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Creation of stable and strictly regulated enzyme switch for signal-on immunodetection of various small antigens

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Recently, we reported a fusion-protein-based immunodetection system comprising the two domains of an antibody variable region as the detectors, each tethered to an interface mutant β-glucuronidase (GUSm) as the reporter, for detecting small molecules via dimerization of dimer activation. However, the poor stability of GUSm and background signal propagation possibly due to spontaneous proteolysis undermined its performance. To solve these problems, we attempted thermostabilization of GUSm by using a previously isolated thermostable mutant GUS_{IV5} as a backbone. After screening several interface mutants, we selected one with M516K/Y517W mutation because it exhibited higher activity after dimerization than the wild-type GUS, while maintaining very low background activity. By using this improved immunosensor, we achieved a two-fold improvement in terms of sensitivity in the detection of 4-hydroxy-3-nitrophenyl acetyl. Moreover, by constructing a new biosensor tethered to a nanobody for caffeine as the detector, we could achieve noncompetitive signal-on detection of caffeine in a practically useful concentration range.

[**Key words:** Antibody fusion protein; Homogeneous immunoassay; Open sandwich immunoassay; Biosensor; Thermostabilization; Nanobody; Oligomer; Protein–protein interaction; Fluorescence immunoassay]

Immunoassays employing the antigen–antibody reactions exhibit high specificities and sensitivities for detecting target substances. Unlike traditional analytical methods, such as liquid chromatography and mass spectrometry, requiring large and costly instruments, immunoassays are simple, convenient, can be performed quickly, and have high specificity and sensitivity (1). Consequently, immunoassays have broad application prospects. Many types of immunoassays, such as sandwich enzyme-linked immunosorbent assay (ELISA), competitive ELISA, and open-sandwich (OS) ELISA (2), have been developed. The last approach has been applied to enzyme-based homogeneous OS immunoassays (3–5) to facilitate the detection of various antigens within a short time. It is complementary to traditional methods, requiring several steps, including incubation, washing, and laborious and error-prone manipulations.

β-Glucuronidase (GUS) is one of the enzymes from the glycosidase family. Its activity can be detected quantitatively and sensitively by catalyzing the breakdown of complex carbohydrates. GUS is a tetramer that can only be activated by the assembly of all its four identical subunits (6). According to a previous study, an *Escherichia coli* β-glucuronidase mutant (GUSm) introduced with a set of interface mutations (M516K/Y517E) prevents the aforementioned self-assembly and yields inactive dimers instead of active tetramers (7).

Recently, an immunosensor system has been developed by using the variable regions of an antibody as a detector tethered to GUSm as a reporter to detect antigens (Fig. 1A) (8). Variable regions represent the polypeptide chain on the N-terminal of the heavy-chain (V_H) and the light chain (V_L) of an immunoglobulin (e.g., IgM, IgG), which varies greatly in its sequence from one antibody to another, and they altogether constitute an antigen binding site (9). The affinity between two variable regions of an antibody (V_H and V_L) is relatively low without antigen binding, but it can increase considerably in the presence of an antigen (open-sandwich immunoassay, OS-IA) (2,10). It has been shown that after dimerization of dimers by means of interaction of antigen-induced variable regions, GUSm activity increased significantly in response to 4-hydroxy-3-nitrophenyl acetyl (NP) (11,12) and osteocalcin (13,14). However, the poor stability of GUSm and background signal propagation possibly due to spontaneous proteolysis hampered the performance of these fusion proteins as robust immunosensors.

Previously, random mutations have been introduced to improve the thermostability of wild-type *E. coli* GUS (15). After several rounds of randomization and selection, a mutant named IV-5 with improved thermostability and activity (N27Y, F51Y, A64V, E115D, D185N, I349F, G368C, N369S, Y517F, Y525F, G559S, K567R, F582Y, Q585H and G601D) was selected. For stabilizing GUSm to lower the background activity and improve the signal, in the present study, we introduce interface mutations to IV-5 based on these previous studies and screen for a suitable thermostabilized reporter enzyme as a new reporter for the immunosensor.

