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Improving the Productivity of Single-Chain Fv Antibody Against c-Met by Rearranging the Order of its Variable Domains

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Single-chain Fv (scFv) antibody against c-Met is expected to be employed in clinical treatment or imaging of cancer cells owing to the important biological roles of c-Met in the proliferation of malignancies. Here, we show that the productivity of scFv against c-Met in *Escherichia coli* is significantly influenced by the orientation of its variable domains. We generated anti-c-Met scFv antibodies with two different domain orders (*i.e.*, V_L-linker-V_H and V_H-linker-V_L), expressed them in the cytoplasm of *E. coli trx/gor* deleted mutant, and compared their specific activities as well as their productivities. Productivity of total and functional anti-c-Met scFv with V_H/V_L orientation was more than five times higher than that with V_L/V_H format. Coexpression of DsbC enhanced the yield of soluble amounts of anti-c-Met scFv protein for both constructs. The purified scFv antibodies of the two different formats exhibited almost the same antigen-binding activities. We also compared the productivities and specific activities of anti-c-Met diabodies with V_H/V_L or V_L/V_H formats and obtained similar results to the case of scFv antibodies.

Keywords: Anti-c-Met scFv, variable domain, domain order, diabody

c-Met, a receptor of hepatocyte growth factor/scattering factor, is recognized as a potential target in developing therapeutic reagents of cancer as well as in targeted therapies owing to its important biological roles in the proliferation of malignant tumor cells [8]. Accordingly, antibody against c-Met is expected to be usefully employed in the clinical treatment or imaging of many cancer cells [13]. In particular, fragment antibodies against c-Met such as anti-c-Met single-chain Fv (scFv) antibody can be suitably utilized for the applications because of their small sizes [7, 17]. In addition, fragment antibodies can be more economically produced compared with whole immunoglobulins since it is possible to produce them in prokaryotic cells such as *E. coli* while retaining their antigen binding capacities [4, 15].

It is important to achieve a large amount of functional anti-c-Met scFv for the practical utilization of the scFv in diagnosis or therapy. We have previously reported that recombinant scFv against c-Met can be functionally expressed in the cytoplasm of *E. coli* [6]. We have succeeded to increase the ratio of soluble scFv fraction to insoluble fraction by employing a host with oxidizing cytoplasm, coexpressing molecular chaperones, and optimizing expression conditions. However, in those studies, the amount of total anti-c-Met scFv produced was very low in spite of employing a pET expression system, which is one of the extensively used systems for the production of recombinant protein because of its high inducibility of target protein [2]. We have done a few experiments to achieve improved productivities of anti-c-Met scFv in *E. coli*. For example, we tried to coexpress tRNAs for rare codons in *E. coli* with anti-c-Met scFv, since our anti-c-Met scFv gene contains a number of rare codons in *E. coli* that can limit the productivity of recombinant protein [12]. However, we could not observe any improvement of the yield of anti-c-Met scFv through that approach. We also tried to produce the anti-c-Met scFv at low temperature to prevent its degradation, but there was no improvement in the productivity of scFv.

The strategy of rearranging the order of the variable domains of scFv has been occasionally attempted to improve the productivity of scFv. Although the approach is not always successful, a few reports have demonstrated that the expression level of target scFv can be increased by varying the orientation of its variable domains [5, 11, 14]. In this report, we show that the expression level of anti-c-Met scFv in the cytoplasm of *E. coli* can be significantly enhanced by simply altering its domain order from V_L/V_H that we

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used in our previous study [6] to V_H/V_L. We constructed anti-c-Met scFv antibodies of V_H-linker-V_L format and V_L-linker-V_H format, and compared their antigen binding activities as well as their productivities. The diabody form of this antibody was also investigated.

