Analytical Biochemistry 451 (2014) 63–68

Contents lists available at ScienceDirect

Analytical Biochemistry

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

A human kringle domain-based fluorescence-linked immunosorbent assay system

Gu Min Jeong [a], Yong Sung Kim [b], Ki Jun Jeong [a,c,*]

[a] Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon 305-701, Republic of Korea
[b] Department of Molecular Science and Technology, Ajou University, Yeongtong-gu, Suwon 443-749, Republic of Korea
[c] Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon 305-701, Republic of Korea

ARTICLE INFO

Article history:
Received 6 November 2013
Received in revised form 4 January 2014
Accepted 31 January 2014
Available online 10 February 2014

Keywords:
Kringle domain
FLISA
GFP
Immunoassay

ABSTRACT

As a non-immunoglobulin protein scaffold, human kringle domain (KD) has attractive properties such as high specificity, stability, and production in bacterial hosts. Here, we developed a rapid and sensitive fluorescence-linked immunosorbent assay (FLISA) system using a fluorescent kringle domain (fluoKD), a fusion protein of a green fluorescent protein (GFP), and a kringle domain variant (KD548). Two kinds of fluoKDs in which KD was fused to the N terminus of GFP (N-fluoKD) or the C terminus of GFP (C-fluoKD) were constructed and characterized. In *Escherichia coli* host, both fluoKDs were produced in high yield and solubility and were successfully purified by a simple procedure. The purified fluoKDs exhibited strong fluorescent activities and high affinities to the target antigen. Furthermore, it was successfully demonstrated that the FLISA with purified fluoKDs allowed for more rapid detection of target antigens with higher sensitivity compared with conventional enzyme-linked immunosorbent assay (ELISA), indicating that a simple, rapid, and sensitive immunoassay system could be developed by using KD instead of antibody or antibody fragments.

Currently, enzyme-linked immunosorbent assays (ELISAs)[1] are widely used for the detection of specific molecules, including proteins, peptides, and sugars, as well as various other small molecules and chemicals. In addition, the immunosorbent assay is an important tool in most protein-based diagnostic platforms [1]. In most immunosorbent assay systems, antibodies are used as capturing reagents because of their high specificity and affinity to target molecules. However, the intrinsic properties of antibodies also create several limitations in the immunosorbent assay systems. In many cases, antibodies are produced in a mammalian host and the production cost is high due to their low productivity and use of expensive media. Even though bacterial hosts can be used for production of antibody proteins, the folding efficiency and purification yield are often poor. In addition, the instability of antibodies in harsh conditions makes them difficult to use in diagnostic systems [2].

To overcome the limitations of antibodies, exploration of alternative protein reagents with the ability of specific binding to target molecules has been stimulated and has led to the development of non-immunoglobulin protein scaffolds. Compared with typical antibodies, non-immunoglobulin protein scaffolds, used as an alternative to antibody proteins, have several advantages: (i) smaller size (<100 amino acids), (ii) simple structure with high stability, and (iii) economic production in bacterial or yeast hosts [3,4]. During the past decade, several types of protein scaffolds, including DARPins, Affibody, Avimers, and Fibronectin, have been developed and successfully engineered for many applications in biotechnology and biomedical research [5]. Recently, we also reported the development of a new non-immunoglobulin scaffold from human plasminogen kringle domain 2 (PgnKD2) [6]. The kringle domain (KD) is composed of 80 amino acids and contains a rigid core structure with three conserved disulfide bonds. Compared with other non-antibody protein scaffolds, KD has several distinctive properties, including high thermal stability (active at 65 °C), higher rigidity via formation of three disulfide bonds, and relatively long

* Corresponding author at: Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology (KAIST), Yuseong-gu, Daejeon 305-701, Republic of Korea. Fax: +82 42 350 3910.
E-mail address: kjjeong@kaist.ac.kr (K.J. Jeong).

[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; KD, kringle domain; FLISA, fluorescence-linked immunosorbent assay; DR5, death receptor 5; fluoKD, fluorescent kringle domain; GFP, green fluorescent protein; N-fluoKD, fluoKD in which anti-DR5 KD548 was fused to the N terminus of GFP; C-fluoKD, fluoKD in which anti-DR5 KD548 was fused to the C terminus of GFP; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; PBSM, PBS containing 10 wt% skim milk; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LOD, limit of detection.

http://dx.doi.org/10.1016/j.ab.2014.01.019
0003-2697/

variable regions in the seven flexible loops (~ 45 amino acid residues; see Fig. 1A). In particular, owing to the long variable regions, KD can be engineered to multivalent variants that might simultaneously interact with two or more targets [7]. The typical structure of KD is strongly sequence tolerant to mutation in the loop region; therefore, it has the potential to be engineered for distinct binding to various target molecules [6,7]. In addition, a functional KD can be produced in *Escherichia coli* host [8] and yeast, including *Pichia pastoris* and *Saccharomyces cerevisiae* [6,7], both of which have high production yields (>5 g/L in *E. coli* host); therefore, the low production cost may be more beneficial when compared with most antibody molecules. In most diagnostic systems based on immunosorbent reaction, the sensitivity and stability are highly dependent on the capturing molecules immobilized on the surface of the diagnostic system. As described earlier, KD has ideal properties for diagnostic systems, and its application toward the development of diagnostic systems is highly desired.