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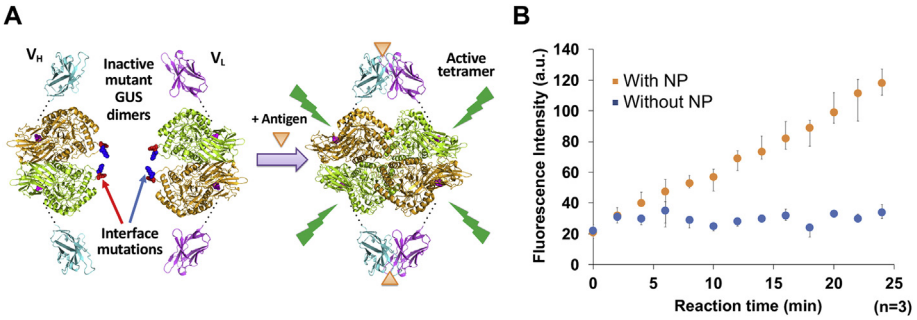

FIG. 1. (A) Scheme of mutant GUS-based immunodetection system. The interdimer mutations (pos. 516 and 517 in blue and red, respectively) and the N-terminus in each monomer (magenta) are shown as spheres. (B) Time course measurement using GUS_{IV5}_KE as a reporter enzyme.

Heavy-chain antibodies are found in camelids consisting of only two heavy chains (16). The variable fragments (V_{HH}) or nanobody separated from a heavy-chain antibody contains an antigen binding site, and it attains full antigen binding function (17). Previously, V_{HH} has been proved to be effective for detecting small haptens (18), including caffeine (19). Interestingly, the reported anti-caffeine V_{HH} showed hapten-induced dimerization, as in the case of V_H and V_L in OS-IA. To demonstrate the broad applicability of the immunosensor owing to the use of the aforementioned thermostabilized reporter enzyme, a new sensor that uses the anti-caffeine V_{HH} as the detector was constructed and evaluated.

MATERIALS AND METHODS

Materials Oligonucleotides and the gene encoding GUS_{IV5}_KE and anti-caffeine V_{HH} (19) were synthesized by Eurofins Japan (Tokyo, Japan). The synthesized GUS_{IV5}_KE gene with the interface mutations (M516K/Y517E) was devoid of the G368C mutation, which can lead to the formation of undesirable disulfide bonds with the antibody V region. KOD-Plus-Neo DNA polymerase was obtained from Toyobo Biochemical (Osaka, Japan). Polymerase chain reaction (PCR) was performed using a Thermo Cycler T3000 (Biometra, Göttingen, Germany). The XL10-Gold *E. coli* strain was used for DNA preparation (Agilent Technologies, Carlsbad, CA, USA). The SHuffle T7 Express lysY strain for protein expression and the restriction enzymes were obtained from NEB Japan (Tokyo, Japan). Anti-His tag antibody (PentaHis) was from Qiagen (Tokyo, Japan). Other chemicals were purchased from Fujifilm-Wako Chemicals (Osaka, Japan), unless indicated otherwise. A fluorescence microplate reader (ClarioStar, BMG Labtech, Ortenberg, Germany) was used to measure fluorescence intensity.

The following nucleotides were used for plasmid construction: GUS_NotBack: 5′-ATAAGAATGCGGCCGCTATGTTAC-GTCCTGTAGAAA-3′; GUS_IV5_XhoFor: 5′-GCCCTCGAGTCATTGTTTGTCTCCCTG-3′; MY_Top: 5′-GCCGGCCTGCACTCAATGTA-TACCGACATGTGGAG-3′; KY_Top: 5′-GCCGGCCTGCACTCAAAGTATACCGA-CATGTGGAG-3′; ME_Top: 5′-GCCGGCCTGCACTCAATGGAGACCGACATGTGGAG-3′; KF_Top: 5′-GCCGGCCTGCACTCAAAGTTCACCGACATGTGGAG-3′; KW_Top: 5′-GCCGGCCTGCACTCAAAGTGGACCGACATGTGGAG-3′; IV5_Bottom: 5′-TGAGTG-CAGGCCGGCTAACG-3′.