To investigate the effect of the order of variable domains on the productivity of anti-c-Met scFv, we first constructed pET24ma-LHscFv and pET24ma-HLscFv, which respectively express the scFv of V_L/V_H format and the scFv of V_H/V_L format, by combining the two variable domains using $(Gly_4Ser)_3$, a linker sequence generally employed for the formation of scFv [1]. For the construction of pET24ma-LHscFv, the V_L and V_H genes of anti-c-Met scFv amplified from pET24ma-scFvMet, a plasmid containing the gene that encodes the anti-c-Met scFv of V_L/V_H form fused with a nonrepeated linker [6], using the respective 5' and 3' primers (5' primer for V_L: GAATTCGAGCTCGTGCTG-ACTCAGTCG; 3' primer for V_L: AGATCCACCACCGC-CAGAGCCACCGCCACCGCCTGTGACGGTCAGCTG; 5' primer for V_H: GGCGGTGGTGGATCTGGTGGCGG-CGGTTCTCA GGAGTCGGTGAAGGAG; 3' primer for V_H: AAGCTTTTATTAGACTGATGGAGCC TTAGG) were fused by overlapping PCR and cloned into pET24ma [9] using EcoRI and HindIII. For the construction of pET24ma-HLscFv, the V_H and V_L genes of anti-c-Met scFv amplified using the respective 5' and 3' primers (5' primer for V_H: GAATTCCAGGAGTCGGTGAAGGAG; 3' primer for V_H: AGATCCACCACCGCCA GAGCCACCGCCACCGAC-TGATGGAGCCTTAGG; 5' primer for V_L: GGCGGTGGT GGATCTGGTGGCGGCGGTTCTGAGCTCGTGCTGA-CTCAG; 3' primer for V_L: AAGCTTTTATTAGTGGTGG-TGGTGGTGGTGGCCTGTGACGGTCAGCTG) were fused and cloned into pET24ma. The T7 tag was added to the N-terminal of the scFv genes, and the expressed scFv antibodies could be detected by a HRP-labeled anti-T7 tag monoclonal antibody in Western blotting or ELISA. We fused hexahistidine at the C-terminus of the respective proteins to purify the fragment antibodies simply through a Ni-NTA column.

The constructed pET24ma-LHscFv and pET24ma-HLscFv were used to transform *E. coli* Origami2 (DE3), which is a strain that has cytoplasmic condition for proper disulfide bond formation [3], and the productivity of each scFv protein was compared. Fig. 1A shows the expression level of each anti-c-Met scFv. The scFv antibodies were induced with 1 mM IPTG at 30°C for 4 h and the amount of total scFv of the soluble fraction as well as insoluble fraction was analyzed by Western blotting using an anti-T7 tag antibody conjugated with horseradish peroxidase (HRP) as described previously [16]. As shown in the figure, the amount of scFv with V_H/V_L domain order was much higher that of the scFv with V_L/V_H format, although it is not easy to tell the degree of improvement quantitatively owing to the limitation of Western blotting for quantification. This result

