Expression of antibody variable region-human alkaline phosphatase fusion proteins in mammalian cells

Authors: Yoshiyuki Sasajima^a, Ryohei Iwasaki^a, Kouhei Tsumoto^{a,c}, Izumi Kumagai^d, Masaki Ihara^{b,1}, and Hiroshi Ueda^{a,b*}

Addresses: ^aDepartment of Chemistry and Biotechnology, and ^bDepartment of Bioengineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan;

^cDepartment of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Bldg. FSB-301, 5-1-5 Kashiwanoha Kashiwa Chiba 277-8562, Japan

^dDepartment of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 6-6-11-606, Sendai, 980-8579, Japan.

*Corresponding author

Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
Tel/Fax: +81-3-5841-7362; E-mail: hueda@chembio.t.u-tokyo.ac.jp

Abbreviations

AP, alkaline phosphatase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fv, antibody variable region; HEL, hen egg lysozyme; MBP, *E. coli* maltose binding protein; NP, 4-hydroxy-3-nitrophenacetyl; OS, open-sandwich; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; SEAP, secreted human placental AP; V_H, antibody H chain variable region; V_L, antibody L chain variable region;

25

¹ Current address: Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano, 399-4598, Japan

1 **Abstract**

2

3 Antibody fragments and their fusion proteins are indispensable tools as immunoassay reagents
4 in diagnostics and molecular/cellular biotechnology. However, bacterial expression of cloned
5 antibody genes with correct tertiary structure is not always guaranteed because of the lack of proper
6 folding machinery and/or post-translational modifications. In addition, frequently used bacterial
7 alkaline phosphatase as a fusion partner generally shows lower specific activity than the
8 mammalian enzyme, which hampers its wider use as a detection reagent. Here we tried to express
9 the fusion proteins of antibody variable region(s) and secreted human placental alkaline
10 phosphatase (SEAP) using mammalian cell culture. As a result, functional V_H-SEAP and
11 single-chain Fv-SEAP fusion proteins were successfully obtained from COS-1 cells, which was
12 confirmed by ELISA and Western blotting. This system will be applicable to the rapid production
13 of various antibody-enzyme fusions suitable for ELISA and open-sandwich ELISA that utilizes
14 antigen-dependent V_H/V_L interaction for antigen quantitation.

15

16 **Key Words**

17 4-hydroxy-3-nitrophenacetyl, alkaline phosphatase, antibody variable region, enzyme immunoassay,
18 fusion protein, open-sandwich immunoassay

19

20

1 **1. Introduction**

2 Antibody fragments and their conjugates are indispensable tools as immunoassay reagents in
3 diagnostics and molecular/cellular biotechnology. Among them, enzyme-labeled antibody is an
4 essential component in immunoassays such as ELISA and Western blotting (Porstmann and Kiessig,
5 1992). To date, they have been produced either by chemical-conjugation, or more recently by
6 expression systems for recombinant antibody-enzyme fusion proteins, mainly by microbial
7 expression systems (Ducancel et al., 1993; Weiss and Orfanoudakis, 1994; Suzuki et al., 1997;
8 McDonagh et al., 2003; Medzihradszky et al., 2004; Alderson et al., 2006; Mayer et al., 2006;
9 Venisnik et al., 2006; Coelho et al., 2007; Mousli et al., 2008). However, chemical conjugation
10 needs multiple steps of reaction/purification that might affect the antibody activity (Ishikawa et al.,
11 1983). On the other hand, the expression of cloned antibody genes by bacteria such as *Escherichia*
12 *coli* with correct tertiary structure is not always guaranteed because of the lack of eukaryotic folding
13 machinery and/or post-translational modifications. Moreover, frequently used bacterial alkaline
14 phosphatase shows lower specific activity than the corresponding mammalian enzymes (Murphy et
15 al., 1995), which hampers its wider use as a detection reagent.

16 As a solution for the above problems of conventional techniques, here we made a system that
17 enables production of antibody variable region-alkaline phosphatase (AP) fusion protein in
18 mammalian cells. As the variable regions, we chose two types: one is a heavy chain variable region
19 V_H that is from antibody (HyHEL-10) for hen egg lysozyme (HEL), and the other is a single chain
20 variable region scFv that recognizes 4-hydroxy-3-nitrophenacetyl (NP) on its own. As an AP for
21 labeling, SEAP, a truncated form of human placental alkaline phosphatase (PLAP) that lacks the
22 membrane-anchoring domain, was chosen because it is readily secreted out of mammalian cells and
23 widely used as a sensitive reporter enzyme. In addition, it was reported that human PLAP shows
24 higher activity than *E. coli* AP (Murphy et al., 1995). As a result, functional V_H-SEAP and
25 ScFv-SEAP fusions were successfully expressed in, and secreted from simian COS-1 cells, and

whose characterization was performed. Also investigated was the effect of the length of interdomain linker between V_H and SEAP of the fusion proteins on the enzyme activity.