Vector construction The GUS_{IV5}_KE gene was amplified by means of PCR and by using the primers GUS_NotBack and GUS_IV5_XhoFor from the synthesized IV-5(C368G/M516K/Y517E) gene as a template. PCR was performed by carrying out 30 denaturation cycles (1 min at 94°C), annealing (1 min at 55°C), extension (1 min at 68°C) with 10 ng of template in a 50 μl reaction mixture containing 50 pmol of each primer and 1 U of KOD-Plus-Neo DNA polymerase. All PCRs in this study were carried out under the same conditions. The product was digested by *Not*I and *Xho*I and inserted into the linearized plasmids pET32-VH(NP)-GS-GUS and pET32-VL(NP)-GS-GUS (8) digested by the same enzymes, each encoding *E. coli* thioredoxin as a solubilization tag and a variable domain of anti-NP antibody to yield the pET32-VH(NP)-GS-GUS-IV5_KE and pET32-VL(NP)-GS-GUS-IV5_KE, respectively.

To create mutant GUS_{IV5}, the GUS_{IV5}_KE template was amplified using one of primers MY_Top, KY_Top, ME_Top, KF_Top, and KW_Top, together with GUS_IV5_XhoFor, to introduce the MY, KY, ME, KF, and KW mutations at sites 516 and 517. The purified fragment was mixed with the overlapping N-terminal fragment amplified by GUS_NotBack and IV5_Bottom, re-amplified with GUS_NotBack and GUS_IV5_XhoFor, and cloned into pET32-VL(NP)-GS-GUS-IV5_KE, as above (Fig. S1A).

For creating a caffeine sensor, the synthesized V_{HH}(Caf) DNA was digested by *Nco*I and *Hin*dIII to obtain V_{HH}(Caf) DNA, which was inserted into the pET32-VH(NP)-GS-GUS-IV5_KW plasmid linearized by the same restriction enzymes to construct the pET32-VHH(Caf)-GS-GUS-IV5_KW vector (Fig. S1B).

Protein expression and purification *E. coli* SHuffle T7 Express lysY cells were transformed using the expression vector and grown at 30°C in LB medium (1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl) containing 100 μg/ml ampicillin. Protein expression was induced by adding isopropyl-thio-β-galactopyranoside to a final concentration of 0.5 mM at a cell density of $A_{600} = 0.5–0.6$. Thereafter, the cells were cultured for 16 h at 16°C and recovered by centrifugation. After disrupting the cells, Talon IMAC resin (Clontech, Takara-Bio, Shiga, Japan) was added to the soluble cellular extracts, which were then washed with Talon washing buffer (Talon buffer supplemented with 0.34 g/l imidazole) and eluted with Talon elution buffer (Talon buffer supplemented with 3.4 g/l imidazole). The expression and purification processes were confirmed by SDS–PAGE (Figs. S2–S5).

Time course measurement After mixing 0.1 μM each of the Trx-V_L(NP)-GUS_{IV5} variants with 50 nM anti-His tag antibody in PBST (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20; pH 7.4), the solution was incubated for 5 min at room temperature. After 0.3 mg/ml 4-methylumbelliferyl-β-D-glucuronide was added to the solution, fluorescence intensity was measured at intervals of 1 min. Three samples from each group were taken, and 100 μl of each sample was added into plate wells. The fluorescent intensity was determined from triplicate samples from three independent experiments at the excitation and the emission wavelengths of 340 nm and 480 nm, respectively.

Thermostability test Trx-V_L(NP)-GUS_{IV5}_KW or Trx-V_L(NP)-GUSm protein (0.1 μM) was incubated at higher temperature up to 80°C for 10 min, respectively. Afterwards, five groups of Trx-V_H(NP)-GUS_{IV5}_KW protein mixed with 50 nM anti-His tag antibody, or Trx-V_L(NP)-GUSm in PBST, were added to PBST along with 0.3 mg/ml 4-methylumbelliferyl-β-D-glucuronide and incubated for 5 min at 25°C. Fluorescence intensity of triplicate samples from three independent experiments conducted using the excitation and emission wavelengths of 340 nm and 480 nm, respectively, was measured at intervals of 1 min. Enzyme activity was calculated for each group of proteins, and the residual activity after treatment was calculated by considering the activity at 25°C as 100%.

