HSA as an immunogen and coating specialist. Wgn (5.0 mg, 0.023 mmol) and 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 6.0 mg, 0.031 mmol) were added to a solution mixture of 30% pyridine (0.5 mL). The reaction mixture was added dropwise to 0.25 mL of 5.0 mg HSA dissolved in water, and thereafter mixed at room temperature for 7 h. The mixture was dialyzed subsequently with five changes of H_2O at 4 °C for three days, and lyophilized to yield 6.30 mg Wgn-HSA conjugate.

2.4. Strains, media, and plasmid vectors

E. coli strain JM109 was used to prepare the different plasmid constructs. *E. coli* strain BL21 (DE3) was used to express Wgn–scFvs. Bacteria were cultivated on lysogeny broth (LB) medium containing 1% (w/v) polypeptone, 0.5% w/v yeast extract, 0.5% (w/v) NaCl, pH 7.2. The pMD 20-T vector (Takara, Kyoto, Japan) was utilized for developing VH and VL genes, while the pET28a (+) vector (Novagen, WI, USA) was used to constructing individual full-length scFv genes.

2.5. Construction, expression, and purification of multiform Wgn–scFv genes

The total RNA (5 μg) was extracted from 3×10^6 hybridoma cells (315A) utilizing the Sepasol RNA I super reagent (Nacalai Tesque Inc., Kyoto, Japan) as directed by the manufacturer. First-strand cDNA was synthesized utilizing random hexamer primers (Amersham Biosciences, Buckinghamshire, UK). The VH and VL genes were amplified by PCR using established antibody-specific primers (Krebber et al., 1997), and the PCR products were cloned into the pMD 20-T vector.

Specific primers for constructing multiform Wgn-scFv genes were planned from the exact sequence of the VH and VL genes. Genes encoding Wgn–scFvs were developed in the VH-linker-VL orientation, where several linkers in the configuration of $(GGGGS)_n$ (n = 3, 5, or 7) were used. Primers for constructing the multiform Wgn–scFv genes are abridged in Table 1. As VH-$(GGGGS)_3$–VL (3L), the VH gene was amplified with primers 1 and 3, while the VL was amplified with primers 2 and 4. The genes encoding VH and VL were fused in the order VH-$(GGGGS)_3$-VL using splicing by overlap extension PCR (SOE PCR) with primers 1 and 2. The VH-$(GGGGS)_n$–VL (n = 5 or 7) was exclusively named 5L and 7L. Construct 5L was built using the primer pairs 1, 5 and 2, 6, whereas 7L was built using the primer pairs 1, 7 and 2, 8, to amplify the VH and VL genes. All Wgn–scFv constructs were sub-cloned into the pET28a (+) expression vector (Novagen, Darmstadt, Germany) in order to use the hexahistidine (His6) epitope tag and

Table 1
List of primers used to amplify anti-Wgn-scFv genes.

No.	Name	Sequences (5′–3′)
1	VH-for-*Eco*RI	CGC GAA TTC CAG GTC CAG CTG CAG CAA CCT GGG
2	VL-rev-*Sal*I	CGC GTC GAC CTA CCG TTT TAT TTC CAG CTT GGT
3	VH-rev-3linker	GGA GCC GCC GCC GCC TGA ACC ACC ACC ACC TTC AGA GAC AGT GAC CAG
4	VL-for-3linker	GGC GGC GGC GGC TCC GGT GGT GGT GGT TCA GAC ATT GTG ATG ACC
5	VH-rev-5linker	GGA ACC ACC ACC ACC GGA GCC GCC GCC GCC TGA ACC ACC ACC ACC TTC AGA GAC AGT GAC CAG
6	VL-for-5linker	GGT GGT GGT GGT TCC GGC GGC GGC GGC TCA GGT GGT GGT GGT TCC GAC ATT GTG ATG ACC
7	VH-rev-7linker	TGA GCC GCC GCC GCC TGA ACC ACC ACC ACC GGA GCC GCC GCC GCC TGA ACC ACC ACC ACC TTC AGA GAC AGT GAC CAG
8	VL-for-7linker	GGC GGC GGC GGC TCA GGT GGT GGT GGT TCC GGC GGC GGC GGC TCA GGT GGT GGT GGT TCC GAC ATT GTG ATG ACC

The restriction sites are underlined.