2. Materials and Methods

2.1. Materials

Oligonucleotides were purchased from Fasmac Co. (Atsugi, Kanagawa, Japan). Restriction and modification enzymes were from Takara-Bio (Otsu, Japan) or Toyobo (Osaka, Japan). Other chemicals were of highest grade available from Wako (Osaka, Japan), or Sigma-Aldrich (Tokyo, Japan) unless otherwise indicated.

2.2. Preparation of the expression plasmids

Before constructing the expression vectors for fusion proteins, an expression vector for His-tagged SEAP was made as follows. First, polyhistidine tag sequence was appended to the C-terminus of secreted human placenta alkaline phosphatase (SEAP) encoded by a mammalian expression vector pSEAP2_control (Clontech, Takara-Bio, Shiga, Japan). To do this, the sequence around the 3' end of SEAP ORF was amplified by standard PCR using SeapHis6p2 (5'-ccgggttactctaga[XbaI]gtcggggcgggccggccaccaccaccaccactgataagatacattgatgag-3') and Mun2EcoFor (5'-ggaattc[EcoRI]ttgttgtaactgtttattgc-3') as primers, and pSEAP2_control as a template. The amplified product was digested with restriction enzymes *XbaI* and *EcoRI*, and inserted to pSEAP2_control digested with *XbaI* and *MfeI*. The nucleotide sequence of the product (hereinafter, pSEAP-His) was confirmed by CEQ-8000 sequencer using CEQ DTCS kit (Beckman-Coulter, Tokyo, Japan).

Next, an antibody heavy chain variable region (V_H) and an IgM-derived intron were inserted to the upstream of SEAP gene as follows. The sequence around the 5' end of mature SEAP sequence was amplified by PCR using pSeapFor

1 (5'-ggaattc[EcoRI]catgg[NcoI]cttaattaa[PacI]ggcgcgcctccgga[BspEI]atcatcccagttgaggaggagaa-3')
 2 and pSeapBk (5'-ccggaattc[EcoRI]atatgggaagcgggtccattgccaggggtat-3') primers, and
 3 pSEAP2_control as a template. The amplified product was treated with *EcoRI* and then inserted
 4 to the *EcoRI* site of pUC-19 (Takara-Bio). To the upstream of SEAP sequence, the μ CH1- μ CH2
 5 intron sequence, which had been amplified from pSV-V 1 using IgMInt5
 6 (5'-ggaattccttaattaa[PacI]cttaag[AflII]taggtttgggggatg-3') and IgMInt3
 7 (5'-ggaattctccgga[BspEI]acctgcagtcaagagaacac-3') primers, was inserted between *PacI* and *BspEI*
 8 sites (pUCintSEAPn). To the upstream thereof, an expression cassette encoding the Ig leader and
 9 anti-lysozyme V_H sequences was inserted from pBSSK-L-VH (Ueda et al., 2000) by digesting it
 10 with *NcoI* and *AflII*, and inserted to the vector digested with the same. To make a cloning site
 11 (*MfeI*) at the 5' of V_H, site-directed mutagenesis was performed by QuikChange mutagenesis kit
 12 (Stratagene) according to the manufacturer, using primers VHquikFor
 13 (5'-ccactcccaggtccaaTtgcaggagtctggccc-3') and VHquikBk (5'-
 14 cgggccagactcctgcaAttggacctgggagtgg-3'), resulting in pUC-VHiSEAPn. To excise a fragment
 15 encoding V_H-intron-N-terminal SEAP, pUC-VHiSEAPn was treated with *EcoR* I and *Nde* I, and
 16 incorporated into pSEAP-His that had been treated with the same, to obtain pVHiSEAPhis. Based
 17 on this plasmid, the anti-lysozyme V_H sequence was replaced using *MfeI* and *AflII* sites, with that of
 18 anti 4-hydroxy-3-nitrophenacetyl (NP) scFv which had been PCR amplified using
 19 pGEM-SCA(Suzuki et al., 1997) encoding the anti-NP scFv (V_L-V_H) as a template, VlamMfeBack
 20 (5'-caggtccaattg[MfeI]gatgctgttgactcaggaatc-3') and VHAflFor2
 21 (5'-tttaagcttaag[AflII]gactcaccgaggaactgtgagagtgg-3') as primers, resulting in pscFvSEAP (Fig.
 22 1). To evaluate the effect of linker length, a peptide linker sequence that encodes 10 amino acids
 23 (L10) was inserted between V_H and SEAP genes of pVHiSEAPhis or pscFvSEAP as follows. The
 24 L10 linker prepared by annealing BspE1linkU (5'- ccggcaccgggtggttctggtggcgggtggaa -3') and
 25 BspE1linkL (5'- ccggttcaccgcccaccagaaccaccgggtg -3') was ligated to the *BspEI*-digested vector,