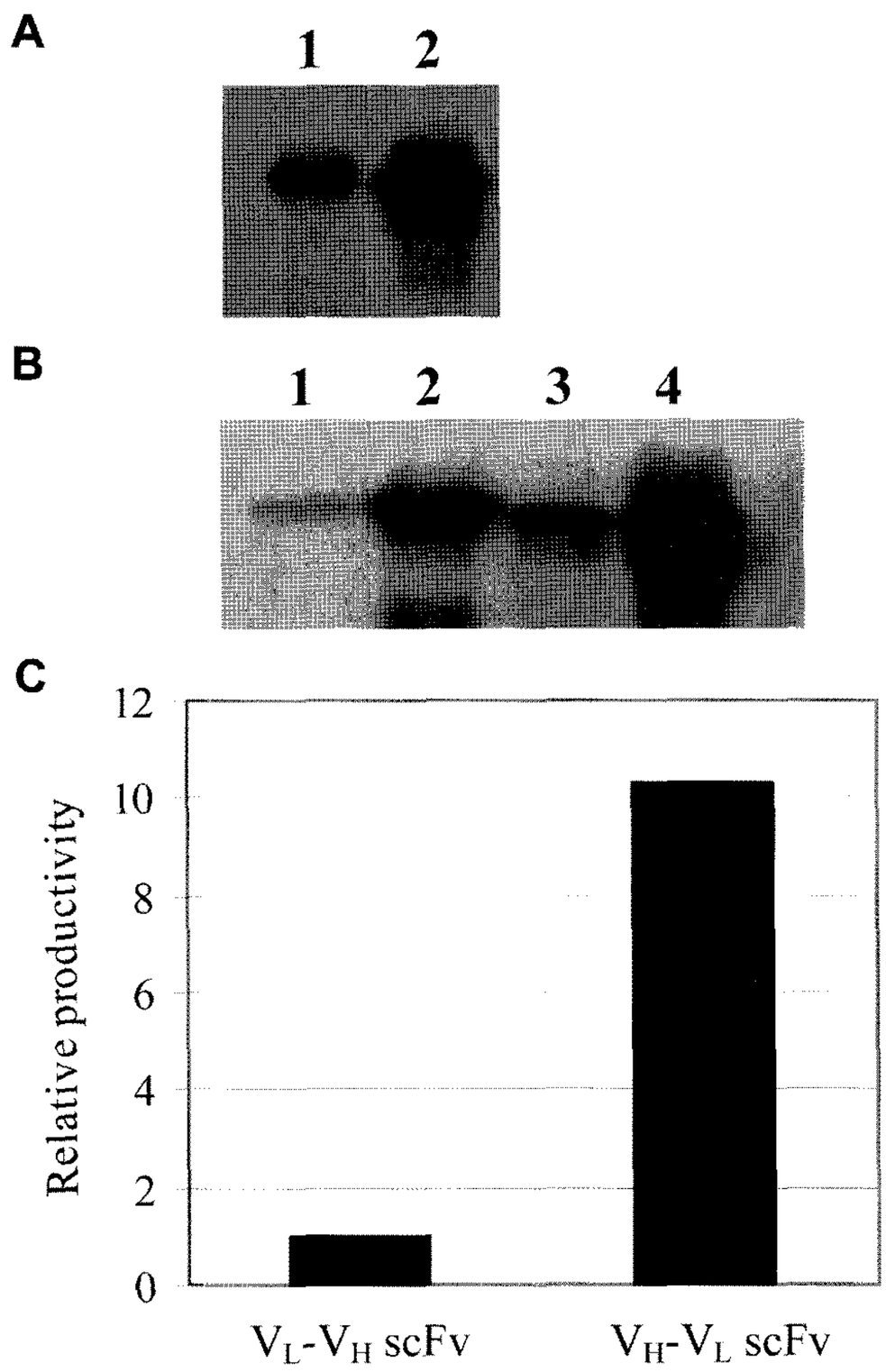

Fig. 1. Effect of domain order on the productivity of anti-c-Met scFv.
A. Western blotting of total proteins for the anti-c-Met scFv antibodies with different domain orders. Lane1, V_L-linker-V_H scFv; lane2, V_H-linker-V_L scFv. **B**. Western blotting of soluble and insoluble fractions for the anti-c-Met scFv antibodies with different domain orders. Lane 1, soluble fraction of V_L-linker-V_H scFv; lane 2, insoluble fraction of V_L-linker-V_H scFv; lane 3, soluble fraction of V_H-linker-V_L scFv; lane 4, insoluble fraction of V_H-linker-V_L scFv. **C**. Relative productivity of produced functional anti-c-Met scFv antibodies with different domain orders in the soluble fraction. Productivity of functional scFv was determined by measuring the activity of scFv in the soluble fraction through ELISA assay as described previously [6] and relative productivities were determined on the basis of the productivity of functional scFv with V_L-linker-V_H.

suggests that the order of variable domains has remarkable influence on the productivity of anti-c-Met scFv.

