

Figure 3. Results of DSC analysis in the absence and presence of biotin. The denaturation temperature was increased in the presence of biotin. (A) cSA WT, mutein 314, and mutein 414. (B) Mutein 083', which showed multiple peaks.

charge and functional moiety (carbamoyl group, $-\text{CONH}_2$) from those of the WT residue, glutamic acid (carboxyl group, $-\text{COOH}$). Our analysis of crystal structural data indicated that the loop and helix near E116 contacts two interfaces of the cSA tetramer (Fig. 5), and mutation of W120 on this helix to lysine was reported to cause dimerization of cSA.³⁰ Although the mutations E116N and E116Q apparently maintain the conformational structure of cSA, changes in electrostatic charge and functional moieties in this region may alter the thermodynamic stability and tetramerization of the resulting mutein. Therefore, our data regarding R84 and E116 strongly suggest that mutations that preserve the electrostatic charge of the original residues and their functional moieties for hydrogen bonds maintain the functions and structures of the proteins for therapeutic application.

Although the cSA muteins seemed to maintain the tetrameric structure of cSA, the T_{MS} of cSA muteins decreased in DSC analyses [Fig. 3(C)], and monomer bands appeared even at low sample-heating temperatures in SDS-PAGE analyses (Fig. 4 and Supporting Information Fig. S6) except for muteins 001 (R84K) and 091 (E116Q). This indicated that each of these mutations slightly decreased the thermal and chemical stabilities of the resulting muteins. However, the decreased tetramerization ability of mutein 083 (E116N only) at refolding was rescued by inducing additional mutations (mutein 030; Y83S, R84K, and E116N) (data not shown). The key for successful refolding is achieving a balance between structural collapse and flexibility of unfolded structures during treatment with denaturant.³¹ Our current results suggest that the various mutations we induced in cSA did not totally disrupt its tetrameric structure but instead likely “loosened” it. Although this looseness slightly decreases the thermal and chemical stabilities of the resulting

mutein, it may increase the flexibility of unfolded structures during exposure to denaturants and improve the refolding efficiency. The FFF-MALS analyses of muteins 314 and 414 indicated larger molecular radii in the absence of biotin (Supporting Information Fig. S3), in support of our hypothesis of increased structural looseness in our cSA muteins.

We constructed cSA muteins that had decreased immunoreactivity but that preserved the tetrameric structures and biotin-binding functions of the WT protein. Previous reports have suggested that human and murine immune systems recognize cSA through a conformational epitope.¹⁸ The mutations induced here were expected to maintain the higher-order structures of cSA; however, these mutations probably also changed the residual structures of side chains exposed on the protein surface. These changes might disrupt the conformation of the cSA epitope and thus achieve immune evasion. In this regard, a low-immunogenicity recombinant of staphylokinase (K35A, E65Q, K74R, D82A, S84A, T90A, E99D, T101S, E108A, K109A, K130T, K135R) had several similar mutations (underlined)¹⁶ to those of

Table III. Results of DSC Analysis

Mutein	T_M (°C)	
	Biotin (-)	Biotin (+)
WT	74.9	109.5
072	73.7	107.8
001	75.7	108.9
083	70.2	109.2
091	76.9	111.1
030	68.2	104.9
040	74.9	107.0
314	66.7	101.0
414	72.9	104.2

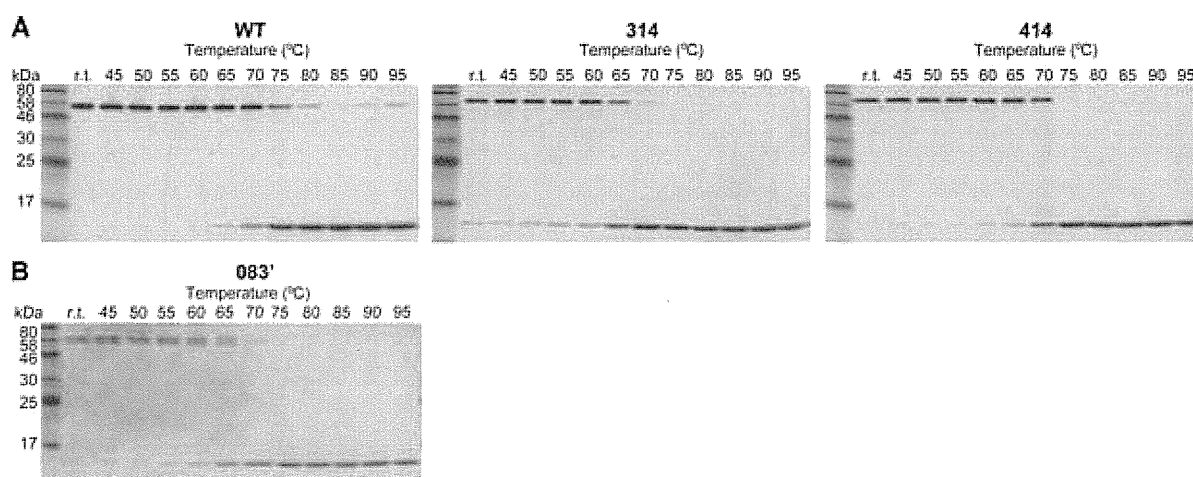


Figure 4. Analysis of stability by using SDS-PAGE. Each sample was prepared as a PBS solution and heated for 3 min at the indicated temperature. (A) cSA WT (left), mutein 314 (middle), and mutein 414 (right). (B) Mutein 083' showed multiple bands at low temperatures.

our hypoinmunogenic cSA muteins. In contrast, the single mutations E116N and E116Q (muteins 083 and 091) increased the immune response to these cSAs (Table I), perhaps due to unexpected effects of structural or thermodynamic changes.

The increased immunoreactivities of muteins 083 and 091 were ameliorated by inducing additional mutations (those in muteins 030, 040, 314, and 414) (Table I). This outcome indicates that looseness of the tetrameric structure might also contribute to increasing immune evasion. This looseness we allude to may generate mobility in the epitope and increase the energy loss associated with antibody binding to prevent immune recognition. Furthermore, the looseness may also contribute to the immunoreactivity correlated to T-cell epitope. It is reported that the high resistance to the lysosomal proteolysis controlled the amount of T-cell epitope and favored its presentation to antigen-presenting cells for longer period, resulting the enhanced immunogenicity of the antigen.^{32–34} The decreased stability of cSAs we discussed here may weaken the immunoreactivity of T-cells. However, in this view point, the immunogenicity *in vivo* animal models should be considered, because cSAs become substantially stable in the presence of biotin.

The current study showed that all of our muteins maintained the tetrameric structure and functions of cSA WT. We propose that mutein 314 and 414 are the candidates for therapeutic applications. Furthermore, in at least cSAs, our findings also might suggest a new strategy for constructing hypoinmunogenic muteins, in which mutations are induced at charged or aromatic residues on the surface of the target protein; the mutations induced should preserve the electrostatic charge and functional moieties of the WT residues. Consequently, these mutations maintain high-order structures and

functions while modifying the residual structures on the surface and generating structural looseness in the resulting protein that leads to immune evasion. Viruses such as picornaviruses are known to escape antibody recognition through substitutions of surface-exposed residues;³⁵ this scenario may be somewhat analogous to the strategy we propose here. Unlike previous strategies such as the construction of chimeric antibodies,¹⁵ our current design does not require knowledge of the human counterpart to a target protein. For therapeutics including pretargeting method, we expect that this design will be applied to the other biotin-binding protein such as rhizavidin, the dimeric avidin,³⁶ or other various proteins, and that the immunogenicity of those designed proteins will be evaluated using animal models.

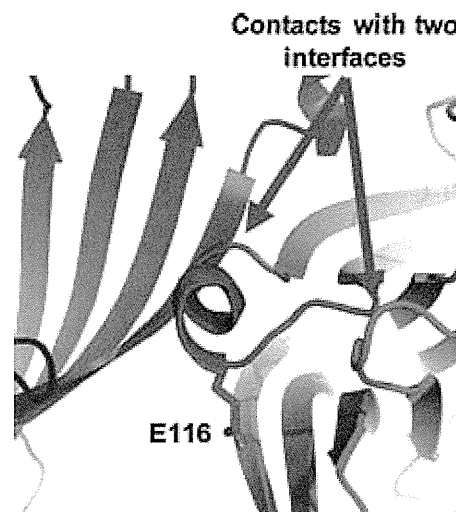


Figure 5. The loop and helix (magenta) near cSA E116 (from Ref. 20) on the interfaces of the tetramer (blue and cyan).

To date, site-directed mutageneses have been performed only to eliminate B-cell epitopes mainly by using alanine,^{16,17} and there are no reliable methodologic guidelines for retaining function and structure in the mutated protein. Our current strategy warrants confirmation through its application to other nonhuman proteins with therapeutic potential.

Materials and Methods

Materials

pET28b vector was obtained from Novagen (Darmstadt, Germany). Tetramethylethylenediamine, bromophenol blue, and 30% (w/v)-acrylamide/bis (37.5:1) mixed Solution was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals including biotin were purchased from Wako (Osaka, Japan). Primers were synthesized by Operon (Tokyo, Japan).