before re-digestion with *BspEI*. The screening of the recombinant colonies with correct insert by colony PCR resulted in pVHi-L10-SEAPhis or pscFv-L10-SEAP.

2.3. Cell culture and transfection

Simian kidney-derived COS-1 cells were cultured in low glucose DME medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% fetal calf serum, penicillin and streptomycin in a humidified incubator at 37 °C and 5% CO₂. COS-1 cells (1.5×10^5 cells) were seeded on a tissue culture treated ϕ 35-mm dish (Iwaki Technoglass, Tokyo, Japan). Twelve to 24 h later, transfection was performed with one of the five vectors and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol specified by the manufacturer.

2.4. Alkaline phosphatase activity of the culture supernatant

The culture supernatants containing the fusion proteins were dispensed at 100 μ l per well of a 96-well half-area white plate (Costar 3693, Corning Japan, Tokyo). Then 90 μ l each of CDP-star chemiluminescent substrate (Tropix, Roche Diagnostics, Tokyo, Japan) was added and incubated for 20 min at room temperature. The luminescence intensity integrated for 5 s was measured using a luminometer Phelios AB-2350 (ATTO, Tokyo, Japan).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The antigen solution (100 μ g/ml NP-BSA or BSA in PBS) was dispensed at 100 μ l per well of a 96-well white plate (Costar 3922) and then stored at 4 °C overnight for immobilization. After washing with PBS containing 0.05% Tween 20 (PBST) three times, 200 μ l each of 20% immunoblock (DS Pharma, Osaka, Japan) in PBS was added and incubated for 2 h at room temperature. After washing, 50 μ l of the culture supernatant and 50 μ l of PBS containing 5%

immunoblock (IPBS) were added, followed by 1 h of incubation at room temperature. After washing, 100 μ l each of CDP-star chemiluminescent substrate was added and measured for the luminescence intensity as above.

To perform open-sandwich (OS) ELISA that detects V_H - V_L interaction, bacterially expressed purified fusion protein that consists of His₆-tagged *E. coli* maltose binding protein (MBP) and anti-HEL V_L (10 μ g/ml in PBS) was dispensed at 100 μ l per well of a 96-well white plate (Costar 3922) and then stored at 4 $^{\circ}$ C overnight for immobilization. After washing with PBST for three times, 200 μ l each of 20% immunoblock in PBS was added and incubated for 2 h at 25 $^{\circ}$ C. After washing, 50 μ l of the culture supernatant and 50 μ l of IPBS with or without HEL were added, and incubated for 1 h at 25 $^{\circ}$ C. After washing, chemiluminescent detection was performed as above.

2.6. Purification and western blotting of expressed proteins

The culture supernatants containing the secreted fusion proteins from the transfected COS-1 cells were purified with Talon metal affinity resin (Clontech), according to the protocol specified by the manufacturer. The buffer was exchanged to Tris-based storage buffer (0.1 M Tris-HCl, 0.1 M NaCl, 1 mM MgCl₂, 20 μ M ZnCl₂, pH 8.0) with Amicon Ultra 30K (Millipore, Tokyo, Japan).

