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A Single-Chain Fv Fragment 2A3 Specific for Native Lysozyme: Isolation from a Human Synthetic Phage Display Antibody Library and Characterization[1]

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We have isolated from a human synthetic phage display library a clone, 2A3, which discriminates native lysozyme from denatured forms. Binding of single-chain Fv fragments (scFvs) of the clone to native hen egg white lysozyme was competitively inhibited by native hen egg white (hew) and human (h) lysozymes. Dot blotting analysis indicated that scFv of the clone did not react with denatured lysozymes. The K_d values for scFv of 2A3 binding to native hew- and h-lysozymes were 3.78×10^{-9} and 9.31×10^{-9} M, respectively, indicating that 2A3 binds more strongly to native hew-lysozyme than to native h-lysozyme. The deduced amino acid sequence of the V_H chain-CDR3 region of 2A3 was RRYALDY, of which the Arg residues at positions 1 and 2 of the CDR3 region were observed to be extremely rare in other antibodies by homology analysis. Based on these observations, site-directed mutagenesis of the RRYALDY-coding region was carried out. The results, combined with biomolecular analyses, demonstrated that Arg residues at positions 1 and 2 of this region were important for native lysozyme-binding.

Key words: human synthetic phage display library, lysozyme, native conformation, single chain Fv fragment.

Animal immunization has provided valuable antisera and monoclonal antibodies for many proteins. However, single-chain Fv fragments (scFvs) are advantageous for mutation, modification, and production by recombinant techniques. The human synthetic phage display antibody library established by Winter *et al.* (*1*) has made it possible to select various scFvs without immunization. The display and selection of scFvs on the surface of phage (*2*, *3*) mimic immune selection (*4*), and scFvs have also been isolated without immunization from repertoires of V genes rearranged *in vivo* (*5*) or *in vitro* (*6*). Such successes suggest that tailor-made antibodies may be synthesized and selected to acquire the desired affinity and specificities (*7*). Antibody variable domains sometimes function poorly as scFvs, either because of instability due to a weak V_H-V_L association, or poor binding due to problems such as interference by the linker sequence (*8*). Thus it seems likely that phage display selects directly for stable scFv (*8*). Recently, antibodies against self-antigens such as p53, thyloglobulin (*9*), and tumor necrosis factor (*10*), and antibodies against small and flexible compounds such as glutathione (*11*), were isolated from a human synthetic phage display antibody library. However, the isolation of scFvs which discriminate among protein conformations has not yet been achieved using the phage antibody library, and these scFvs might be helpful to study conformational diseases recently argued (*12*, *13*).

In this study, we have focused on isolation from the human synthetic phage display antibody library of scFvs that can distinguish between protein conformations using hen egg white lysozyme and human lysozyme. Here we report the isolation and characterization of a phage library-derived scFv, which we call 2A3, that specifically binds only to lysozyme proteins having a native conformation.

MATERIALS AND METHODS

Materials—A human synthetic phage display antibody library prepared by Nissim *et al.* (*9*) was kindly provided by

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Abbreviations: CBB, Coomassie Brilliant Blue; CDR, complementarity-determining region; DAB, 3,3′-diaminobenzidine tetrahydrochloride; ELISA, enzyme-linked immunosorbent assay; hew-lysozyme or hew-L, hen egg white lysozyme; h-lysozyme or h-L, human lysozyme; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside; k_{ass} and k_{diss}, association and dissociation rate constants; K_d, dissociation constant; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; p.f.u., plaque-forming units; RU, resonance unit; scFv, single-chain Fv fragment; V_H, heavy chain-variable region.