Antigen dose-dependency measurements Antigens were prepared under gradient concentrations (0–100 μM for NP and 5-iodo-NP (NIP), 0–10 μM for caffeine) in PBST and mixed with the Trx-V_H(NP)-GUS_{IV5}_KW and Trx-V_L(NP)-GUS_{IV5}_KW proteins or the Trx-V_{HH}(Caf)-GUS_{IV5}_KW protein (0.1 μM each in PBST) and 0.3 mg/ml 4-methylumbelliferyl-β-D-glucuronide in PBST. After 20 min incubation at room temperature, the fluorescence intensity of the abovementioned mixtures was measured. Dose-response curves were fitted to a four-parameter logistic equation by using Prism 7 (GraphPad Software, San Diego, CA, USA), and the EC_{50} values were calculated from the curve. The limit of detection (LOD) was obtained as the estimated antigen concentration of the mean blank value plus 3 SDs.

RESULTS

Construction of immunosensor using IV-5 mutant GUS with M516K/Y517E mutations (GUSm IV-5) as reporter enzyme First, the GUSm IV-5 (GUS_{IV5}_KE) gene encoding the interface mutation (M516K/Y517E) was fused to each variable domain of anti-NP antibodies by using a $(GGGGS)_3$ interdomain linker to express fusion proteins Trx-V_H(NP)- GUS_{IV5}_KE and Trx-V_L(NP)-GUS_{IV5}_KE (Fig. S1A). For comparison with the previous immunosensor based on GUSm without thermostabilizing mutations, antigen-induced and background activities of the

GUSm and the GUS_{IV5}_KE systems were compared in a time course experiment conducted using a fluorescence microplate reader.

After expression in *E. coli.* and purification (Fig. S2), an equimolar mixture of Trx-V_H(NP)- GUS_{IV5}_KE/Trx-V_L(NP)-GUS_{IV5}_KE and Trx-V_L(NP)-GUSm/Trx-V_L(NP)-GUSm was mixed with a small-molecule antigen NP and a fluorescent substrate, and the resulting fluorescence intensity was measured at intervals of 2 min (Fig. 1B). Compared with the previous data using GUSm as a reporter (8), the background signal of the new reporter GUS_{IV5}_KE was attenuated significantly in the absence of NP, which led to an increase the signal/background ratio from 2.5 to more than 6, based on the endpoint fluorescence. However, the activity of the GUS_{IV5}_KE system after mixing with NP was approximately 20-fold lower than that of the GUSm system. This means that the GUS_{IV5}_KE system requires considerably more time to yield a similar signal when it is used in the assay.

Screening of GUS IV-5 interface mutants with high signal/background ratio upon dimerization We reasoned that the low activity of GUS_{IV5}-KE could probably be ascribed to the more rigid structure of IV-5 compared to that of the wild-type GUS. To increase the antigen-responsive activity of GUS_{IV5} mutants to be used as reporters, we attempted to back-mutate the interface residues at 516 and 517 (K, E) to the wild-type residues (M, Y) and further replaced them with similar residues. To screen the desired mutants, the anti-His tag antibody was used to homodimerize the V_L-fused dimers with the His_6 tag at the N-terminal of V_L to imitate the presence of an antigen (Fig. 2A). Including GUSm as the control, GUS_{IV5}_MY (M516/Y517), GUS_{IV5}_ME (M516/Y517E), GUS_{IV5}_KY (M516K/Y517), and GUS_{IV5}_KW (M516K/Y517W) were expressed in *E. coli*, and they were successfully purified to homogeneity (Figs. S3 and S4). By contrast, the mutant GUS_{IV5}_KF was not expressed well and was not subjected to further evaluation.