The purified proteins were separated by SDS-PAGE (80 min, 20 mA) using 5-20% gradient polyacrylamide gel, and were transferred to a nitrocellulose membrane (90 min, 80 mA). The membrane was incubated in 100% immunoblock (DS Pharma) for 1 h at 25 $^{\circ}$ C. After three washings with TBS-T (20 mM Tris-HCl, 0.15 M NaCl, 0.05 % Tween-20, pH7.6) for 10 min, the membrane was incubated in PentaHis Ab-HRP conjugate (Qiagen, Tokyo, Japan) diluted at 1/5000 in CanGetSignal Solution 2 (Toyobo, Osaka, Japan) for 1 h at 25 $^{\circ}$ C. After washing, the membrane was incubated with West Pico substrate (Pierce, Thermo) for 5 min at 25 $^{\circ}$ C. Luminescent image was taken with a LAS-4000 mini luminescent image analyzer (Fujifilm, Tokyo, Japan), and the amount of each fusion protein was quantified using Multi-Gauge program (ver 3.0) using

C-terminally His₆-tagged MBP expressed in *E. coli* as a control (HU, unpublished).

2.7. Kinetic measurements of the proteins

Alkaline phosphatase activity of the purified proteins was assayed as the initial hydrolysis rate of *p*-nitrophenyl phosphate (pNPP, Wako, Osaka) to *p*-nitrophenol. The proteins diluted to the concentration between 0.31 nM to 0.69 nM in DEA buffer (1 M diethanolamine, 1 mM MgCl₂, 20 μM ZnCl₂, pH 9.8) were dispensed at 100 μl per well of a 96-well microplate (Costar 9018). Then 100 μl of pNPP at 0.05 mM to 10 mM in DEA buffer were added to each sample. The absorbance at 405 nm was measured every 1 min for 30 min, and initial reaction velocity was calculated from the linear part of the time-course. The formation of *p*-nitrophenol was calculated from the molar absorption coefficient ($\epsilon = 10080 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$). The Michaelis constant (K_m) and V_{max} were determined from the plot of initial reaction velocities (v) versus substrate concentration (pNPP), applying the equation of $v = V_{max}[S]/(K_m+[S])$ using the curve-fit function of Kaleida Graph (Synergy Software, MA). The catalytic rate constant (k_{cat}) was calculated as the ratio of $V_{max}/[E]$.

3. Results

3.1. Construction of expression vectors

The secreted alkaline phosphatase (SEAP) gene is one of most popular reporter genes to monitor the activity of eukaryotic promoters and enhancers. We used pSEAP2_control plasmid as a basis of our fusion protein expression vectors, which ensures an efficient transcription of its mRNA as well as its replication in COS cells owing to its SV40 early promoter/enhancer. First, pSEAP-His was designed to incorporate an in-frame His₆-tag sequence at the downstream of SEAP gene, so that the expressed SEAP protein has six histidines at the C-terminal end. pVHiSEAPHis and pscFv-SEAP are pSEAP-His appended with the sequence for either anti-lysozyme HyHEL-10

1 V_H or anti-NP scFv, each preceded by an IgH leader sequence, at the upstream of mature SEAP
2 open reading frame with an intervening intron, respectively. pVHi-L10-SEAPhis and
3 pscFv-L10-SEAP are the variants of pVHiSEAPhis and pscFvSEAP, respectively, with an extra
4 flexible linker sequence encoding 10 residues
5 (NH₂-Gly-Thr-Gly-Gly-Ser-Gly-Gly-Gly-Thr-COOH) at the 5' of SEAP ORF. In these vectors, a
6 pair of cloning sites (*Mfe*I and *Afl*II) was introduced to facilitate the exchange of antibody variable
7 region domains (Fig. 1).

9 3.2. Expression of SEAP-His, V_H -SEAP and scFv-SEAP

10 In order to express the target proteins, the five expression vectors constructed were transfected to
11 simian COS-1 cells. Since all these vectors have SV40 replication origin, they should give high
12 copy number in COS-1 cells, thus lead to high productivity of the encoded proteins. To confirm the
13 expression of the recombinant proteins, 144 h after transfection, the culture supernatants were
14 subjected to the enzyme activity measurement using CDP-star chemiluminescent substrate (Fig. 2).
15 As a result, the three culture supernatants showed significantly higher signals than that of
16 non-transfected COS-1 cells, suggesting the efficient expression of the proteins with SEAP domain.