Cloning, expression, and purification of recombinant proteins

cSA (residues 13–139 of streptavidin) WT and its muteins were cloned into pET28b vector by using forward primer 5'-GCGGAAGCTGGTATCACTG-3' and reverse primer 5'-GCTAGCAGCAGAAGGCTT AAC-3'. A His₆-tag was fused to the C-terminus. *E. coli* Rosetta2 (DE3) cells were transformed with cSA plasmids (WT and muteins) and grown at 28 °C in 2 × YT Broth containing 50 µg mL⁻¹ kanamycin. Expression was induced with 1.0 mM Isopropyl β-D-1-thiogalactopyranoside at O.D.₆₀₀ = 0.8 and then the cells were cultured at 37 °C. Cells were harvested 18 h later by centrifugation (7000 × *g*, 10 min, 4 °C) and suspended in a buffer comprising 500 mM NaCl and 20 mM Tris-HCl (pH 8.0 at 4 °C). The suspension was sonicated (model UD-201, TOMY, Tokyo, Japan) followed by centrifugation (6000 × *g*, 30 min, 4 °C). The pellet containing inclusion bodies was washed with wash buffer (2% Triton X-100 and 50 mM Tris-HCl pH 8.0 at 4 °C), acetone, and water purified by reverse osmosis. The pellet then was suspended in buffer A (6 M guanidinium HCl, 500 mM NaCl, 20 mM Tris-HCl; pH 7.9 at 4 °C) containing 5 mM imidazole, followed by shaking overnight at 4 °C. The supernatant was collected by centrifugation (40,000 × *g*, 30 min, 4 °C) and loaded onto a Ni-NTA resin (Qiagen, Valencia, CA) equilibrated with buffer A containing 5 mM imidazole. Then, the resin was washed with buffer A containing 5 mM imidazole and then buffer A containing 30 mM imidazole. cSA (WT and muteins) were eluted by using buffer A containing 50 mM imidazole and buffer A containing 300 mM imidazole. Eluates were dialyzed against buffer B (200 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA; pH 8.0 at 4 °C) containing 6 M guanidinium HCl for 12 h followed by dialyses against buffer B containing 3 M guanidi-

nium HCl and 2 M guanidinium HCl for 6 h each and buffer B with 0.4 M L-arginine containing 1 M guanidinium HCl and 0.5 M guanidinium HCl for 12 h each. Then the dialysate was dialyzed three times against buffer B for more than 4 h each, followed by centrifugation (6000 × *g*, 30 min, 4 °C) and filtration over Millex-GP (0.22 µm, PES, 33 mm, sterile; Millipore, Bedford, MA). The supernatant was concentrated by using Amicon Ultra-15 (Millipore, Bedford, MA) and loaded onto a 26/60 Superdex column (GE Healthcare, Piscataway, NJ) equilibrated with buffer B. Eluate fractions were analyzed by using SDS-PAGE, pooled and stored at 4 °C.

FFF-MALS analyses of cSAs

FFF-MALS analyses for the estimation of protein size and weight were done in a Wyatt Eclipse separation system (Wyatt Technology, Santa Barbara, CA). This instrument was equipped with refractive index and MALS detectors. Protein samples were concentrated to 1.8 mg mL⁻¹ and supplemented with excess biotin, when needed. The focused flow rate was 3 mL min⁻¹. Separation was performed by using a cellulose membrane with a nominal molecular weight cutoff of 5 kDa. At the elution step, the channel flow was kept at a constant rate of 1 mL min⁻¹ and the cross-flow decreased linearly from 3 to 0 mL min⁻¹ in 15 min. Buffer B was used for these analyses. The eluted peaks were detected with a refractive index detector, and the molecular weights of these peaks were calculated by using MALS measurement. Data were analyzed with the program ASTRA ver. 5.3 (Wyatt Technology, Santa Barbara, CA) to determine molecular weights.

ITC analyses of cSAs

The thermodynamics of biotin binding were measured by using a iTC₂₀₀ instrument (MicroCal, Northampton, MA). Purified cSAs were dialyzed against PBS overnight. Biotin was dissolved in the same buffer as that of the cSAs to obtain a 500 µM stock solution. This stock solution was adjusted to a concentration tenfold higher than that of each mutein tested. In a calorimeter cell, 9–20 µM cSAs was titrated with biotin solution at 25 °C. Thermography data were analyzed by using the software package ORIGIN (Light Stone, Tokyo, Japan), and titration curves were fitted to a one-site binding isotherm.

DSC analyses of cSAs

Heat capacity curves were measured by using a VP-DSC ultrasensitive scanning calorimeter (MicroCal, Northampton, MA). The heating rate was 1 K min⁻¹. The sample cell was filled with approximately 0.4 mL of PBS containing 20–50 µM cSA or its muteins. When necessary, samples were supplemented with excess biotin. The data were analyzed by using the software package ORIGIN. The buffer baseline was

subtracted from the raw data, which were normalized by protein concentration to obtain thermodynamic parameters and then fitted to two-state thermal transition models.

SDS-PAGE analyses of cSAs

SDS-PAGE analyses were performed as described previously with slight modification.^{28,29} Samples of cSAs were prepared as 6 μL aliquots of 9–12 μM PBS solutions and then heated for 3 min at the indicated temperature by using a GeneAmp 9700 PCR system (Applied Biosystems, Tokyo, Japan). Immediately thereafter, 12 μL of sample buffer (80 mM SDS, 62.5 mM Tris-HCl, 8% glycerol; pH 6.8 at room temperature) and 2 μL of 1 mg mL^{-1} bromophenol blue solution containing 10% glycerol were added to the heated samples. The samples were placed on ice and then loaded onto a 15% polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at 150 V for 80 min. Gels were stained with 0.5% Coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid for more than 10 min and destained with water.

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Effects of subclass change on the structural stability of chimeric, humanized, and human antibodies under thermal stress

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Abstract: To address how changes in the subclass of antibody molecules affect their thermodynamic stability, we prepared three types of four monoclonal antibody molecules (chimeric, humanized, and human) and analyzed their structural stability under thermal stress by using size-exclusion chromatography, differential scanning calorimetry (DSC), circular dichroism (CD), and differential scanning fluoroscopy (DSF) with SYPRO Orange as a dye probe. All four molecules showed the same trend in change of structural stability; the order of the total amount of aggregates was IgG1 < IgG2 < IgG4. We thus successfully cross-validated the effects of subclass change on the structural stability of antibodies under thermal stress by using four methods. The T_h values obtained with DSF were well correlated with the onset temperatures obtained with DSC and CD, suggesting that structural perturbation of the CH2 region could be monitored by using DSF. Our results suggested that variable domains dominated changes in structural stability and that the physicochemical properties of the constant regions of IgG were not altered, regardless of the variable regions fused.

Keywords: antibody; circular dichroism; differential scanning calorimetry; differential scanning fluoroscopy; subclass change; thermodynamic stability

Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; DSF, differential scanning fluoroscopy; DTT, dithiothreitol; HMWS, high-molecular-weight species; LMWS, low-molecular-weight species; MRW, mean residue weight; SEC, size-exclusion chromatography; VH, variable regions of heavy chains; VL, variable regions of light chains.

Additional Supporting Information may be found in the online version of this article.

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Introduction

Monoclonal antibodies (mAbs) are very versatile reagents and are under extensive development for therapeutic use. To date, more than 30 therapeutic antibodies have been approved for clinical use and more than 240 molecules are under development.¹⁻³

Antibodies are heterodimers composed of two heavy chains and two light chains, which are linked through disulfide bonds. As classified on the basis of the constant region of the heavy chain there are five classes of antibody, namely IgA, IgD, IgE, IgG, and IgM. The constant heavy regions of IgA, IgD, and IgG have three IgG domains and a hinge region to

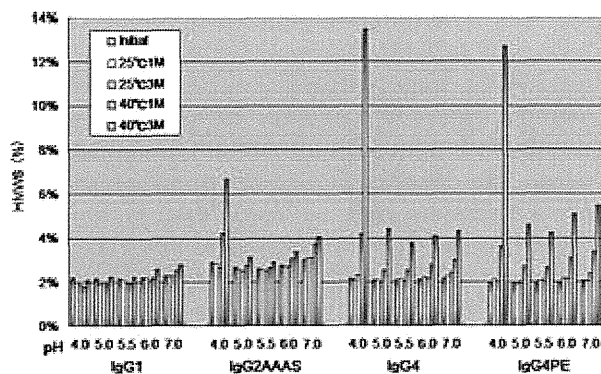


Figure 1. Size-exclusion high-performance liquid chromatographic analysis of percentages of HMWS as a function of pH after storage at 25 or 40°C for 1 or 3 months.

give flexibility. Despite this range of choice of antibody classes to select from in the development of therapeutic antibodies, all recombinant antibodies under development to date have belonged to the IgG class because it has the highest serum half-life among all the classes.^{4,5}

Four subclasses of IgG antibody have been identified, and each subclass has, in principle, highly homologous constant region sequences. However, the abilities of the IgGs in the four subclasses to trigger effector functions differ distinctly. For therapeutic applications the subclass needs to be carefully chosen; to achieve the desired therapeutic effects and avoid side effects, subclass selection is primarily dictated by the effector functions needed. IgG1 and IgG3 have greater ability to activate antibody-dependent cellular cytotoxicity and complement-dependent cellular cytotoxicity than do IgG2 and IgG4.^{6–8} If the effector function is required to eliminate targets (e.g., to destroy tumor cells in oncology applications), one would expect IgG1 or IgG3 to be used. However, in practice, the IgG3 subclass has not been used as a therapeutic candidate owing to its short half-life and long hinge region, which makes it susceptible to proteolysis, along with its allotypic polymorphism.⁹ Most of the therapeutic antibodies approved to date have belonged to the IgG1 subclass; only a few IgG2 and IgG4 antibodies have reached the market.³ Nevertheless, subclasses IgG2 and IgG4 are increasingly being developed as therapeutic antibodies because of their blocking or inhibitory functions.⁹ Selection of antibody subclass is therefore considered increasingly critical in the development of therapeutic antibodies.⁹

Products have been developed that have reduced effector functions compared with those seen with the IgG1 subclass.^{10–12} IgG2 and IgG4 have unique physicochemical properties. Human IgG2 antibodies can form covalent dimers and structural isoforms via disulfide shuffling *in vivo*.^{13–17} IgG4 antibodies can form half-antibodies (i.e., those with one heavy

chain and one light chain) that lack inter-heavy chain disulfide bonds and form intra-chain disulfide bonds. Recent studies have shown that a half-antibody can exchange with another IgG4 half-antibody, resulting in the production of a bispecific antibody.^{18–20} Moreover, IgG2 and IgG4 are unstable in terms of tendency to aggregate.^{21,22} Among the physicochemical properties of antibodies, homogeneity and stability are particularly critical issues limiting the successful development of therapeutic antibodies.