Although the expression level of the anti-c-Met scFv was dramatically improved by altering the domain order from V_L/V_H to V_H/V_L, it does not necessarily guarantee the higher productivity of functional anti-c-Met scFv, since the orientation of variable domains can also change the specific activity of the recombinant protein. Thus, we measured the activity of each anti-c-Met scFv in the soluble fraction as well as the amount of soluble scFv produced for the samples obtained in the above experiment. Fig. 1B shows

that the amount of soluble scFv with V_H/V_L was much higher than that of V_L/V_H format. It is estimated that the amount of soluble scFv with V_H/V_L format is five to ten times higher than that of V_L/V_H format within the limitation of Western blot analysis. The total relative functional activity of the V_H/V_L form was approximately 10 times higher than that of the V_L/V_H form in the same amount of soluble extract (Fig. 1C), which roughly matches with the protein yield of soluble anti-c-Met scFv shown in Fig. 1B. These results suggest that the productivity of soluble and functional anti-c-Met scFv can be improved by reversing the domain order. These results also indirectly indicate that the specific binding activity of anti-c-Met scFv is not significantly affected by its domain order.

To check the effect of domain order on the specific activity of anti-c-Met scFv exactly, we purified the recombinant proteins and measured their specific binding activities. As shown in Fig. 1B, the amount of soluble fraction was much lower than that of insoluble fraction for both constructs under the investigated condition, which results in the limited yield of soluble scFv. Thus, we attempted to produce the scFv antibodies under the previously optimized condition for the production of soluble functional anti-c-Met scFv [6].

The recombinant scFv antibodies were induced with 0.05 mM IPTG at 20°C for 6 h with the coexpression of DsbC, a molecular chaperone containing the disulfide bond isomerase activity [10]. After the cell lysis, we measured the binding activity of scFv in the same amount of cell extracts and compared the amount of soluble fraction and insoluble fraction of each molecule. Fig. 2A shows that the ratio of soluble fraction to insoluble fraction for both constructs increased in the optimized condition compared with the results in Fig. 1B. The productivity of functional scFv of V_H/V_L was approximately 5 times higher than that of V_L/V_H in the optimized condition (Fig. 2B), confirming again that the productivity of V_H/V_L format is much higher than that of V_L/V_H format.

We produced the fragment antibodies under the optimized condition and purified them by the Ni^{2+}-NTA system. *E. coli* Origami2 (DE3) harboring the respective plasmids for anti-c-Met scFv and diabody and pBAD-DsbC was grown to OD_{600} of 0.6 at 20°C in 100 ml of LB medium, and the protein was induced with 0.05 mM IPTG for 6 h at 20°C. The cell extracts were incubated with 5 mg of Ni-NTA HisBind Resin (Novagen) for 3 h at 4°C, and the resin was loaded into a column, and washed with 2×4 ml of washing buffer (50 mM phosphate buffer, pH 8.0; 300 mM NaCl; 50 mM imidazole). The target protein was eluted with 1 ml of elution buffer (50 mM phosphate buffer, pH 8.0; 300 mM NaCl; 250 mM imidazole). The imidazole was removed by diafiltration. We could obtain samples of scFv showing more than 95% purity. Using the purified scFv