18 3.3. Open-sandwich ELISA with V_H -SEAP fusion proteins

19 To test the binding and catalytic activities of V_H -SEAP in the culture supernatant, open-sandwich
20 ELISA was performed. The interaction between the two antibody variable regions (V_H and V_L) of
21 anti-hen egg lysozyme (HEL) antibody HyHEL-10 was reported to be weak in the absence of HEL,
22 but significantly strengthened in presence of HEL (Ueda et al., 1996). To date, a number of antigens
23 were detected by using this property with immobilized V_L and enzyme-labeled V_H proteins (Suzuki
24 et al., 1999; Suzuki et al., 2000; Lim et al., 2007; Ihara et al., 2009). To confirm that the expressed
25 V_H -SEAP fusion proteins retain the characters of both antigen dependent V_L binding as well as

enzyme activities, AP activity bound to the MBP-fused V_L-immobilized plate was measured in the presence or the absence of antigen. Among the culture supernatants containing V_H-SEAP, V_H-L₁₀-SEAP, SEAP-His, or no fusion protein, only those with V_H-SEAP and V_H-L₁₀-SEAP showed significant HEL-dependent increase in chemiluminescence signal (Fig. 3A). On the contrary, negligible HEL-dependent signal was observed for those with pSEAP-His or mock transfectants. Hence, it was suggested that antigen-dependent V_H-V_L interaction was successfully detected with V_H-SEAP and V_H-L₁₀-SEAP proteins, in other words, we could perform open-sandwich ELISA with these proteins.

3.4 scFv-SEAP fusion proteins work as an enzyme-labeled antibody

To confirm that scFv-SEAP fusions retain both antigen-binding and catalytic activities, AP activity was measured in a conventional ELISA format. The culture supernatants of COS-1 cells into which either pscFv-SEAP, scFv-L₁₀-SEAP or pSEAP-His had been transfected were incubated in microplates coated with the conjugated antigen NP-BSA or BSA as a control. In the cases of scFv-SEAP and scFv-L₁₀-SEAP, the signals in the wells coated with NP-BSA were significantly higher than those with BSA (Fig. 3B). In the cases of SEAP-His and the negative control (culture supernatant of non-transfected COS-1 cells), no significant difference was observed between the signals for NP-BSA and BSA. The results suggest that the scFv-SEAP fusion proteins secreted from COS-1 cells retain both antigen binding and AP activities, and functions as an enzyme-labeled antibody.

3.5. Purification and characterization of the fusion proteins

To further characterize the produced proteins, the five culture supernatants were subjected to purification using a cobalt affinity resin through their C-terminal His₆-tag. To confirm the integrity of each protein, Western blotting with anti-His tag antibody was performed (Fig. 4). For each fusion

protein, a distinct single band at around 70 k (SEAP-His), 80 k (V_H -SEAP/ V_H -L₁₀-SEAP) and 100 k (pscFv-SEAP/scFv-L₁₀-SEAP) was detected, respectively, which well corresponds to its expected molecular weight.

From the imaging analysis, the calculated amounts of recovered proteins were 12.6, 1.8, 2.3, 3.1 and 2.6 pmol (0.88, 0.14, 0.15, 0.31 and 0.26 μ g) per ml culture for SEAP-His, V_H -SEAP, V_H -L₁₀-SEAP, scFv-SEAP and scFv-L₁₀-SEAP, respectively.

3.6. Kinetic parameters of the fusion proteins

For the quantitative evaluation of enzyme activity, the kinetic analysis of the purified proteins was performed. Using pNPP as a substrate, the initial velocities of the reaction at different substrate concentrations were measured by the color development, and the kinetic parameters K_m and V_{max} were calculated from the curve-fit of v-s plot assuming Michaelis-Menten steady-state kinetics (Fig. 5). The k_{cat} values were calculated from V_{max} and the protein concentration. The data are summarized in Table. 1. The calculated K_m values for V_H -SEAP, V_H -L₁₀-SEAP, scFv-SEAP and scFv-L₁₀-SEAP remain almost the same as that for SEAP-His. On the other hand, the k_{cat} values for the antibody-SEAP fusion proteins were 13.9-38.7% that of SEAP-His. The somewhat lower k_{cat} for the fusion proteins were possibly due to the steric hindrance of SEAP active site brought by the N-terminally fused variable domains (see discussion). This was also supported by the higher k_{cat} values of the longer-linker variants, which suggest lesser steric hindrance in these proteins. The k_{cat} values for SEAP fused with scFv were almost two-fold higher than that with V_H , which might suggest lower negative effect of scFv on SEAP than V_H alone.