Here, we focus on the effects of changes in IgG subclass on the conformational and physicochemical stability of antibodies. We prepared three types of four antibody molecules, namely chimeric, humanized, and human. From our results, we concluded that changes in variable domains dominate changes in thermodynamic stability and that the trends in thermodynamic stability caused by changes due to subclass switching are in principle identical among antibody molecules.

Results

Size-exclusion chromatography and SDS-PAGE

To compare differences in aggregation and degradation tendency among subclasses, we investigated aggregation and degradation behavior in the pH range of 4.0–7.0 at 25°C and 40°C. Samples taken at different incubation times were analyzed by using size-exclusion chromatography (SEC).

We tried to construct and analyze subclass-substituted molecules of mAb-A, mAb-B, mAb-C, and mAb-D. Here, we first give the results for mAb-C, followed by those of the other antibody molecules.

Supporting Information Figures 1 to 1–6 give representative SEC chromatograms of mAb-C (chromatographic UV profile) for various glutamate formulations as a function of pH. The intact non-aggregated antibody (monomer) was eluted at approximately 31.5 min. Soluble aggregate (high-molecular-weight species, HMWS) was eluted earlier, and degraded species (low-molecular-weight species, LMWS) were eluted later. The total amounts of degraded species were enhanced with increasing incubation time at 40°C.

All subclasses were most stable at pH 5.5, and the total HMWS content was enhanced with increasing pH in the range 5.5–7.0. In the case of IgG1, the lower pH samples exhibited greater physical stability, as evidenced by less aggregation. In contrast, IgG2AAAS, IgG4, and IgG4PE showed greater aggregation at lower pH (pH 4.0 and 5.5).

We examined the percentages of HMWS, as revealed by SEC analysis as a function of pH after storage at 25°C or 40°C for 1 or 3 months (Fig. 1). IgG1 showed no increase in HMWS content over time at pH 4.0 at 40°C from time zero (Initial) to 3

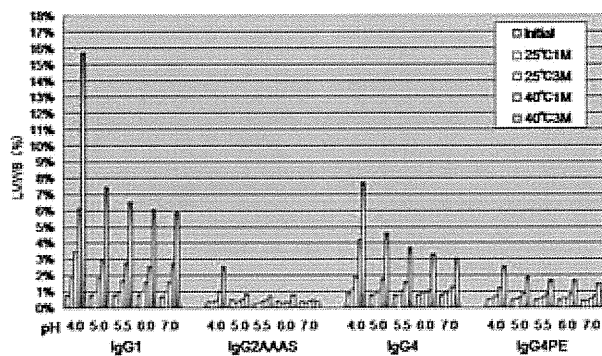


Figure 2. Size-exclusion high performance liquid chromatographic analysis of percentages of LMWS as a function of pH after storage at 25 or and 40°C for 1 or 3 months.

months. In contrast, IgG2 (IgG2AAAS) and IgG4 (IgG4PE) showed an increase in HMWS content over time at 40°C during the 3-month period at all pH values. The rate of aggregation was strongly dependent on the antibody subclass (IgG1 vs. IgG2 vs. IgG4). At 40°C, all IgG1s were resistant to low-pH aggregation, but the IgG2s and IgG4s were aggregated.

We examined the percentages of LMWS, as revealed by SEC analysis as a function of pH after storage at 25°C or 40°C for 1 or 3 months (Fig. 2). All subclasses were most stable at pH 5.5–6.0 and showed decreasing LMWS content with increasing pH in the range 4.0–6.0. IgG1 samples exhibited more LMWS than did IgG2 and IgG4 samples, revealing the lower chemical stability of IgG1. The amount of LMWS was highest at pH 4.0 for each subclass, although to differing extents. Reducing and non-reducing SDS-PAGE showed a high-molecular-weight ladder (data not shown); this was consistent with the heterogeneity observed within the aggregates upon SEC.

The same trend was observed for other antibodies: examples for mAb-D are given in Supporting Information Figure 1 (1-7 to 1-11).

Differential scanning calorimetry

To determine whether antibodies of different subclasses exhibited different thermodynamic stabilities, we obtained differential scanning calorimetry (DSC) thermograms for antibodies of three different subclasses, including mutations that had the same variable region. We constructed and analyzed subclass-substituted molecules of mAb-A, mAb-B, mAb-C, and mAb-D. Here, we first describe the results for mAb-C, followed by a discussion of the other mutants.

We examined the profiles of temperature-induced unfolding of all subclasses of mAb-C (IgG1, IgG2AAAS, IgG4, IgG4PE) under the same solvent conditions (Fig. 3). Table 1 lists the observed thermal unfolding midpoint (T_m) values for mAb-C. In

the case of mAb-C, we prepared IgG1, IgG2AAAS, IgG4, and IgG4PE. Thermal unfolding of mAb-C G1 resulted in three partially overlapping transitions with T_m values of about 73, 78, and 84°C, respectively, at pH 5.5 under optimized formulation conditions. The results showed good agreement with previous results from an IgG1 Mab, in which the three transitions were assigned to unfolding of the CH2 domain, the antigen-binding fragment (Fab), and the CH3 domain.^{23,24} For mAb-C G2AAAS, G4, and G4PE, the first transition shifted to the low temperature side compared with that for IgG1. The change in the number of transitions in members of the IgG subclass with the same variable regions of heavy and light chains (VH/VL region) suggests that independent unfolding of the CH2, CH3, and Fab domains is a general property of IgGs.

The apparent Fab T_m values of mAb-C in the IgG1 and IgG2AAAS formats were similar to each other ($\Delta T_m < 0.3^\circ\text{C}$), whereas the apparent Fab T_m values in the IgG4 and IgG4PE formats were about 1°C different from the Fab T_m values of mAb-C in the IgG1 and IgG2AAAS formats. Among the four antibodies with the same variable domains and different subclasses, the T_m values of the Fab fragments differed in the order of $\text{IgG1} \geq \text{IgG2} \geq \text{IgG4}$ and IgG4PE.

The same trend was observed for the other antibodies, namely mAb-A, mAb-B, and mAb-D: a list of observed T_m values is given in Supporting Information Tables 1–III and Supporting Information Figure 2. Average values of T_m for the four antibodies were calculated and plotted against pH (Fig. 4). The CH1 and Fc sequences of the IgG subclasses have some amino acid differences and different disulfide-bonding patterns (data not shown); this might suggest why the effects on the profiles of antibodies with different variable domains were identical.

Changing pH had marked effects on the DSC profiles of each antibody molecule reported here (Table 1, Supporting Information Tables 1–III). A decrease in pH resulted in broader endotherms that occurred at lower temperatures. Notably, the CH2

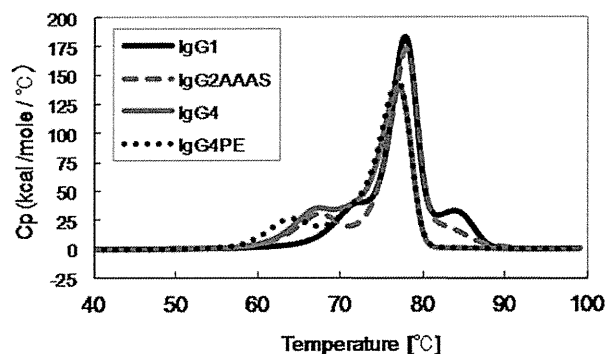


Figure 3. Temperatures inducing melting (unfolding) of all subclasses of mAb-C at pH 5.5, as measured by using DSC.

Table 1. Differential scanning calorimetry measurements of melting transition of mAb-C in all subclasses as a function of pH.

Subclass	domain	pH	T _m (°C)				
			4.0	5.0	5.5	6.0	7.0
G1	CH2		60.10	69.41	72.55	73.23	73.72
	Fab		73.91	77.23	77.87	78.05	78.06
	CH3		79.07	83.55	83.88	84.05	84.02
G2AAAS	CH2		52.78	64.55	67.73	69.24	70.50
	Fab		72.58	77.19	78.04	77.58	77.99
	CH3		NA	NA	NA	NA	NA
G4	CH2		54.76	64.07	67.40	69.04	69.90
	Fab		71.07	76.55	77.05	76.36	76.06
	CH3		NA	NA	NA	NA	NA
G4PE	CH2		53.77	61.42	64.08	65.79	67.74
	Fab		71.25	76.55	76.90	76.27	75.56
	CH3		NA	NA	NA	NA	NA

NA, not available; melting transition was not detected or well defined.

domain displayed greater pH dependence of thermal stability than did the CH3 domain; ΔT_m was larger for IgG4 than for IgG1 and IgG2.