Dr. G. Winter. Hen egg white lysozyme was purchased from Sigma Chemical (St. Louis, USA), and h-lysozyme was prepared by the method described previously (*14*). Bovine serum albumin and ampicillin were from Sigma Chemical. CNBr-activated Sepharose 4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Bacterial Strains—*Escherichia coli* strain TG-1 [K12, *Δ(lac-pro), supE, thi, hsdD5/F'traD36, proA⁺B⁺, lacIᵠ, lacZΔM15*] was used for rescue of the phage. Non-suppressor *E. coli* strain HB2151 [K12, *ara, Δ(lac-pro), thi/F'proA⁺B⁺, lacIᵠZΔM15*] and *E. coli* JM109 [*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)/F' (traD36, proAB⁺, lacIᵠ, lacZΔM15)*] were used for preparation of scFvs by secretion, and *E. coli* BL21(DE3)pLysS [F^-, *ompT, hsdS$_B$($r_B^-m_B^-$), gal(cI857, Ind1, Sam7, nin5, lacUV5-T7gene1), dcm(DE3), pLysS(Cm^R)*] was used for preparation of scFvs from cells by osmotic shock.

Preparation of the Conjugates with Native and Denatured Lysozymes—The coupling of native lysozymes to CNBr-activated Sepharose 4B was carried out according to the manufacturer's instructions (Amersham Pharmacia Biotech, Sweden). To remove antibodies that also recognized denatured lysozymes, conjugates with denatured lysozyme were prepared by the method of Nigam *et al.* (*15*). The conjugates with native and denatured lysozymes (1 ml) were packed in FLEX columns (KONTES) (0.7 cm × 4 cm), respectively and used in the selection process.

Rescue of Phagemid Particles from the Library—The human synthetic phage display antibody library used for the selection of scFvs is described by Hoogenboom and Winter (*6*). The library (*9*) contains a diverse repertoire of human V_H genes from 50 germline V_H gene segments having variable CDR3 regions of 4–12 residues in length in combination with the human Vλ3 light chain gene segment. Rescue of phagemid particles is described by Hirose *et al.* (*11*).

Selection of Phages Binding Native hew-Lysozyme or h-Lysozyme by Panning and Affinity Chromatography—Phage particles obtained above were subjected either to panning for binding using Nunc Maxisorp immuno test tubes (Gibco BRL, UK) coated with native lysozymes (*5, 9*) or to affinity purification using a native lysozyme-bound Sepharose 4B column. Native lysozymes were coated in PBS (0.1 M NaCl, 33 mM Na_2HPO_4, 17 mM NaH_2PO_4) at a concentration of 50 μg/ml overnight. Panning was carried out as described by Hirose *et al.* (*11*), and phages were selected by column chromatography as described by Griffiths *et al.* (*16*).

Screening and Sequencing of Clones—Phages were rescued from single colonies of the infected *E. coli* suppresser strain TG-1 using VCS-M13 helper phage (*17*), and soluble scFvs were prepared from the *E. coli* non-suppresser strain HB2151 (*5*). ELISA was used to screen phages and soluble scFvs for binding to native lysozymes, respectively. The 96-well plates for ELISA were coated with native lysozyme (10 μg/ml). The binding of phages to native lysozyme was detected using HRP/anti-M13 monoclonal conjugate (Amersham Pharmacia Biotech, Sweden), and that of soluble scFvs was detected with the mouse monoclonal antibody 9E10 (Boehringer Mannheim GmbH, Germany) (4 μg/ml), which recognizes the tag peptide c-myc (*18*). Peroxidase-conjugated anti–mouse Fc antibody (Sigma Chemical) was used as the secondary antibody.

The nucleotide sequences were determined by the dideoxy method (*19*) using a PRISM Dideoxy Terminator kit (Amersham Pharmacia Biotech, Sweden) and an Amersham Pharmacia Biotech 373A DNA sequencer.

Dot Blotting—Native forms of hew- and h-lysozymes were spotted onto the PVDF membrane (BIO-RAD, California, USA), and some of the lysozyme spots were denatured after binding by treatment with 6 M urea containing 1 M β-mercaptoethanol. The scFvs were detected as described by Hirose *et al.* (*11*). Peroxidase activity was detected using DAB and hydrogen peroxide.