After mixing with anti-His tag antibody and fluorescent substrate, time-dependent fluorescence intensities were measured at intervals of 1 min. From the slopes of these plots, the enzyme activities of GUS_{IV5} mutants with or without antibodies were evaluated. The catalytic activity of mutant GUS_{IV5} mixed with the anti-His antibody was compared to that of GUS_{IV5} without the antibody to obtain the signal/background (S/B) ratio of each mutant. Compared with the original GUSm (Fig. 2B), both single back-mutants GUS_{IV5}_ME and GUS_{IV5}_KY showed similar antibody-induced activity to the antibody-independent activity of GUS_{IV5}_MY revertant (Fig. 2C–E). However, GUS_{IV5}_KY showed a lower background activity, resulted in higher signal/background (S/B) ratio. We then decided to further engineer this mutant while keeping 516K, and GUS_{IV5}_KW was found to show the highest S/B ratio of 13 during the 10-min period (1–11 min after substrate addition) (Fig. 2F). Surprisingly, GUS_{IV5}_KW had the highest catalytic activity when dimerized by the antibody, while maintaining very low background activity when not dimerized. Moreover, when GUS_{IV5}_KW was incubated with the antibody and the substrate for a longer period of up to 20 min, the apparent enzyme activity increased, but the background activity did not change (Fig. 2G). This resulted in an average S/B ratio of more than 42 during the 20-min reaction. The higher activity of GUS_{IV5}_KW might be because of the increased stability of the enzyme and the fact that the mutation G559S included in IV-5 is known to increase GUS activity (20). Hence, GUS_{IV5}_KW was found to be a suitable reporter enzyme that can improve the sensitivity and reduce the background of this immunosensor system.

Thermostability test of GUS_{IV5}_KW To confirm that the thermostability of GUS_{IV5}_KW improved compared to that of GUSm, the effect of incubation at higher temperatures on the remaining enzyme activity of Trx-V_L fused enzymes was tested (Fig. 3). Consequently, after incubation at high temperatures