In this study, we developed a fluorescence-linked immunosorbent assay (FLISA) system using a KD variant (Fig. 1B). As a model KD variant, KD548 was used and engineered to have high affinity to human death receptor 5 (DR5), a receptor protein present on the surface of cancer cells [6]. For the fluorescent signal, two fluorescent kringle domains (fluoKDs) were constructed where anti-DR5 KD548 was fused to the N terminus or C terminus of green fluorescent protein (GFP) (N-fluoKD or C-fluoKD, respectively) with a flexible $(Gly_4Ser)_2$ linker between GFP and KD548. After production and purification in *E. coli* cultivation, both fluoKDs were applied to the FLISA system and their fluorescent intensity and binding activity were analyzed. We also successfully demonstrated that the fluoKD-based FLISA system has high sensitivity compared with a conventional ELISA system.

Materials and methods

Bacterial strains and plasmid

All bacterial strains used in this work are listed in Table 1. *E. coli* XL1-Blue was used as a host cell for gene manipulation and plasmid maintenance. *E. coli* SHuffle Express, which is engineered to promote disulfide bond formation in the cytoplasm by constitutive expression of DsbC in cytoplasm, was used for production of both fluoKDs and GFP. Even though KD has three disulfide bonds, the correctly folded KD can be produced in cytoplasm of this strain. Polymerase chain reaction (PCR) was performed with the C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using PrimeSTAR HS Polymerase (Takara Bio, Shiga, Japan). The nucleotide sequences of all primers used in this study are listed in Table S1 of the online Supplementary material. A GFP gene was amplified from pGFP-mut2 [9] by PCR with primers CF-F4, CF-F5, CF-F6, and GFP-R1. The PCR product was digested with restriction enzymes *Xba*I and *Hin*dIII and then cloned into the same enzyme sites of pMoPac1 [10], yielding pGM-GFP. For the expression of N-fluoKD in which

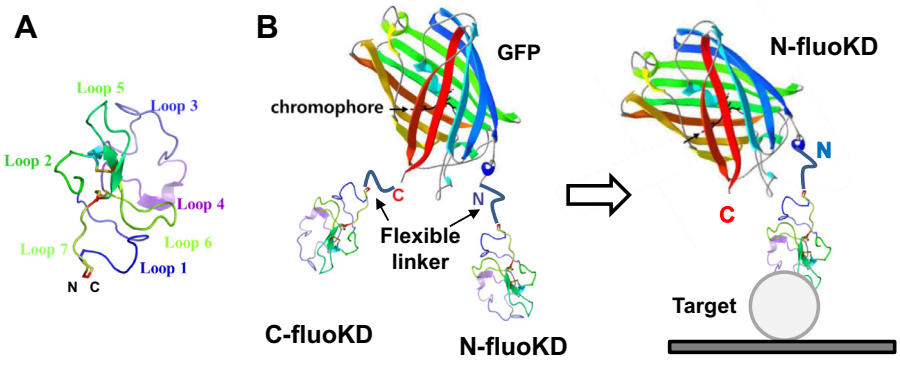

Fig.1. (A) Schematic structure of KD (PDB entry code: 1I5K). It has a rigid core via three disufide bonds and seven flexible loops. (B) Concept of KD-based FLISA system. The fluoKDs in which KD is linked to the N or C terminus of GFP can bind to target antigens with specific affinity, and the fused GFP gives fluorescent signal for detection of target molecules.

Table 1
Bacterial strains and plasmids used in this study.

E. coli strains or plasmids	Description	References
E. coli strains		
XL1-Blue	(*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^r)]	Stratagene[a]
BL21(DE3)	F$^-$ ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagene[b]
SHuffle Express	*fhuA2 [lon] ompT ahpC gal* λ*att::pNEB3-r1-cDsbC* (SpecR, *lacI*^{q}) Δ*trxB sulA11 R(mcr-73::miniTn10–*Tet^S*)2 [dcm] R(zgb-210::Tn10-*Tet^S*) endA1* Δ*gor* Δ*(mcrC-mrr)114::IS10*	New England Biolabs[c]
Plasmids		
pMoPacI	ColE1 origin, Cm^r, lac promoter, lacI^q	[10]
pET21b-DR5	6×His tag-DR5	[4]
pPICZα-KD548	human KD548 gene	[4]
pGFPmut2	GFPmut2 gene	[9]
pGM-GFP	pMoPac1 derivative, 6×His tag-GFP	This study
pGM-N-fKD	pMoPac1 derivative, 6×His tag-N-fluoKD	This study
pGM-C-fKD	pMoPac1 derivative, 6×His tag-C-fluoKD	This study

[a] Stratagene, Santa Clara, CA, USA.
[b] Novagene, Madison, WI, USA.
[c] New England Biolabs, Ipswich, MA, USA.