Previous characterization of the Fc fragment has identified an early transition due to unfolding of the CH2 domain followed by thermal unfolding of the CH3 domain.²⁵ Previous studies indicate that the CH3 region of IgG1 has an extremely high T_m compared with other domains.²⁵ Of the three human IgG subclasses (IgG1, IgG2, and IgG4), IgG1 has the most stable Fc, based on the T_m of its CH2 and CH3 domain.²⁴ This subclass dependence is correlated with reduced thermodynamic stability of IgG2 and IgG4 CH2 relative to IgG1 CH2. Regardless of subclass, we found that thermodynamic stability of CH2 was an important determinant of the stability and aggregation of Fc and intact antibody molecules under acidic conditions. Some of these findings have already been reported.^{26,27} Notably, also, the unfolding transitions of the CH2 and CH3 domains for all four IgG1 constructs were identical as described in

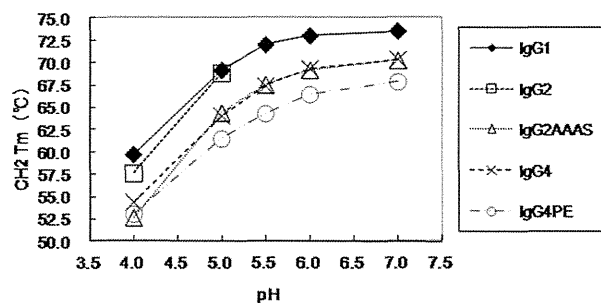


Figure 4. DSC measurements of CH2-domain melting transition of antibodies as a function of pH. IgG1, 4 (mAb-A, B, C, D); IgG2, 2 (mAb-A, -B); IgG2AAAS, 2 (mAb-C, -D); IgG4, 2 (mAb-C, -D); IgG4PE, 4 (mAb-A, -B, -C, -D).

the results. This trend was also observed for the other subclasses, namely IgG2, IgG2AAAS, IgG4, and IgG4PE; in contrast, the Fab unfolding transitions were highly variable.

Circular dichroism

We used far-UV circular dichroism (CD) to compare the secondary structures of the subclasses (Supporting Information Figs. 3-1 to 3-3). First, we give the results for mAb-C, followed by a discussion of those for the other antibody molecules.

The CD spectra of all subclasses of mAb-C before and after heating are shown in Supporting Information Figure 3-1. The subclasses showed little difference in terms of far-UV CD before heating (Supporting Information Fig. 3-1a), regardless of pH (data not shown). The spectra were characterized by a single negative peak with a minimum at a wavelength of 217 nm, suggestive of the expected β -sheet structure of the immunoglobulin fold.²⁸ These observations suggested that subclass changes did not alter the overall secondary structure.

To probe the thermal stability, we monitored the signal at 217 nm from 25 to 100°C and compared the thermal stabilities of the subclasses (Fig. 5). In all of the ellipticity-temperature profiles there were two steps at which the intensity decreased, suggesting that there were two distinct temperatures at which changes in secondary structure occurred. At pH 4.0, the antibody had a slightly lower transition-onset temperature than at other pH values. The transition-onset temperatures of the antibody at pH 5.0 and 6.0 were comparable, and the ellipticity did not change until the temperature reached approximately 60°C, whereas a much lower onset temperature was observed at pH 4.0. At pH 4.0, the major

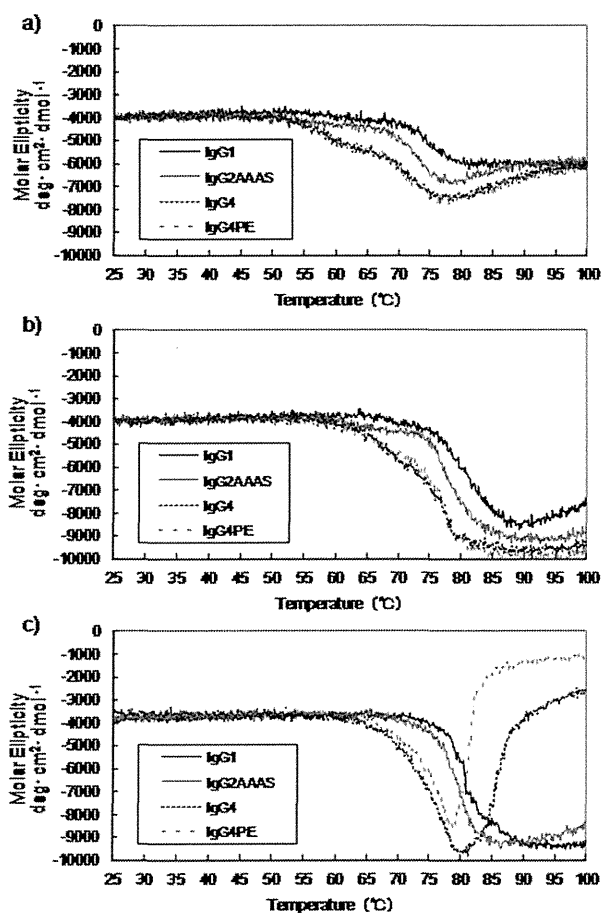


Figure 5. Effect of temperature and pH on molar ellipticity at a wavelength of 217 nm (a, pH 4.0; b, pH 5.5; c, pH 6.0) in the case of mAb-C.

changes occurred at 55°C, followed by a second step at about 70°C, in all subclasses. The transition-onset temperatures were in the order of $IgG4 < IgG2 < IgG1$. Furthermore, in the case of the IgG4 formats, precipitation was observed after heating at pH 6.0. The temperature values corresponded well to the T_m values observed for the structural transitions in the DSC experiments. IgG4 has the lowest thermal stability among the subclasses reported here.

Next, we obtained CD spectra of different subclasses of the other three antibodies, mAb-A, mAb-B, and mAb-D; the results for mAb-A and mAb-D are shown in Supporting Information Figures 3-2, 3-3 and 4-1, 4-2, respectively. The same trends as with mAb-C were observed for these antibodies.

Differential scanning fluoroscopy

The typical differential scanning fluoroscopy (DSF) profile of an IgG molecule consists of a sharp sigmoid-like increase to the maximum level, followed by a decrease in fluorescence intensity. This profile represents changes in the environment of the dye. The initial increase in fluorescence most likely results from exposure of the dye to the hydrophobic

area of the protein during thermal unfolding; the degree of exposure depends on the rate of transition from the folded to the unfolded state. Fluorescence quenching at increased solution temperature—a nonspecific transition—also occurs and contributes to the overall sigmoidal shape.^{29–31} Here, we used SYPRO Orange as a probe in the DSF system.

We investigated four mAb molecules: mAb-A and mAb-B (IgG1, IgG2, IgG4PE) and mAb-C and mAb-D (IgG1, IgG2AAAS, IgG4, IgG4PE). We used a variety of formulation pH values to generate DSF profiles for each mAb's subclasses.

Here, we give the results for mAb-C as an example. The fluorescence profiles and temperatures of hydrophobic exposure (T_h) of each subclass-changed IgG are shown in Supporting Information Figures 5-1 to 5-5 and Table II, respectively. Comparison of fluorescence profiles at pH 5.5 revealed that the fluorescence intensity increased at about 55°C; after two-step transitions it then decreased at about 80°C. These findings were similar to the results reported previously.^{29,30} The changes in intensity started in the order of IgG4PE, IgG4, IgG2AAAS, and IgG1. IgG1 had the highest T_{h1} value, followed by IgG2AAAS, IgG4, and IgG4PE. The order was almost the same in the case of the T_{h2} values.

Next, we examined the effects of pH on changes in T_{h1} and T_{h2} values. In all subclass-changed IgGs the T_{h1} and T_{h2} values were highest at neutral pH; a decrease in pH led to a decrease in values. Notably, our DSF analyses showed that mutations into IgG2 and IgG4 led to decreases in T_h values, and that the T_{h1} value of IgG2AAAS at pH 4.0 was lower than that of IgG4. These results are in good accordance with those obtained from DSC. The trends were similar for the other mAbs.

Discussion

Here, we reported the effects of changes in antibody subclass on stability in terms of molecule conservation and on structural stability. We focused on four antibody molecules (chimeric, humanized, and human), and three subtypes were constructed for each of the four antibodies. We substituted another subclass for the constant region of each antibody. These subclass-changed molecules had the same variable domains but different constant regions; we could therefore evaluate how subclass affected the physicochemical properties of IgG.

The results of SEC and SDS-PAGE revealed clear differences in the physical and chemical degradation of various IgGs. IgG1s were more susceptible to fragmentation, whereas IgG2s and IgG4s were more susceptible than IgG1 to aggregation at low pH. Degradation of human IgG1 at low pH originates from non-enzymatic digestion of the upper hinge region (i.e., EPKSCDKTHT; digested site is

Table II. Temperature of Hydrophobic Exposure (T_h) Values of Antibodies Obtained From Differential Scanning Fluoroscopy Measurements, as a Function of pH

Subclass		T_m ($^{\circ}\text{C}$)					
		pH	4.0	5.0	5.5	6.0	7.0
Mab-A							
G1	Th1		63	68	69.5	70	70.5
	Th2		73	74.5	74.5	NA	NA
G2	Th1		62.5	68	69.5	70	70.5
	Th2		71.5	73	NA	NA	NA
G4PE	Th1		56.5	60	61	61.5	62
	Th2		67.5	69.5	70	70	70
Mab-B							
G1							
Mab-C	Th1		62.5	67.5	69	69.5	70
	Th2		74	75	75	74.5	74
G2	Th1		61.5	67	69	70	70.5
	Th2		73.5	74.5	74.5	74.5	NA
G4PE	Th1		56.5	60	60.5	61	62
	Th2		73	74	74	73.5	73.5
Mab-C							
G1	Th1		63.5	68	69.5	70	70.5
	Th2		76.5	78	78	78	78
G2AAAS	Th1		58.5	63.5	65	66	66
	Th2		74.5	76.5	77	77.5	77.5
G4	Th1		59	62.5	64	65	65.5
	Th2		74	75.5	76	76	75.5
G4PE	Th1		56.5	60.5	61.5	62	62.5
	Th2		74	75.5	75.5	75.5	75.5
Mab-D							
G1	Th1		63	68	69	70	70
	Th2		79	81	81.5	82	82.5
G2AAAS	Th1		58	63	65	65.5	66
	Th2		75	82.5	82.5	83	83
G4	Th1		58	62.5	64.5	65.5	65.5
	Th2		78	81	81	81.5	81.5
G4PE	Th1		56.5	60.5	61.5	62.5	63
	Th2		78.5	81	81.5	81.5	81.5

NA, not available; melting transition was not detected or well defined.