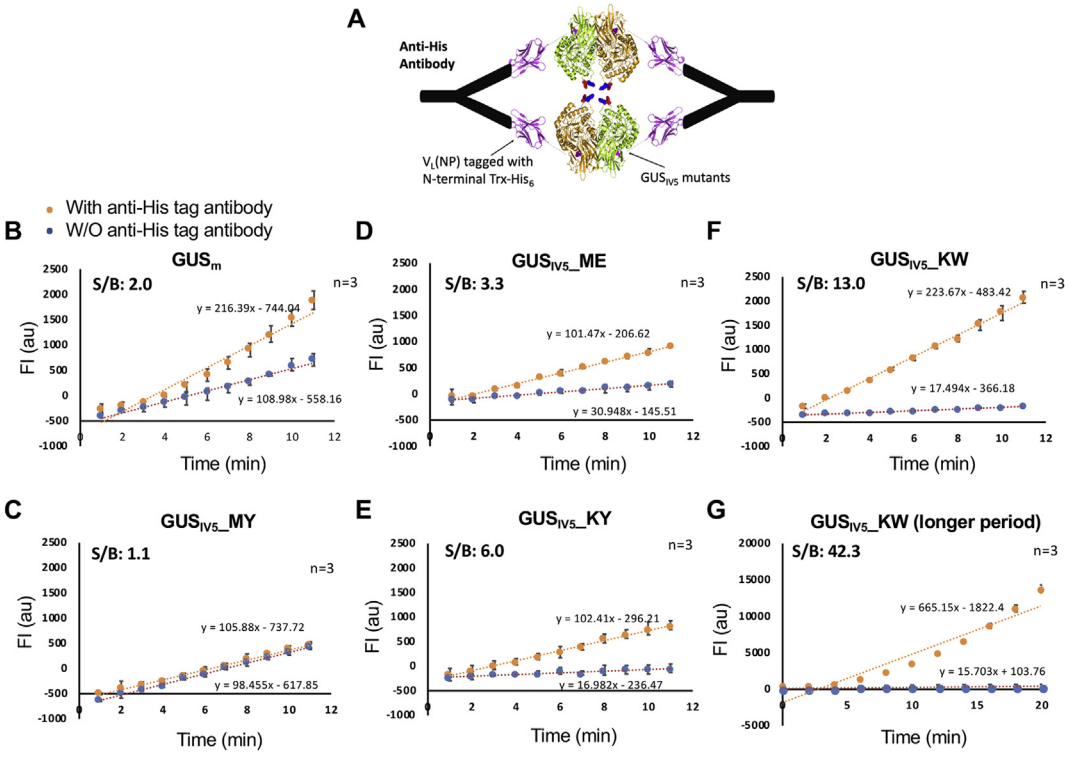

FIG. 2. Screening of better interface mutant based on GUS_{IV5}. (A) Scheme of antibody dimerization experiment. (B–G) Time course activity measurement of Trx-V_L-fused GUS mutants. (B) The original GUSm, (C) GUS_{IV5}_MY, (D) GUS_{IV5}_ME, (E) GUS_{IV5}_KY, (F) GUS_{IV5}_KW, and (G) GUS_{IV5}_MY measured for longer time period. In each panel, the curve fit equations to derive the reaction rates with and without antibody and derived S/B ratio are shown.

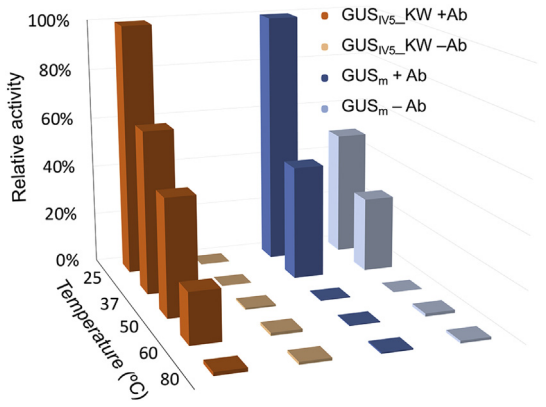

FIG. 3. Thermostability tests of Trx-V_H-GUS$_{IV5}$_KW and Trx-V_H-GUSm by measuring remaining activity (%) at 25°C after treatment at indicated temperatures for 10 min.

(37–80°C) for 10 min, Trx-V_L-fused GUS$_{IV5}$_KW was more active in the presence of anti-His antibody and less active in the absence of the antibody compared to the previous Trx-V_L-fused GUSm. Even in the presence of the antibody, GUSm lost its activity completely after 10-min incubation at 50°C. On the contrary, GUS$_{IV5}$_KW retained more than 20% of its activity after 10-min incubation at 60°C, and it retained some activity even after treatment at 80°C. The background activity of GUS$_{IV5}$_KW remained low during all the aforementioned treatments.

Improved sensitivity in NP detection Because the background activity of the immunosensor system decreased considerably after replacing the reporter enzyme with a thermostabilized GUS mutant, the sensitivity of this immunosensor in detecting its target was expected to improve. To prove this, previously used targets NP and its derivative 5-iodo NP (NIP) were used in an antigen dose-dependency test conducted on GUS$_{IV5}$_KW fusion proteins. After mixing the Trx-V_H(NP)-GUS$_{IV5}$_KW/Trx-V_L(NP)-GUS$_{IV5}$_KW equimolar mixture with NP or NIP in different concentrations and incubating it for 18 min, we measured its fluorescence intensity by adding the substrate. In the dose-dependency curves, we observe an increase in GUS enzyme activity corresponding to the increase in antigen concentration (Fig. 4). The NIP detection sensitivity was roughly 10 times higher than the NP detection sensitivity, which agrees well with the 10-fold difference in the dissociation constants K_d for binding of NP and NIP with the antibody used by Yokozeki et al. (3). After curve fitting, the EC_{50} values of the GUS$_{IV5}$_KW immunosensor for NP and NIP detection were 2.2 ± 0.6 μM and 0.17 ± 0.02 μM, respectively; the LOD values for the detection of NP and NIP were 50 nM and 3.1 nM, respectively. The sensitivity of the IV5_KW