4. Discussion

In this study, we could successfully prepare two types of functional antibody variable

1 region-SEAP fusion proteins by mammalian cell culture. According to the kinetic parameters
2 determined, the K_m values for the antibody-SEAP fusion proteins are similar to that for SEAP-His,
3 and also to the reported value for PLAP (0.36 mM) (Kozlenkov et al., 2002). This indicates that the
4 SEAP domain of each protein should fold correctly to the native conformation. On the other hand,
5 the k_{cat} values for V_H -SEAP (109 s^{-1}), V_H -L₁₀-SEAP (165 s^{-1}), scFv-SEAP (252 s^{-1}) and
6 scFv-L₁₀-SEAP (302 s^{-1}) were lower than that for SEAP-His (780 s^{-1}). Considering the similar K_m
7 values, this might be due to the steric hindrance brought by the N-terminally fused domain(s). In
8 fact, the k_{cat} values for the longer linker variants were markedly higher than those of the
9 corresponding fusion protein with shorter linker. Probably, the longer linker reduced the steric
10 hindrance and improved the k_{cat} . However, ironically, the k_{cat} values for SEAP fused with scFv were
11 almost two-fold higher than those fused with smaller V_H . Possibly, in the cases of SEAP fused with
12 V_H , the exposed hydrophobic V_L interface of V_H , which is normally buried inside scFv, has a
13 greater propensity to interact with SEAP, so that greater inhibition was observed when fused with
14 V_H alone. Nevertheless, the k_{cat} values for mammalian expressed V_H -SEAP fusions were still higher
15 than those for bacterially expressed V_H -PhoA fusions ($43\text{-}70\text{ s}^{-1}$) (Suzuki et al., 2000). Also, the
16 k_{cat} values for scFv-SEAP fusions were significantly higher than that of bacterially expressed
17 scFv-PhoA (103 s^{-1}) (Suzuki et al., 1997).

18 From the crystal structures determined, the distance between the active site and the N-terminus
19 of mammalian AP is shorter than that of *E. coli* AP (Kim and Wyckoff, 1991; Du et al., 2001). This
20 might be reflected to the enhanced interference of the SEAP fusions by the tethered domain(s)
21 compared to the corresponding PhoA fusions. However, potential merits of N-terminal fusion of
22 antibody domains include conservation of native antibody N-terminal sequence, as well as its
23 application to the detection of spliceosome-mediated *trans*-splicing (Iwasaki et al., 2009). In
24 addition, use of intestinal AP with higher specific activity (Murphy et al., 1995) instead of SEAP
25 will further improve the usefulness of the system. In fact, we have observed several-fold higher k_{cat}

1 of intestinal AP than SEAP, when expressed similarly in COS-1 cells (Sasajima et al., in
2 preparation). In combination with such improvements, our system will enable the production of
3 various enzyme-labeled antibodies that are useful in conventional and open-sandwich ELISAs, as
4 well as in immunoblots and immunostaining.

5

6 **Acknowledgments**

7

8 This study was supported in part by Japan Science and Technology Agency (PRESTO program),
9 and by Grant-in-Aid for scientific research (B20360368) from JSPS, Japan. We also thank
10 FUJIFILM Co. for the financial support.

11

1 **References**

- 2
- 3 Alderson, R.F., Toki, B.E., Roberge, M., Geng, W., Basler, J., Chin, R., Liu, A., Ueda, R., Hodges,
4 D., Escandon, E., Chen, T., Kanavarioti, T., Babé, L., Senter, P.D., Fox, J.A. and
5 Schellenberger, V. (2006) Characterization of a CC49-based single-chain
6 fragment-beta-lactamase fusion protein for antibody-directed enzyme prodrug therapy
7 (ADEPT). *Bioconjug Chem* 17, 410-8.
- 8 Coelho, V., Dervedde, J., Petrusch, U., Panjideh, H., Fuchs, H., Menzel, C., Dübel, S., Keilholz,
9 U., Thiel, E. and Deckert, P.M. (2007) Design, construction, and in vitro analysis of
10 A33scFv::CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy
11 in colon cancer. *Int J Oncol* 31, 951-7.
- 12 Du, M.H.L., Stigbrand, T., Taussig, M.J., Ménez, A. and Stura, E.A. (2001) Crystal structure of
13 alkaline phosphatase from human placenta at 1.8 Å resolution. *J. Biol. Chem.* 276,
14 9158-9165.
- 15 Ducancel, F., Gillet, D., Carrier, A., Lajeunesse, E., Ménez, A. and Boulain, J. (1993) Recombinant
16 colorimetric antibodies: construction and characterization of a bifunctional F(ab)₂/alkaline
17 phosphatase conjugate produced in *Escherichia coli*. *Biotechnology* 11, 601-605.
- 18 Ihara, M., Suzuki, T., Kobayashi, N., Goto, J.-i. and Ueda, H. (2009) Open-sandwich enzyme
19 immunoassay for one-step noncompetitive detection of corticosteroid 11-deoxycortisol.
20 *Anal. Chem.* 81, 8298-8304.
- 21 Ishikawa, E., Imagawa, M., Hashide, S., Yoshitake, S., Hamaguchi, Y. and Ueno, T. (1983)
22 Enzyme labeling of antibodies and their fragments for enzyme immunoassay and
23 histochemical staining. *J. Immunoassay* 4, 209-327.
- 24 Iwasaki, R., Kiuchi, H., Ihara, M., Mori, T., Kawakami, M. and Ueda, H. (2009) Trans-splicing as a
25 novel method to rapidly produce antibody fusion proteins. *Biochem. Biophys. Res.*