T_m , thermal unfolding midpoint.

underlined)³²⁻³⁵; these earlier findings are in good agreement with our results. It is plausible to assume that human IgG2 is more resistant than IgG1 to non-enzymatic proteolysis for the following reasons: (1) it is shorter at the hinge region; (2) the hinge region has a more rigid structure owing to the presence of four S-S bonds; and (3) the amino acid sequence of human IgG2 is distinctly different from

that of human IgG1. Aggregate formation increased with decreasing pH, and the order of the total amounts of aggregates within the pH range 4.0 to 7.0 was IgG1 < IgG2 < IgG4. IgG4 was unstable in the acidic pH range. Our results are consistent with those described previously.^{21,26,27,36,37} Moreover, in these previous studies the pH-dependent effects of subclass changes on the physicochemical stability of

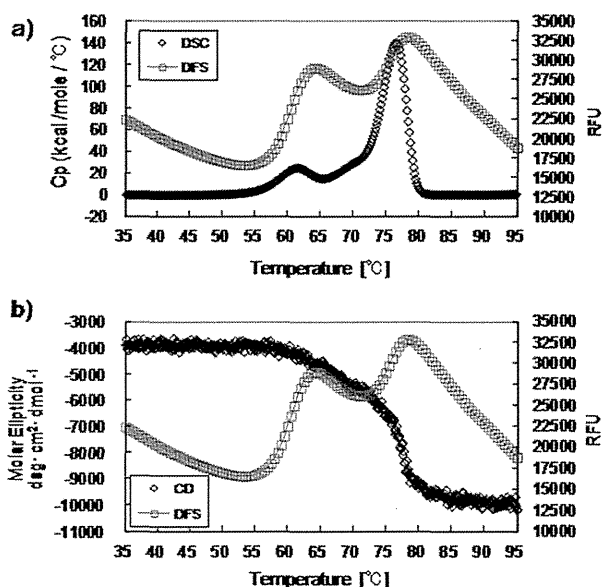


Figure 6. Comparison of thermal unfoldings of mAb-C IgG4PE at pH 5.0, as monitored by (a) DSC and DSF, and (b) CD and DSF.

antibodies were almost identical to the changes produced by each antibody variable domain reported here.

Development of antibody formulations with increased concentrations is desirable in biotherapeutics³⁸, however, aggregation capacity should be carefully evaluated in the case of high-concentration formulations. It has been suggested that aggregates of antibodies might be correlated with immunogenicity.^{39,40} The choice of subclass might determine the aggregation tendency of the antibodies to be constructed.

We compared the thermal unfolding of each subclass-changed mAb-C, as monitored by CD, DSC, and DSF (Fig. 6). The DSC profile for subclass-changed mAb-C showed two well-separated transitions. For multi-domain proteins such as antibodies, multiple DSC transitions usually suggest reduced inter-domain interactions. Similar to DSC, DSF was able to distinguish two separate transitions in the thermal unfolding of mAb-C. At several test pHs, the inflection point of the DSF curve corresponded to the peak of the DSC curve and the CD thermogram, with only a small onset. The small onset was expected, because DSF monitors the exposure of hydrophobic residues, whereas DSC measures the change in excess heat capacity changes (ΔC_p) and CD measures the change in secondary structure. The T_h value derived from DSF was, in principle, well correlated with the onset temperature value obtained from DSC or CD. For the other three mAbs, we observed profiles identical to those of mAb-C. We therefore proposed the T_h value obtained from DSF as a parameter for evaluating the thermodynamic stability of antibodies.

DSC is one of the methods most commonly used to characterize conformational stability on the basis of differences in thermal stability.^{31,41} We compared the normalized DSC thermograms of IgG molecules and DSF data (Supporting Information Fig. 6-1 to 6-3). The T_h obtained with DSF was slightly lower than the first T_m determined by DSC under all experimental conditions. At pH 4.0, the DSF profile (Supporting Information Fig. 6) seemed similar to the transition corresponding to that of the CH2 domain, which had the lowest melting temperature for these mAb molecules, as determined by DSC. The CH2 domain was more sensitive to pH changes than the other IgG domains; this was consistent with results reported previously.⁴² We plotted the correlation between T_h and the CH2 unfolding temperature T_m for different molecules (Fig. 7). A linear relationship between T_h and CH2 unfolding temperature T_m was observed. The transition of the Fab domain did not vary at this pH, as analyzed by both DSC and DSF (data not shown). At higher pH values the transition of the CH2 domain overlapped with that of the Fab domain; on the other hand, Fab domain transition did not change under the pH range tested.

Direct comparison of DSF and DSC demonstrated that both methods detected the same trends in thermostability at different pH values and the same relative stabilities of the domain. T_h was consistently lower than T_m determined by DSC. We successfully cross-validated the effects of subclass changes on the structural stability of antibodies under thermal stress by using four methods. The T_h values for the CH2 domain obtained by DSF were well correlated with the onset temperatures obtained by DSC and CD, suggesting that structural perturbation of the CH2 region can be monitored by using DSF. Our evaluation of the structural stability

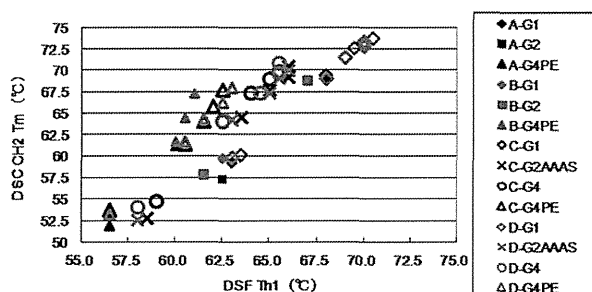


Figure 7. Correlation between DSF T_{h1} (temperature of hydrophobic exposure 1) and differential scanning calorimetry (DSC) CH2 T_m (thermal unfolding midpoint) values. DSF T_{h1} values were determined as the midpoint of the first fluorescence transition in DSF profiles by using the first derivative curves. DSC CH2 T_m values were determined as the lowest melting temperature in DSC thermograms. Linear regression yielded a linearity correlation coefficient of $r^2 = 0.9822$ – 0.9975 .

of subclass-changed antibodies suggested that the Fc region of each subclass dominates the physicochemical characteristics of the molecules. All four molecules showed the same trend in change in structural stability, in the order of IgG1 > IgG2 > IgG4. Taken together, our findings suggest that the state of the CH2 region—the most unstable one in the domains of antibodies—is strongly correlated with the structural stability of antibodies and that aggregation tendency under acidic conditions originates from the structural stability of the CH2 domain of each subclass. Our DSF analyses suggest that exposure of the hydrophobic region upon thermal stress is correlated with the tendency of antibodies to aggregate.

Our results suggest that a tendency toward poorer physicochemical quality of antibodies is associated with the subclass type and not with the variable domain. The physicochemical properties of antibodies may therefore be conserved even if the variable domains have been exchanged with those of other IgGs. This concept might help us to select appropriate subclasses. Notably, we can exchange IgG subclasses by appropriately predicting changes in the physicochemical properties of the antibodies. Our results should provide valuable insights into antibody design using appropriate subclasses and into the process of antibody production in accordance with the physicochemical properties of different subclasses.

Materials and Methods

Materials

All IgG antibodies used were manufactured by Kyowa Hakko Kirin (Tokyo, Japan). All antibodies were expressed in Chinese Hamster Ovary cells under essentially identical conditions. Secreted antibodies were recovered from the culture medium and purified by using a series of chromatographic and filtration steps. Carbohydrate structures bound to all antibodies were confirmed by mass-spectroscopy, demonstrating that no major differences have been observed (data not shown).

Four IgG antibodies with different variable regions, mAb-A (human), mAb-B (human), mAb-C (chimeric), and mAb-D (humanized) have been used in this study. IgG4PE contained an amino acid point mutation of Ser228Pro and Leu235Glu in the heavy chain of IgG4 (EU-index numbering scheme used) to prevent half-antibody formation⁴³ and reduce antibody-dependent cytotoxicity.⁴⁴ IgG2AAAS contained an amino acid point mutation of Val234Ala, Gly237Ala, and Pro331Ser in the heavy chain of IgG2 to reduce effector functions.^{45–47} Thus, the amino acid sequences of the light chains and VH regions in each set were identical, but their subclasses were different (IgG1, IgG2 or IgG2AAAS, IgG4, or IgG4PE). Their isoelectric points were in

the range of 8–9. The antibody solution contained 10 mM sodium glutamate, 262 mM D-sorbitol, and 0.05 mg/mL polysorbate 80. Glutamate was selected from the buffer compounds commonly used in clinical antibody formulation. D-sorbitol was selected from the tonicity agents commonly used in antibody formulation. Polysorbate 80 was added to prevent protein particle formation. All excipients met the criteria of the monographs in the United States Pharmacopeia and National Formulary.

All evaluated antibodies were buffer-exchanged into formulations of the desired pH values by using a desalting column (NAP25 column, GE Healthcare U.K., Buckinghamshire, England), and their concentrations were adjusted to 5.0 mg/mL. The formulated antibody solutions were sterilized with a 0.22- μ m filter, and 1 mL of each solution was placed into a sterilized USP-type 5-mL glass vial, which was sealed with autoclaved rubber stopper. The prepared samples were stored in a temperature-controlled incubator at 25 or 40°C for 1 or 3 months before SEC analysis and SDS-PAGE.

Size-exclusion high-performance liquid chromatography.