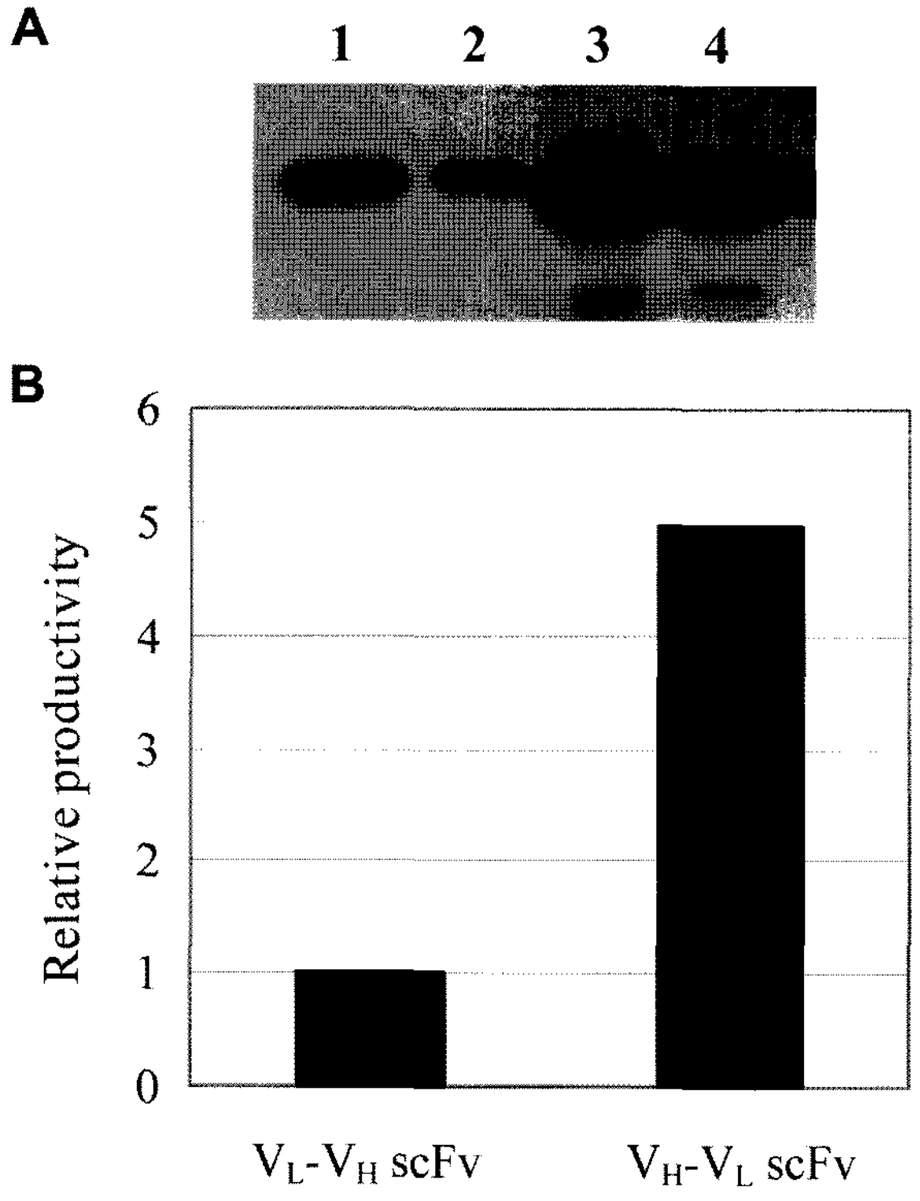

Fig. 2. Effect of DsbC coexpression on the productivity of anti-c-Met scFv.

A. Western blotting of soluble and insoluble fractions for the anti-c-Met scFv antibodies with different domain orders expressed with DsbC. Lane 1, soluble fraction of V_L-linker-V_H scFv; lane 2, insoluble fraction of V_L-linker-V_H scFv; lane 3, soluble fraction of V_H-linker-V_L scFv; lane 4, insoluble fraction of V_H-linker-V_L scFv. **B**. Relative productivity of produced functional anti-c-Met scFv antibodies with different domain orders in the soluble fraction. Productivity of functional scFv was determined by measuring the activity of scFv in the soluble fraction and relative productivities were determined on the basis of the productivity of functional scFv with V_L-linker-V_H.