Commun. 384, 316-321.

Kim, E.E. and Wyckoff, H.W. (1991) Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. *J. Mol. Biol.* 218, 449-464.

Kozlenkov, A., Manes, T., Hoylaerts, M.F. and Millán, J.L. (2002) Function assignment to conserved residues in mammalian alkaline phosphatases. *J Biol Chem* 277, 22992-9.

Lim, S.-L., Ichinose, H., Shinoda, T. and Ueda, H. (2007) Noncompetitive detection of low molecular weight peptides by open sandwich immunoassay. *Anal. Chem.* 79, 6193-6200.

Mayer, A., Francis, R.J., Sharma, S.K., Tolner, B., Springer, C.J., Martin, J., Boxer, G.M., Bell, J., Green, A.J., Hartley, J.A., Cruickshank, C., Wren, J., Chester, K.A. and Begent, R.H. (2006) A phase I study of single administration of antibody-directed enzyme prodrug therapy with the recombinant anti-carcinoembryonic antigen antibody-enzyme fusion protein MFECPI and a bis-iodo phenol mustard prodrug. *Clin Cancer Res* 12, 6509-16.

McDonagh, C.F., Beam, K.S., Wu, G.J., Chen, J.H., Chace, D.F., Senter, P.D. and Francisco, J.A. (2003) Improved yield and stability of L49-sFv-beta-lactamase, a single-chain antibody fusion protein for anticancer prodrug activation, by protein engineering. *Bioconjug Chem* 14, 860-9.

Medzihradszky, K.F., Spencer, D.I., Sharma, S.K., Bhatia, J., Pedley, R.B., Read, D.A., Begent, R.H. and Chester, K.A. (2004) Glycoforms obtained by expression in *Pichia pastoris* improve cancer targeting potential of a recombinant antibody-enzyme fusion protein. *Glycobiology* 14, 27-37.

Mousli, M., Turki, I., Kharmachi, H. and Dellagi, K. (2008) Genetically engineered colorimetric single-chain antibody fusion protein for rapid diagnosis of rabies virus. *Dev Biol (Basel)* 131, 483-91.

Murphy, J.M., Tibbitts, T.T. and Kantrowits, E.R. (1995) Mutations at positions 153 and 328 in *Escherichia coli* alkaline phosphatase provide insight towards the structure and function of

mammalian and Yeast alkaline phosphatases. *J. Mol. Biol.* 253, 604-617.

Porstmann, T. and Kiessig, S.T. (1992) Enzyme immunoassay technique: An overview. *J. Immunol. Methods* 150, 5-21.

Suzuki, C., Ueda, H., Mahoney, W. and Nagamune, T. (2000) Open Sandwich Enzyme-Linked Immunosorbent Assay for the quantitation of small haptens. *Anal. Biochem.* 286, 238-246.

Suzuki, C., Ueda, H., Suzuki, E. and Nagamune, T. (1997) Construction and characterization of an anti-hapten ScFv-alkaline phosphatase chimeric protein. *J. Biochem.* 122, 322-329.

Suzuki, C., Ueda, H., Tsumoto, K., Mahoney, W., Kumagai, I. and Nagamune, T. (1999) Open Sandwich ELISA with VH- / VL- alkaline phosphatase fusion proteins. *J. Immunol. Methods* 224, 171-184.

Ueda, H., Kawahara, M., Aburatani, T., Tsumoto, K., Todokoro, K., Suzuki, E., Nishimura, H., Schueler, P.A., Winter, G., Mahoney, W.C., Kumagai, I. and Nagamune, T. (2000) Cell-growth control by monomeric antigen; the cell surface expression of lysozyme-specific Ig V-domains fused to truncated Epo receptor. *J. Immunol. Methods* 241, 159-170.