To detect soluble aggregates and fragments, size-exclusion high-performance liquid chromatography was performed on an Alliance 2795 device equipped with a 2487 UV detector (Waters Corporation, Mildford, MA) and a TSK G3000SWXL 7.8 \times 300 mm² column (Tosoh Biosep., Tokyo, Japan). Separation was performed with a mobile phase of 20 mM K₂HPO₄/KH₂PO₄ and 500 mM NaCl (pH 7.0) at a flow rate of 0.5 mL/min at a constant 25°C. A diluted sample was injected to obtain a total loading amount of 200 μ g, and detection was performed at a wavelength of 215 nm. The resulting chromatograms were analyzed by integrating the area under each eluting peak by using Empower 2 Chromatography Data System software (Waters Corporation) and recorded as percentages of HMWS and LMWS.

SDS-PAGE. IgG samples were run on Novex 8% to 16% Tris-glycine 1.0 \times 15-well precast SDS-PAGE gels (Invitrogen, Carlsbad, CA). All samples were diluted to 1 mg/mL or 0.1 mg/mL with formulation buffer and diluted further to 0.1 mg/mL or 0.01 mg/mL with a 4 \times solution consisting of 69 mM Tris-HCl (pH 7.0), 2.2% SDS, 0.04% bromophenol blue, and 22.2% glycerol buffer; under reducing conditions 111.1 mM dithiothreitol (DTT) was added to the 4 \times solution. The sample load was 1 μ g or 0.1 μ g. The molecular weight marker (Mark12: 200- to 2.5-kDa range) was purchased from Invitrogen.

Far-UV CD. The IgG formulations were diluted to 0.25 mg/mL with formulation buffer and quantified with a Jasco J-820 CD spectrometer in combination

with a Jasco PTC-423S temperature controller (Jasco International, Tokyo, Japan) in quartz cuvettes with a path length of 1 mm at 25°C. Far-UV spectra were collected by continuous scanning from 200 to 260 nm at a scanning speed of 10 nm/min, a response time of 1 s, a bandwidth of 1 nm, a sensitivity of 100 m, steps of 0.5 nm, and an accumulation of three scans. Spectra Analysis Software (Version 1.53.04, Jasco) was used to background-correct the spectra for the spectrum of the respective buffer. Data were calculated as mean residue ellipticity based on mean amino acid residue weight. The mean residue ellipticity was determined as $[\theta]_{\text{mrw}, \lambda} = (\text{MRW} \times \theta_{\lambda}) / (10 \times c \times d)$, where MRW is the mean residue weight, θ_{λ} is the observed ellipticity (in millidegrees) at wavelength λ , c is the protein concentration in mg/mL, and d is the path length in cm. Thermal studies were conducted by raising the temperature in 0.5°C intervals from 25 to 100°C at a rate of 60°C/h. Far-UV CD spectrum after heating have been measured by incubation of the sample at 25°C for 15 min. The molar ellipticity at 217 nm was monitored for changes in the relative content of the β -sheet structure of the immunoglobulins.

DSC. The thermal stability of individual domains was evaluated by using DSC. Measurements were performed on a 1.0 mg/mL IgG solution using a capillary VP-DSC system (MicroCal LLC, Northampton, MA) with a cell volume of 0.135 mL. Temperature scans were performed from 25 to 100°C at a scan rate of 1°C/min. A buffer–buffer reference scan was subtracted from each sample scan before concentration normalization. Baselines were created in Origin 7.0 (OriginLab, Northampton, MA) by cubic interpolation of the pre- and post-transition baselines.

DSF. DSF was used to monitor IgG unfolding during temperature melting. A 96-well microplate was used during DSF, with each well containing 19.5 μ L of IgG sample and 0.5 μ L of SYPRO Orange (Invitrogen Inc.) that had been diluted from the purchased stock to 1:125 in water. The final dye concentration was 1/5000 of that of the initial product. A CFX96 Real-Time PCR instrument (Bio-Rad Laboratories, Hercules, CA) was used, and the “FRET” channel setting was used to record fluorescence changes during DSF measurement. Samples were incubated at 20°C for 3 min before the melting, during which time the temperature was increased from 35 to 95°C in 0.5°C increments, with an equilibration time of 10 s at each temperature. The hydrophobic exposure temperature, $T_{\text{m}1}$ or $T_{\text{m}2}$, was reported as an indication of the transition midpoint of protein unfolding. The first-order derivative curves and T_{m} values were determined by using CFX Manager software (Bio-Rad Laboratories).

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Secretory production of single-chain antibody (scFv) in *Brevibacillus choshinensis* using novel fusion partner

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Abstract Halophilic β -lactamase (BLA) has been successfully used as a novel fusion partner for soluble expression of aggregation-prone foreign proteins in *Escherichia coli* cytoplasm (Appl Microbiol Biotechnol 86:649–658, 2010b). This halophilic BLA fusion technology was applied here for secretory expression in *Brevibacillus*. The “*Brevibacillus* in vivo cloning” method, recently developed by Higeta Shoyu group, for the construction and transformation of *Brevibacillus* expression vectors facilitates efficient screening of the production conditions of *Brevibacillus* expression system. Two single-chain antibodies (scFv), HyHEL-10 single chain scFv (scFvHEL) and anti-fluorescein single chain scFv (scFvFLU), were successfully secreted to culture supernatant as a fusion protein with halophilic BLA. The scFvHEL-His, purified after cleavage of BLA portion with thrombin, was fully active: it formed a stoichiometric complex with the antigen, lysozyme, and inhibited the enzymatic activity. The scFvFLU-His, similarly expressed and purified, stoichiometrically inhibited

fluorescence intensity of fluorescein. The molecular mass of scFvHEL-His was determined to be 27,800 Da by light scattering measurements, indicating its monomeric structure in solution.

Keywords Halophilic β -lactamase · Single-chain antibody (scFv) · *Brevibacillus choshinensis* · Fusion protein · Net negative charge · High solubility

Introduction

Proteins from halophilic bacteria function normally and remain stable in varying salt concentrations (DasSarma et al. 2006; Ventosa et al. 1998). They possess unique amino acid sequences characterized by high content of acidic amino acid residues and resultant high net negative charges at physiological pH (Elcock and McCammon 1998; Mevarech et al. 2000; Tokunaga et al. 2004; Yamaguchi et al. 2011, 2012; Yonezawa et al. 2007). These characteristics confer halophilic proteins' high aqueous solubility and resistance to aggregation at high salt concentrations. For example, β -lactamase (BLA) from *Chromohalobacter* sp. 560 was soluble both in the native and denatured state, resulting in high refolding efficiency from the denatured structures (Arakawa et al. 2010; Tokunaga et al. 2004, 2006a). Reflecting high solubility and efficient folding under variable salt concentrations, BLA was efficiently expressed as a native active protein in *Escherichia coli* cytoplasm (Tokunaga et al. 2004) despite being a secretory protein. These ideal properties of BLA have enabled us to develop a novel BLA–fusion protein expression system for soluble expression in *E. coli* (Tokunaga et al. 2010a, b).

Monoclonal antibodies occupy a large fraction of biopharmaceutical proteins (Yamada 2011). One of the disadvantages.

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of using the whole antibody is its large size, which limits penetration into diseased areas (Beck et al. 2010; Demarest and Glaser 2008; Kontermann 2010). Binding of Fc domain of the whole antibody to cell surface Fc receptor also limits the circulation and mobility. Smaller version of antibody, lacking Fc domains, can overcome these problems and also open an opportunity for intracellular delivery. One of them is a construct linking two variable domains, i.e., heavy and light chains, of antibody with a flexible spacer, called single-chain antibody (scFv) (Stockwin and Holmes 2003; Wörn and Plückthun 2001). Such scFv molecules normally retain the original antigen specificity and affinity. Conjugation of scFv with cytotoxic agents is intensely developed for site-specific delivery of anti-cancer agents (Beckman et al. 2007; Ottiger et al. 2009). It is increasingly apparent, however, that the mammalian secretion system, which is a major workhorse for the production of monoclonal antibodies (Chon and Zerbis-Papastoitis 2011; Shukla and Thommes 2010) and other secretory proteins, is rather ineffective for antibody fragments due to both folding and aggregation problems (Andersen and Reilly 2004; Humphreys and Glover 2001; Ottiger et al. 2009). Thus, bacterial cells, in particular *E. coli*, have been used for production of scFv, but mostly as inclusion bodies (Andersen and Reilly 2004; Humphreys and Glover 2001). Various solubilization and refolding technologies have been developed using denaturants or detergents (Kudou et al. 2011; Lilie et al. 1998; Tsumoto et al. 2003). Such refolding technologies have been applied to various antibody fragments, often with limited success (Fujii et al. 2007; Fursova et al. 2009; Kurucz et al. 1995; Tsumoto et al. 1998). Novel expression technologies of soluble and active antibody fragments should be a valuable option for reliable production of this important class of proteins.

Brevibacillus choshinensis expression system is a well-established host–vector system for the production of foreign proteins, especially secretory proteins (Mizukami et al. 2010; Takagi et al. 1989; Yashiro et al. 2001). Recently, a simple and highly efficient cloning technique, *Brevibacillus* in vivo cloning method (BIC method), has been developed for construction and transformation of *Brevibacillus* expression vectors, making this *Brevibacillus* system more comprehensive and user friendly. We here report expression of scFv using *Brevibacillus* secretory expression system and two fusion partners, halophilic BLA or P45 that is an endogenous secretory protein of *Brevibacillus*.

Materials and methods

Bacterial strain and culture medium

B. choshinensis strain HPD31-SP3 (FERM BP-8479) was used as a host bacterium. TMN medium was used to culture

transformants. The composition of TMN is as follows: 1 % glucose, 1 % Polypeptone (Nihon Pharmaceuticals, Tokyo, Japan), 0.5 % meat extract (Kyokuto Pharmaceuticals, Ibaraki, Japan), 0.2 % yeast extract (Difco, Detroit, MI, USA), 0.001 % FeSO₄·7H₂O, 0.001 % MnSO₄·4H₂O, 0.0001 % ZnSO₄·7H₂O, pH 7.0, and 50 µg/ml neomycin.