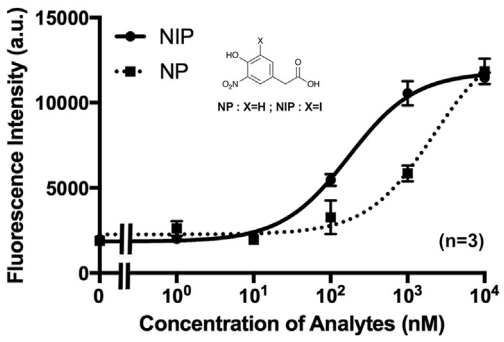

FIG. 4. Antigen (NP, NIP) dose-dependency of immunodetection system using Trx-VH/VL-GUS$_{IV5}$_KW as reporters. The signal from substrate was subtracted as a background.

system was approximately double that of the GUSm system (EC_{50} = 4.8 ± 0.7 μM for NP and 0.44 ± 0.05 for NIP) (8).

Detection of caffeine by antigen-dependent dimerization of single-domain antibody To test the applicability of this system for the detection of practically useful antigen, the V_{HH} fragment of the anti-caffeine antibody was fused to GUS$_{IV5}$_KW by a $(GSSS)_3$ linker to obtain the expression vector of Trx-V_{HH}(Caf)-GUS$_{IV5}$_KW (Fig. S1B). After expression and purification (Fig. S5), Trx-V_{HH}(Caf)-GUS$_{IV5}$_KW protein was mixed with and without 1 μM caffeine, and the enzyme activities of the two mixtures were monitored by adding a fluorescent substrate (Fig. 5A). As expected, the fluorescence signal caused by the recovery of enzyme activity kept increasing after substrate addition (Fig. 5B). The result indicated that the increase in the enzyme activity of GUS$_{IV5}$_KW induced by the V_{HH}-antigen binding was observed continuously by fluorescence measurement. Owing to the low background signal of GUS$_{IV5}$_KW, the S/B ratio in terms of fluorescence intensity increased as the incubation time increased. In the antigen dose dependency measurement (Fig. 5C), antigen concentration was related positively to the fluorescence signal that indicates enzyme activity. After curve fitting, the EC_{50} value of this system for caffeine detection was estimated to be 1.2 ± 0.11 μM, and the LOD was estimated to be 40 nM. It was revealed that by using GUS$_{IV5}$_KW as a reporter enzyme, caffeine can be detected quantitatively with a very low background signal.

DISCUSSION

In this work, the performance of the GUS-based immunosensor system was improved by introducing thermostabilizing mutations to the reporter enzyme GUS. The obtained mutants with new intersubunit mutations were confirmed to be stabilized and showed low background activity without the anti-His tag antibody as a dimerizer. Although the wild-type GUS was fairly stable at room temperature, owing to the intersubunit mutations, the stability of GUSm decreased, resulting in its degradation during the reaction, probably because of contaminated proteases (Fig. S6). Although the underlying mechanism of this degradation-induced catalytic activation is unclear and might warrant further clarification, we speculate that the cleavages at or around the solvent-exposed GUSm interface near the mutated site (516-KE-517) allowed the enzyme to form an active tetramer, and this partially degraded complex accumulated as the reaction proceeded.

The IV-5 mutant with the original interface mutation pair KE showed very low activity upon dimerization compared with GUSm with the same mutation pair. This can possibly be attributed to its more rigid structure, which hinders its efficient dimerization, or a disturbance in the structure of the catalytic site after dimerization. The screening of IV-5 mutants with a back-mutated interface suggested that the KY pair is a more active mutant, and based on this result, we screened the KW pair as a highly active mutant when dimerized. Notably, the GUS$_{IV5}$_KW after dimerization with the anti-His tag antibody showed a similar or higher catalytic turnover rate (1.25 s^{-1}) for this substrate compared to that of the wild-type GUS (1.19 s^{-1}) (Figs. S7 and S8). This would be partly because the mutation G559S near the active site is included in IV-5, which was reported to increase GUS activity (20). It is also interesting that another GUS mutant with higher thermostability, namely, GUS-TR3337 with six mutations (Q493R, T509A, M532T, N550S, G559S, and N566S) also retained G559S mutation, while no other mutations matched those of IV-5 (21). Owing to its activity, GUS$_{IV5}$_KW has great potential for use in detection systems requiring a strong single active enzyme signal, such as digital immunoassay (22).