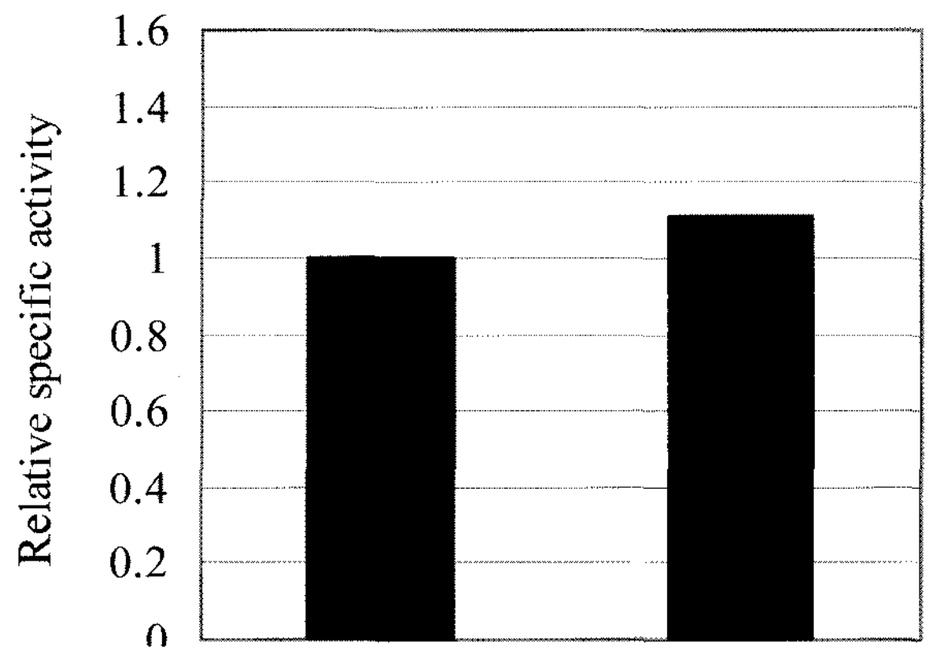

Fig. 3. Relative specific activities of the purified anti-c-Met scFv antibodies with different domains.

Specific activity was determined by dividing the activity of purified scFv by the amount of purified scFv and relative specific activities were determined on the basis of the specific activity of scFv with V_L-linker-V_H.

antibodies, we measured their specific activities (*i.e.*, binding activity per protein). Fig. 3 shows the relative specific activities of the scFv antibodies, illustrating that the domain order does not affect the specific activity of anti-c-Met scFv as we had expected from the results in Fig. 1.

Diabody, a dimer of scFv, is one of the multimeric fragment antibodies intensively studied owing to its higher binding affinity to antigen than a monomeric scFv [1]. They can be easily constructed by simply engineering the linker length between V_H and V_L [1]. To investigate whether the order of variable domains of anti-c-Met antibody has influence on the productivity and specific activity of diabody format against c-Met, we constructed pET24ma-LHdiabody and pET24ma-HLdiabody, which respectively express anti-c-Met diabodies of V_L/V_H and V_H/V_L formats. Construction of the diabodies was designed by combining two variable domains by using a 5 amino acids linker (Gly_4Ser), which is a generally used sequence for the formation of diabodies [1]. For the construction of pET24ma-LHdiabody, the V_L and V_H genes of the anti-c-Met scFv gene were amplified using the respective 5' and 3' primers (5' primer for V_L: GAATTCGAGCTCGTGCTGACTCAGTCG; 3' primer for V_L: AGAGCCACCGC CACCGCCTGTGACGGTCAGCTG; 5' primer for V_H: GGTGGCGGTGGCTCTCAGG AGTC-GGTGAAGGAG; 3' primer for V_H: AAGCTTTTATTAG-ACTGATGGAGCCTT AGG), and the domains were fused and cloned into pET24ma. For the construction of pET24ma-HLdiabody, the V_H and V_L genes of the anti-c-Met scFv gene were amplified using the respective 5' and 3' primers (5' primer for V_H: GAATTCCAGGAGTCGGTGAAGGAG; 3' primer for V_H: AGAGCCACCGCCACCGACTGATGGAGCCT-TAGG; 5' primer for V_L: GGTGGCGGTGGCTCTGAGC-TCGTGCTGACTCAG; 3' primer for V_L: AAGCTTTTA-TTA GTGGTGGTGGTGGTGGCCTGTGACGGTC-AGCTG).