Construction of *Brevibacillus* expression plasmids

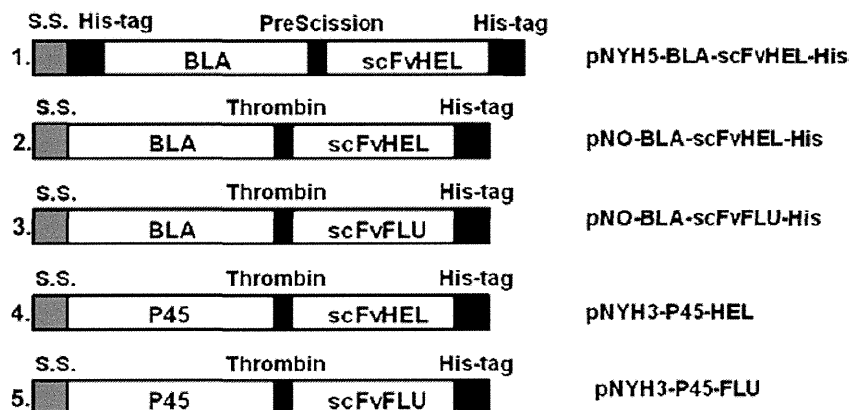
We have tested two scFv genes, HyHEL-10 single chain scFv (scFvHEL) derived from lysozyme neutralizing monoclonal antibody (Tsumoto et al. 1994) and 4M5.3 single-chain scFv (scFvFLU) developed against fluorescein (Midelfort et al. 2004). To construct *Brevibacillus* expression plasmids, we used the BIC method, recently developed by the Department of R & D, Higeta Shoyu Co., Ltd (see detail in “Results” section). Transformation was performed according to the manufacturer’s instruction (TaKaRa Code HB200). Five expression vectors (i.e., construct 1, 2, 3, 4, and 5 in Fig. 1) were constructed as follows.

pNYH5-BLA-scFvHEL-His (construct 1): vector plasmid pNYH5BLA, consisting of P5 promoter and modified secretion signal of *Brevibacillus* major cell wall protein, 6× His-tag, HaBLA, PreScission protease site, multi-cloning site, and 8× His-tag, was amplified with forward primer (Bb5BLA-F) 5'-GGATCCTGGACCTTGGAAACA GAACTTCCAA-3' and reverse primer (Bb5BLA-R) 5'-CTCGAGGAATTCCCCGGGAAGCTTACCCAT-3'. Template DNA containing scFvHEL gene was amplified with forward primer (Bb-scFv-HEL F) 5'-CAAGGTCCAGGATCCGATATCGTCCTGACCCAGAG-CCCGGCGACC-3' and reverse primer (Bb-scFv-HEL R) 5'-GGGGAATTCCTCGAGTTAATGGTGATGGTGATGGTGACCCGCGGA-3'.

The underlined sequences in primers are overlapped with both ends of vector sequence (*Bam*HI-*Xho*I site of pNYH5BLA multi-cloning site). Both amplified fragments were transformed to competent cells and plated out to TM-neomycin plate.

pNO-BLA-scFvHEL-His (construct 2): pNO326 was used as the vector for the expression of BLA-scFvHEL-His. pNO326 is a derivative of pNY326 and has only one translation start site (pNY326 has two initiation sites placed in frame, resulting in the generation of two consecutive secretion signals that possess extra 31 amino acids in length over pNO326). The nucleotide sequence coding BLA-scFvHEL-hisx6 was amplified by PCR with the following primer set: forward (R2L6blafor), 5'-CCCATGGCTTTCGCTCAAG ACGACGCGTCGGACAG-3'; and reverse (his6rev), 5'-CATCCTGTTAAGCTTAATGGTGATGGTGATGGTG-3'. pBF-BLA-scFvHEL-CHis, BLA-fusion vector for *E. coli*, was used as the template. Both primers have 15-bp overlapping sequences (underlined) with the ends of the vector,

Fig. 1 Schematic illustration of scFv-expression vectors. *BLA* halophilic β -lactamase, *scFvHEL* scFv for hen egg white lysozyme, *scFvFLU* scFv for fluorescein, *S.S.* signal sequence for secretion, *His-tag* histidine tag, *PreScission* PreScission protease cleavage site, *Thrombin* thrombin cleavage site, *P45* *Brevibacillus* P45 protein



resulting in recombination between the vector and the insert upon transformation.

The vector was also amplified by PCR to prepare a linearized configuration using the following primer set: forward (326for), 5'-AAGCTTAACAGGATGCGGGG GAG-3'; and reverse (R2L6rev), 5'-AGCGAAAGCCAT GGGAGCAA-3'. Both amplified fragments were transformed to competent *B. choshinensis* cells.

pNO-BLA-scFvFLU-His (construct 3): the same primer set, R2L6BLAfor and his6rev, was used to amplify the gene coding for BLA-scFvFLU-His. pBF-scFvFlu-CHis was used as the template. Other procedures were the same as those described in the preparation of pNO-BLA-scFvHEL-His construct.

pNYH3-P45-HEL (construct 4): first, P45 expression plasmid, pNYH3P45, was constructed. P45 gene was amplified from *B. choshinensis* genome with the following primer set: forward (P45for), 5'-GAACACAAGGTCATGATGAA GATGGAGAGAATA-3'; and reverse (P45rev), 5'-CATCCTGTTAAGCTTAATGGTGATGGTGATGGTGTT GTCCGTCGTGGTTTCTT-3'. The amplified gene was cloned into pNY326 by BIC method to make pNYH3P45. As a next step, pNYH3P45 was linearized by PCR with the following primer set: forward (his6for), 5'-CACCATCAC CATCACCATTA-3'; and reverse (P45rev2), 5'-TTGTTCC GTCGTGGTTTCTT-3'. With the following primer set, LVPRGS (thrombin recognition sequence)-HEL gene was amplified: forward (HELthrombfor), 5'-AAGAAACC ACGACGGAACAactgtccacgtgatccGATATCGTCCTG ACCCAGAG-3'; and reverse, his6rev. The underlined part of the forward primer corresponds to the C-terminal part of P45 and the sequence written with small letters corresponds to LVPRGS. pNO-BLA-scFvHEL-His was used as the template. The PCR product was mixed with linearized pNYH3P45 and then transformed into *B. choshinensis* competent cells.

pNYH3-P45-FLU (construct 5): with the following primer set, LVPR-scFvFlu gene was amplified: forward (Fluthrombfor), 5'-AAGAAACCACGACGGAACA actgtccacgtgatccGATGTGGTGATGACCCAGAC-3'; and reverse, his6rev. pNO-BLA-scFvFLU-His was used as the

template. The other procedures were the same as those used for pNYH3P45-scFvHEL-His construction.

Expression and purification of scFv fusion and scFv proteins

Expression of *Brevibacillus* transformants was carried out in test tube culture (3 ml), flask batch culture (~200 ml), or controlled jar fermenter culture (2 l). Cells were grown in TM-neomycin medium in a test tube or flask for 2–3 days at 30 °C with gentle shaking, and culture supernatant was collected by centrifugation. The scFv-His fusion proteins and scFv-His proteins derived, upon protease cleavage, from fusion proteins were purified by immobilized metal affinity chromatography (Ni-NTA, Novagen and His-Trap, GE Healthcare) using 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PN buffer) as a standard buffer solution. Culture supernatant was mixed with an equal volume of 2-fold concentrated PN buffer and directly applied to His-Trap column, equilibrated with PN buffer, at a flow rate of 1–2 ml/min. After extensive washing of the column with PN buffer, proteins were eluted in stepwise manner with PN buffer containing 20, 50, 100, 200, and 300 mM imidazole. Fractions containing scFv fusion proteins (eluted in 100–200 mM imidazole) were collected and dialyzed against thrombin buffer (50 mM Tris-HCl buffer, pH 8.0, 0.1 M NaCl, and 0.5 mM CaCl₂). The dialyzed sample was digested with bovine thrombin (GE Healthcare) at 22 °C for 16 h and then applied to Ni-NTA column equilibrated with PN buffer. The bound scFv-His protein was eluted with PN buffer containing imidazole in a stepwise manner (20, 50, 100, 200, and 300 mM).

Size exclusion chromatography (SEC) and SEC–multi-angle laser light scattering (MALS) experiments

SEC analysis was performed using Superdex 75 10/300 GL column (1 × 30 cm; GE Healthcare, Uppsala, Sweden) with

0.1 M Na–phosphate buffer, pH 6.8, containing 0.2 M arginine as a standard buffer solution at a flow rate of 0.8 ml/min. Arginine has been shown to prevent non-specific binding of proteins to the SEC column (Ejima et al. 2005).

Molecular mass determination by SEC–MALS was carried out at 25 °C with Superdex 75 10/300 GL column with 0.1 M phosphate buffer, pH 6.8, supplemented with 0.2 M arginine (here arginine refers to arginine hydrochloride) at a flow rate of 0.8 ml/min. The eluting solution from the SEC column was monitored by DAWN-Heleos II multi-angle laser light-scattering detector (Wyatt Technology, Santa Barbara, CA, USA) and UV detector at 280 nm (Hitachi L2400, Tokyo, Japan). The UV absorbance was converted to the protein concentration based on the extinction coefficient of $2.2 \text{ cm}^2 \text{ mg}^{-1}$. Data collection and processing were performed using the Wyatt Technology ASTRA 5 software version 5.3.4. Scattering data obtained at 18 angles were used to construct the Debye plots by Zimm's method (Andersson et al. 2003).