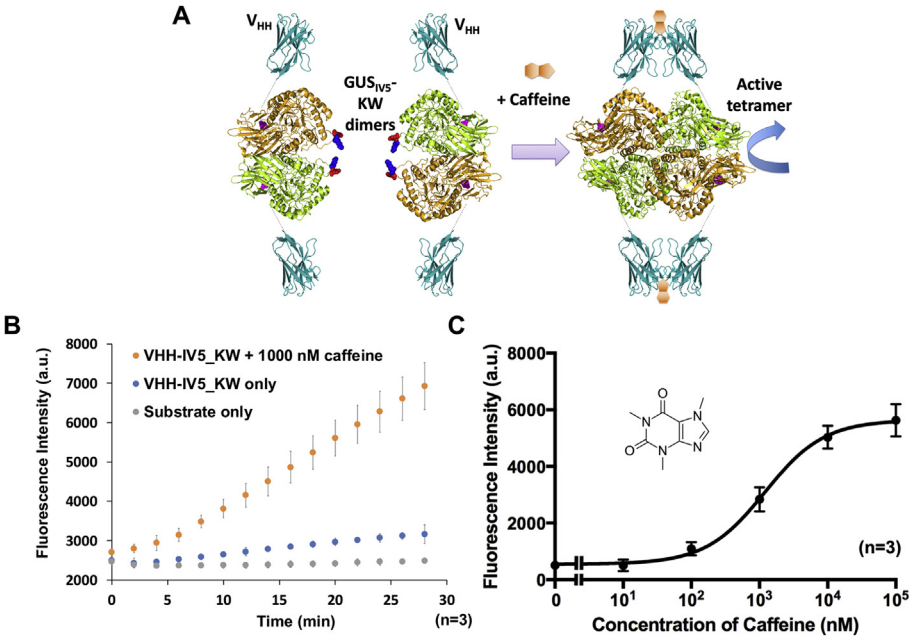

FIG. 5. V_{HH}-based caffeine detection system using Trx-V_{HH}-GUS_{IV5}_KW as a reporter. (A) Scheme, (B) time course measurement and (C) caffeine-dose-dependent GUS activity. The signal from substrate was subtracted as a background in panel B.

The V_{HH} fragment for caffeine used this time has an interesting property that it preferentially forms dimer when the antigen caffeine exists. The observed energy for surface burial of a caffeine in V_{HH} is almost 3-fold more than a typical 1:1 small molecule to protein binding, which means the 1:2 ratio of caffeine to V_{HH} is more thermodynamically stable, and the major existence of 1:2 caffeine/V_{HH} complex was experimentally confirmed (19). Although this property has been applied for making cell-based sensors (23,24), as far as we know, ours will be the first example of its use as a protein-based sensor.

Compared with the original immunosensor system, the reported system with GUS_{IV5}_KW reporters exhibited a weaker background signal without the antigen and higher sensitivity in the detection of antigen NP. Owing to the weak background signal, the incubation time can be lengthened to obtain signals from the antigen at low concentrations. Moreover, quantitative detection of caffeine by using this immunosensor demonstrates its practical utility. Caffeine is present in many widely consumed beverages, and it can be toxic to adults at intake levels exceeding 500 mg per day (25). Considering that the caffeine concentration of tea is approximately 140 μM and that in coffee is approximately 5 mM (26), the proposed immunosensor can detect caffeine in common foods and beverages.

Owing to its improved thermostability, the modified immunosensor will be useful under harsh environments and will have a longer shelf life. Considering the convenience and rapidity of this immunosensor system in the detection of multiple targets, it can be used widely in food and environmental evaluations and medical diagnostic settings.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.015.

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