Figs. 4A, 4B, and 4C show the expression levels of produced diabodies, productivities of functional diabodies, and relative specific activities of the purified diabodies, respectively. In the experiments, induction of target proteins was done under the same condition used in Fig. 2. As shown in Fig. 4A, the amount of produced anti-c-Met diabody of V_H/V_L form was more than two times higher than that of V_L/V_H form both in the soluble fractions and insoluble fractions. The productivity of functional anti-c-Met diabody of V_H/V_L form was approximately three times higher than that of V_L/V_H form (Fig. 4B), which matches well with the results in Fig. 4A. Fig. 4C shows that the domain order does not affect the specific activity of anti-c-Met diabodies. These results indicate that the productivity of anti-c-Met diabody can also be improved by changing the order of variable domains without affecting the binding activities to antigen.

In this study, we have demonstrated that the order of variable domains is a crucial factor for the productivity of scFv or diabody against c-Met. The yield of scFv or diabody against c-Met was significantly enhanced by reversing

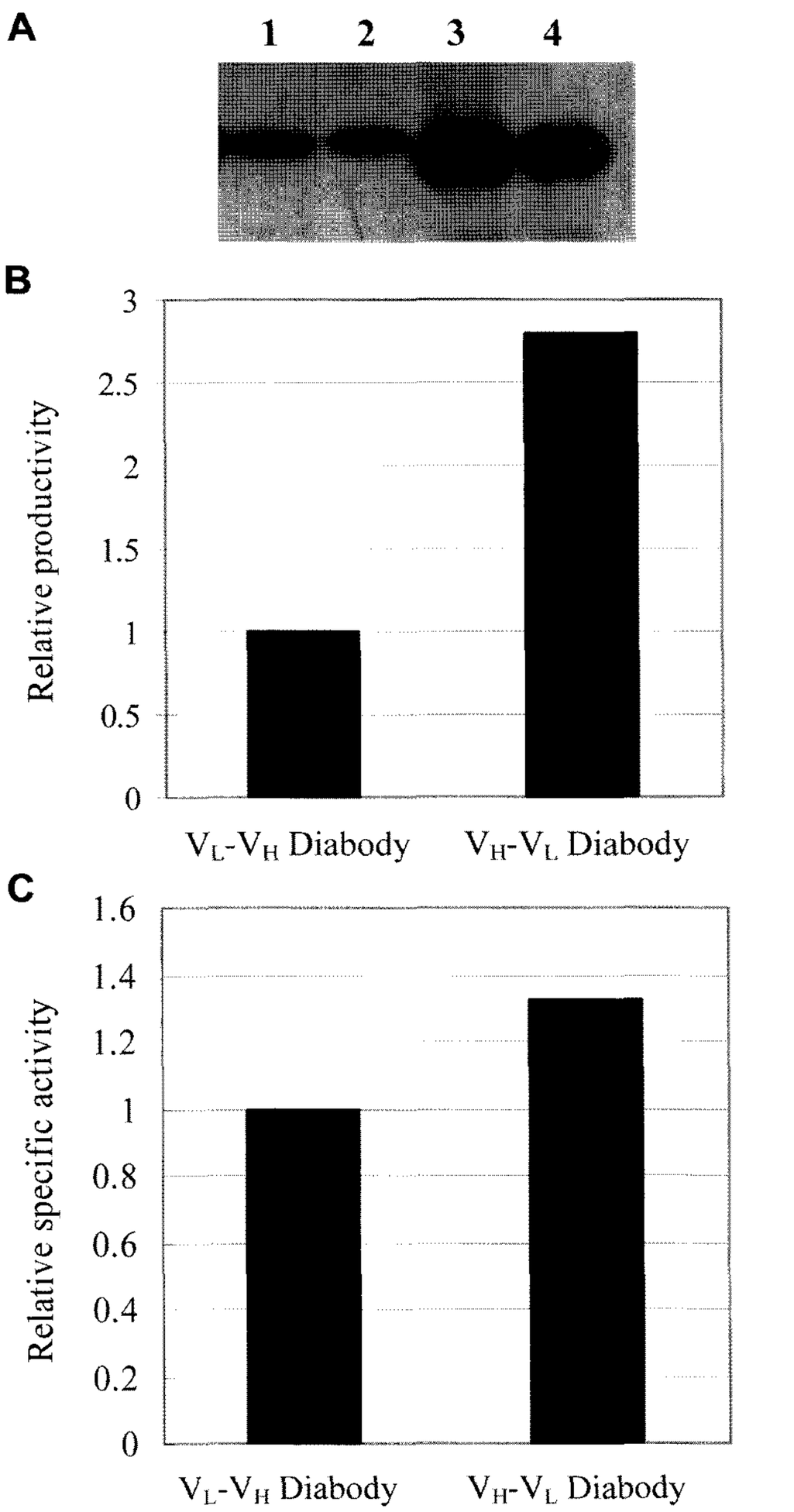

Fig. 4. Effect of domain order on the productivity and specific activity of anti-c-Met diabody.
A. Western blotting of soluble and insoluble fractions for the anti-c-Met diabodies with different domain orders expressed with DsbC. Lane 1, soluble fraction of V_L-linker-V_H diabody; lane 2, insoluble fraction of V_L-linker-V_H diabody; lane 3, soluble fraction of V_H-linker-V_L diabody; lane 4, insoluble fraction of V_H-linker-V_L diabody. **B**. Relative productivity of produced functional anti-c-Met diabodies with different domain orders in the soluble fraction. Productivity of functional diabody was determined by measuring the activity of diabody in the soluble fraction and relative productivities were determined on the basis of the productivity of functional diabody with V_L-linker-V_H. **C**. Relative specific activities of the purified anti-c-Met diabodies with different domains. Specific activity was determined by dividing the activity of purified diabody by the amount of purified diabody and relative specific activities were determined on the basis of the specific activity of diabody with V_L-linker-V_H.