Ueda, H., Tsumoto, K., Kubota, K., Suzuki, E., Nagamune, T., Nishimura, H., Schueler, P.A., Winter, G., Kumagai, I. and Mahoney, W.C. (1996) Open sandwich ELISA: A novel immunoassay based on the interchain interaction of antibody variable region. *Nat. Biotechnol.* 14, 1714-1718.

Venisnik, K.M., Olafsen, T., Loening, A.M., Iyer, M., Gambhir, S.S. and Wu, A.M. (2006) Bifunctional antibody-Renilla luciferase fusion protein for in vivo optical detection of tumors. *Protein Eng Des Sel* 19, 453-460.

Weiss, E. and Orfanoudakis, G. (1994) Application of an alkaline phosphatase fusion protein system suitable for efficient screening and production of Fab-enzyme conjugates in *Escherichia coli*. *J. Biotechnol.* 33, 43-53.

Table. 1. Kinetic parameters for the proteins. The kinetic parameters k_{cat} and K_{m} were calculated based on Michaelis-Menten steady-state kinetics by using s-v plot shown in Fig. 5.

	k_{cat} (1/s)	K_{m} (mM)
SEAP-His	780.0 ± 15.3	0.384 ± 0.026
V _H -SEAP	108.5 ± 1.4	0.329 ± 0.015
V _H -L ₁₀ -SEAP	165.3 ± 1.9	0.383 ± 0.015
scFv-SEAP	251.9 ± 2.3	0.352 ± 0.012
scFv-L ₁₀ -SEAP	301.9 ± 2.0	0.352 ± 0.008

1 **Figure Legends**

2

3 **Figure 1.** Schematic structure of the expression vectors: (A) pSEAP-His (B) pVHiSEAPHis and
4 pVHi-L10-SEAPHis, (C) pscFv-SEAP and pscFv-L10-SEAP. The constructs were designed to
5 express secreted human placental alkaline phosphatase (SEAP) and its fusion proteins with
6 C-terminal his-tag in mammalian cells. Each vector encodes SV40 early promoter at the upstream
7 and SV40 enhancer at the downstream of ORF. (D) The nucleotide and peptide sequences of the
8 linkers A and B. pVHiSEAPHis and pscFv-SEAP have linker A encoding three amino acid residues
9 upstream of the SEAP. pVHi-L10-SEAPHis and pscFv-L10-SEAP have linker B, encoding 13
10 amino acid residues.

11 **Figure 2.** AP activity of the fusion proteins. Enzyme activity of the culture supernatants of the cells
12 transfected with each vector was measured by chemiluminescence. The culture supernatant of
13 non-transfected cells was taken as a negative control. The average of three measurements with error
14 bars for 1 SD is shown.

15 **Figure 3. A,** Open Sandwich ELISA for HEL. Culture supernatant (50 l) was mixed with PBS (50
16 l) containing 5% immunoblock and 100 µg/ml HEL (filled column) or no antigen (empty column),
17 and incubated in microplates coated with V_L. **B,** Antigen-binding ELISA. Culture supernatant (50
18 l) was mixed with PBS containing 5% immunoblock (50 l), and incubated in microplates coated
19 with NP-BSA (filled column) and BSA (empty column). Bound fusion protein was assayed by
20 measuring alkaline phosphatase activity. The values are the average of three independent
21 measurements, with error bars showing 1 S.D.

22 **Figure 4.** Western blot of the proteins. Lanes 1-5 show purified proteins for SEAP-His, V_H-SEAP,
23 V_H-L₁₀-SEAP, scFv-SEAP, and scFv-L₁₀-SEAP, respectively. The positions of molecular weight
24 are indicated. The positions for SEAP-His, V_H-SEAP and scFv-SEAP are shown.

25 **Figure 5.** Michaelis-Menten steady-state kinetics analysis of AP activity. SEAP-His (filled circles),

1 V_H -SEAP (filled squares), V_H -L₁₀-SEAP (open triangles), scFv-SEAP (open circles) and
2 scFv-L₁₀-SEAP (asterisks) are shown. The initial velocities of the reaction at different substrate
3 concentrations were measured by color development.

4

5

Figure(s)

Figure 1. Sasajima et al.

Figure 2. Sasajima et al.

Figure 3. Sasajima et al.

Figure 4. Sasajima et al.

Figure 5. Sasajima et al.