Inhibition Assay by ELISA—Detection of phage or scFvs bound to native lysozyme was performed by ELISA as described by Hirose *et al.* (*11*).

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out in the V_H-CDR3 region by PCR using an *Nco*I site at the 5′ end and a *Not*I site at the 3′ end. A region from the *Nco*I site to a mismatch site and a region from a mismatch site to the *Nco*I site were amplified independently. The primers with the restriction sites, scFv-NCOI and scFvNOTI, were used for PCR amplification, and their oligonucleotide sequences were 5′-AGCCGGCCATGGCCCAGGT-3′ and 5′-AGATGAGTTTTTGTTCTGCGG-3′, respectively (the *Nco*I site for scFvNCOI and a part of the *Not*I site for scFvNOTI are underlined). The primers used for the mutation are shown in Table I. Mutations were confirmed by sequence analyses as described. All the oligonucleotides were synthesized by Amersham Pharmacia Biotech.

Construction of Expression Plasmids—The wild-type DNA in pHEN1-Vλ3 (*6*) was replaced with the mutated DNAs described above, and the plasmids obtained were used for expressing wild-type and mutant scFvs, whose binding activity was determined by ELISA. To detect the binding of scFvs to lysozyme using the BIACORE system, wild-type and mutated 2A3 DNAs were also inserted to pET22b(+) (Novagen, Madison, USA) using the *Nco*I and *Not*I sites, and pET22b(+)-wild, pET22b(+)-R1L, pET22b(+)-R2L, pET22b(+)-R1K, pET22b(+)-R2K, and pET22b(+)-L5M were constructed.

Expression and Purification of scFvs with His-tag—Six plasmids of pET22b(+)-series were transformed into *E. coli* BL21(DE3)pLysS, and transformants were grown at 37°C with shaking. When the OD at 600 nm reached 0.6 to 0.9, IPTG was added at a final concentration of 1 mM and incubation was continued for 3 h. Cells were collected by centrifugation, and scFvs were extracted from cells by the osmotic shock method according to Asada *et al.* using Triton X-100 in place of NP-40 (*20*). Soluble scFvs were ob-

TABLE I. **List of the primers for mutations.**

R1L	F	5′-T G T G C A A G A C T G C G G T A T G C G T T G-3′
	R	5′-C G C A T A C C G C A G T C T T G C A C A G T A-3′
R2L	F	5′-G C A A G A A G G C T G T A T G C G T T G G A T-3′
	R	5′-C A A C G C A T A C A G C C T T C T T G C A C A-3′
R1K	F	5′-T G T G C A A G A A A G C G G T A T G C G T T G-3′
	R	5′-C G C A T A C C G C T T T C T T G C A C A G T A-3′
R2K	F	5′-G C A A G A A G G A A G T A T G C G T T G G A T-3′
	R	5′-C A A C G C A T A C T T T C C T T C T T G C A C A-3′
R5M	F	5′-C G G T A T G C G A T G G A T T A T T G G G G C-3′
	R	5′-C C A A T A A T C C A T C G C A T A C C G C C T-3′

Mutation sites are underlined. F, forward primer; R, reverse primer.

tained by centrifugation and purified using a Ni^{2+} column, which was prepared with Chelating Sepharose (Amersham Pharmacia Biotech) for use with the BIACORE system.

Biomolecular Binding Interaction—The binding of selected scFvs to immobilized lysozyme was measured in surface plasmon resonance (SPR) units (RU) using a Pharmacia BIACORE 1000 instrument (Biacore, Sweden). The proteins, such as hew-lysozyme, h-lysozyme, and BSA, were covalently coupled to the matrix in the flow cell of a CM5 sensor chip *via* amine groups, according to the manufacturer's instructions. The scFvs and their mutants were delivered to the protein-coated flow cell to observe the binding. The detailed methodology for the estimation of association and dissociation rate constants using the BIACORE system has been described by Karlsson *et al.* (*21*). The dissociation constant (K_d) of scFvs for each lysozyme was calculated from the equation $K_d = k_{diss}/k_{ass}$.