the order of their variable domains from V_L/V_H to V_H/V_L without losing their specific activities. Our results would be helpful to design and produce the anti-c-Met fragment antibodies on a large scale, enhancing the applicability of the protein. The results of our study also indicate that the domain order of variable regions is one of the important factors to be considered in the design of fragment antibodies, as previously reported [5, 11, 14].

During this study, we have wondered why the domain order can dramatically affect the productivity of the fragment antibodies against c-Met. Among the papers that reported the effect of domain order on the yield of fragment antibodies, one paper suggested the possibility of ribonucleic cleavage of the target mRNA or mistranslation [14]. They could detect the fragmentations of scFv against lysozyme of V_H/V_L form when the target protein was expressed using the cell-free transcription/translation system, which was not observed in V_L/V_H form. We also attempted to express the anti-c-Met scFvs with different order of domains using the cell-free transcription/translation system, but we could not observe any fragmentations. In addition, we could not observe any differences of expression level of scFv between scFv of V_H/V_L and scFv of V_L/V_H in the cell-free experiment. These results suggest that the factors different between the *in vivo* expression system and the *in vitro* expression system may be involved in the different expression level of anti-c-Met scFv caused by the order of its variable domain. To understand more exactly how the exchange of anti-c-Met scFv variable domain gives rise to the different productivity of anti-c-Met scFv, further studies on the transcription rates, mRNA stabilities, translation rates, and protein stabilities of the scFv antibodies in the cytoplasm of *E. coli* should be performed.

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