Antibody activity assay for scFvHEL and scFvFLU proteins

Hen egg white lysozyme (0.043 nmol) was incubated with purified scFvHEL–His protein or purified scFvHEL–His fusion protein (BLA or P45 fusion) at different molar ratios at 25 °C for 1 h. After incubation, the cell lytic activity of lysozyme was measured as follows. *Micrococcus lysodeikticus* cell suspension ($A_{450} \sim 0.9$) in 50 mM Na–phosphate buffer, pH 6.4, was mixed with enzyme solution in a cuvette placed in a photometer. The lysis of *M. lysodeikticus* cells was monitored from turbidity decrease at 450 nm. It has been demonstrated that the HyHEL-10, from which scFvHEL was derived, stoichiometrically binds to lysozyme and thereby inhibits its activity (Tsumoto et al. 1994). Binding of scFvFLU to fluorescein was examined as follows. Fluorescein (final concentration of 0.6 μM) was added into 200 μl of 0.1 M Tris–HCl buffer, pH 7.4, containing scFvFLU–His or its fusion proteins at different molar ratios, and incubated at 25 °C for 1 h. After incubation, fluorescence intensity was measured with excitation at 480 nm and emission at 515 nm by a microtiter plate reader (Infinite M200 FA). The scFvFLU (4 M5.3) binds to fluorescein and inhibits fluorescence emission (Kudou et al. 2011). All results shown here are an average of two to three independent experiments with a deviation less than 10 %.

Others

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done according to Laemmli (Laemmli 1970). Protein concentration was measured, unless described otherwise, by bicinchonic acid method (Smith et al. 1985) and Pierce 660 nm protein assay reagent (Thermo Scientific, Rockford, IL, USA).

Results

Construction of expression vectors for scFv fusion proteins

We have examined the expression of two scFv genes, HyHEL-10 single-chain scFv and anti-fluorescein single-chain scFv. *Brevibacillus* expression plasmids were constructed based on the BIC method. Both linearized vector and target gene DNA were amplified by PCR with addition of short overlapping sequences (~15 bp) between vector and insert gene at 5' and 3' ends, and both fragments were transformed at once to *Brevibacillus* competent cells. The homologous recombination in *Brevibacillus* cells between overlapping sequences at 5' and 3' ends of vector and target gene fragments produces whole expression vector (target-gene-inserted vector) in vivo. Once several linearized vector DNA cassettes with different promoters and secretion signals were prepared, target gene amplified with short overlapping sequences of 5' and 3' ends of vector sequence was readily inserted to construct a series of expression vectors. Highly efficient vector construction was obtained by this BIC method: DNA sequencing data verified that more than 80 % of isolated transformants on neomycin-selection plate harbored correct plasmid which had been formed by in vivo recombination between vector and insert DNA fragments.

Several constructs were explored for expression of two scFv, as summarized in Fig. 1. They all contain signal sequence (S.S.) to direct extracellular expression and proteolytic cleavage site for either PreScission or thrombin digestion. The C-terminal His-tag can be used to purify scFv after proteolytic removal of the N-terminal fusion partner. Two fusion partners were inserted N-terminal to the scFv to enhance soluble expression, i.e., halophilic BLA and *Brevibacillus* P45 protein. P45 is a soluble secretory protein of *Brevibacillus*. Although its function is totally unknown, it appears to be a stress protein, as seen in elevated level under stress conditions. Even under normal conditions, its secretion efficiency is high. Both high solubility and expression may lead to efficient soluble expression of target proteins when fused to P45, making it a potential fusion partner.

Expression and purification of BLA-fusion protein and scFv after cleavage with protease

First three BLA-fusion constructs, i.e., construct 1, 2, and 3 described in Fig. 1, were expressed in test tube cultures along with the vector control. Figure 2a shows the SDS–PAGE of the culture supernatant fraction. The expected molecular mass of these three constructs are at ~70 kDa. As expected, vector control showed no prominent band at 70 kDa, which should make identification of expressed fusion protein easier. With these three constructs, a band at ~70 kDa is clearly seen (shown by dots), indicating that these fusion proteins are

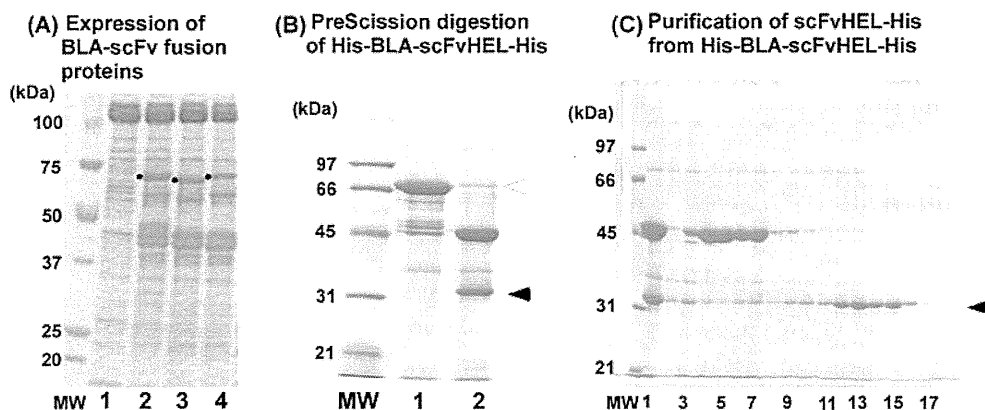


Fig. 2 Expression of BLA-scFv fusion proteins. **a** Expression of BLA-scFv fusion proteins in test tube culture. Cells were grown in 3 ml culture medium in test tube at 30 °C for 3 days. Lane 1 negative control with blank vector, pNO326; 2 pNYH5-BLA-scFvHEL-His; 3 pNO-BLA-scFvHEL-His; 4 pNO-BLA-scFvFLU-His. Dot shows fusion proteins expressed. Each 4 μ l of culture supernatant was applied. **b** Thrombin digestion of purified His-BLA-scFvHEL-His fusion protein. Lane 1 partially purified fusion protein with His-Trap column, 2 after

PreScission digestion. White arrowhead—fusion protein; black arrowhead—scFvHEL-His protein. **c** Purification of scFvHEL-His from PreScission-digested fusion protein with Ni-NTA column. Black arrowhead—scFvHEL-His protein. In this chromatography, 50 mM Tris-HCl, pH 8.0, was used instead of standard PN buffer. Lane 1 sample of before column, 2–5 washed fraction. Imidazole-eluted fractions, 10 mM (lanes 6–8), 20 mM (lanes 9–11), 50 mM (lanes 12–14), and 100 mM (lanes 15–17), are shown

expressed in culture supernatant fractions. There appear to be a few proteins that are also expressed with the constructs, perhaps corresponding to the fragments of the fusion proteins or *Brevibacillus* proteins induced by the expression of heterologous proteins. It should be noted here that a greater mass of the fusion makes easier detection on staining than the expression of smaller scFv alone.

An attempt was made to purify BLA-fusion protein encoded on construct 1 from the culture supernatant by His-Trap column using a stepwise imidazole elution. Figure 2b (lane 1) shows the SDS-PAGE of the partially purified fraction, indicating that the 70-kDa band corresponding to His-BLA-scFvHEL-His protein (white arrowhead) was highly enriched in this fraction. This fraction was digested with PreScission protease, resulting in cleavage into 45-kDa and 31-kDa bands (lane 2), corresponding to His-BLA and scFvHEL-His (black arrowhead). His-BLA (39.8 kDa) apparently migrated as a ~45 kDa protein. Such an unusual slow mobility on SDS-PAGE has been seen in many halophilic proteins and is due to aberrant SDS binding (Tokunaga et al. 2006b, 2008). The protease digested fraction was further purified by Ni-NTA column using stepwise imidazole elution. As shown in Fig. 2c, the earlier eluting fractions from the column (lane 4 to lane 7) contained His-BLA and the later fractions contained scFvHEL-His (lane 12 to lane 15, black arrowhead). It appears that scFvHEL-His binds more strongly to Ni-NTA than His-BLA and is thus fairly free from the latter protein.

Brevibacillus jar fermenter culture and purification of scFv fusion and scFv proteins

Having established above that BLA-fusion produces protease cleavable scFvHEL protein, construct 2 and 3 that lack

the N-terminal His-tag was expressed in controlled jar fermenter culture of *Brevibacillus*. Figure 3a shows the SDS-PAGE of the culture supernatant (each 1 μ l was applied) in which BLA-scFvHEL-His (lane 2, shown by white arrowhead) and BLA-scFvFLU-His (lane 1, black arrowhead) were clearly expressed around 70 kDa. The amount of fusion proteins secreted was estimated to be ~0.5 mg/ml judged by the band intensity of SDS-PAGE. The resultant culture supernatant was applied to His-Trap column as described in the “Materials and methods” section. Purified BLA-scFvHEL-His was subjected to thrombin digestion and then Ni-NTA chromatography. Figure 3b shows SDS-PAGE of fractions eluted by stepwise imidazole elution. In this case, BLA eluted in early fractions (lanes 2 and 3), as expected from no N-terminal His-tag for this construct. The later fractions contained relatively pure scFvHEL-His (lane 12 to lane 17). Fractions 15 and 16 were pooled for subsequent analysis. The final products of purified BLA-scFvHEL-His fusion and scFvHEL-His proteins are shown in Fig. 3c, lanes 1 and 2, indicating purification to homogeneity.

A similar experiment, i.e., His-Trap purification of the fusion protein, thrombin cleavage, and Ni-NTA purification of the cleaved scFvFLU-His, was done for BLA-scFvFLU-His construct (construct 3) and the results are shown in Fig. 3d in which fairly pure preparations of BLA-scFvFLU-His (lane 1) and scFvFLU-His (lane 2) were also obtained. However, it should be noted that this construct showed batch dependence of product quality and hence more work is required for optimal cell culture conditions. The problem appears to be due to greater aggregation tendency of scFvFLU protein.