RESULTS

Selection of Phage Specific for Native Conformation of Lysozyme and Sequencing—Phages directed against native lysozyme were selected from the human synthetic phage display antibody library established by Nissim *et al.* (*9*). Clones of interest were screened by panning using native hew- and h-lysozymes as antigens. We amplified clones with native lysozyme-binding activities by several rounds of panning and detected them by ELISA. Positive clones with strong binding (> 0.5 at 415 nm) were selected. Isolated phages were then loaded successively onto native followed by denatured hew-lysozyme affinity columns. Phages bound to the native hew-lysozyme column were eluted and amplified. Amplified hew-lysozyme binders were then loaded onto the denatured hew-lysozyme column to remove phages also recognizing denatured hew-lysozyme. Pass fractions were again amplified. These operations were repeated several times for both hew- and h-lysozymes.

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5'— GAG GTG CAG CTG GTG GAG TCT GGG GGA GGT GTG   33
     E  V  Q  L  V  E  S  G  G  G  V

GTA CGG CCT GGG GGT TCC TTG AGA ATC TCC TGT GCA GCT   72
 V  R  P  G  G  S  L  R  I  S  C  A  A

TCT GGA TTC ACC TTT GAT GAT TAT GGC ATG AGC TGG GTC   111
 S  G  F  T  F  D  D  Y  G  M  S  W  V
                   CDR 1

CGC CAA GCT CCA GGG AAG GGG CTG GAG TGG GTC TCT GGT   150
 R  Q  A  P  G  K  G  L  E  W  V  S  G

ATT AAT TGG AAT GGT GGT AGC ACA GGT TAT GCA GAC TCT   189
 I  N  W  N  G  G  S  T  G  Y  A  D  S
                   CDR 2

GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG   228
 V  K  G  R  F  T  I  S  R  D  N  A  K

AAC TCC CTG TAT CTG CAA ATG AAC AGT CTG AGA GCC GAG   267
 N  S  L  Y  L  Q  M  N  S  L  R  A  E

GAC ACA GCC GTG TAT TAC TGT GCA AGA AGG CGG TAT GCG   306
 D  T  A  V  Y  Y  C  A  R  R  R  Y  A
                            CDR 3

TTG GAT TAT TGG GGC CAA GGT ACC CTG GTC — 3'         336
 L  D  Y  W  G  Q  G  T  L  V
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Fig. 1. **The nucleotide sequence of the V_H region of 2A3 and deduced amino acid sequence.** CDR 1, CDR 2, and CDR 3 are underlined. The nucleotide sequence has been deposited in DDBJ (accession number: AB049915).

Finally, several phages that bound native lysozyme but not denatured forms were successfully obtained for hew-lysozyme, though not for h-lysozyme (data not shown).

Next, we determined the nucleotide sequences of the coding regions of the selected clones by the dideoxy method and compared the deduced amino acid sequences. From these, we selected one clone 2A3 (Fig. 1), which was a native hew-lysozyme binder with RRYALDY as a V_H-CDR3 amino acid sequence. Based on the report of Tomlinson *et al.* (*22*), the V_H segment of the clone was derived from the V_H3 family, and the germline of 2A3 was from the DP-32 lineage.

Binding Specificities of 2A3—To examine the ability of selected 2A3 to bind to lysozyme, we performed an inhibition ELISA. The results shown in Fig. 2A indicate that the clone recognizes both native hew- and h-lysozymes, but not BSA. The results were confirmed by dot blotting using scFvs derived from 2A3. Polyclonal scFvs antiND, which were isolated from the same library and bind to both native and denatured lysozymes, were used as a control. As shown in Fig. 2B, denatured hew- and h-lysozymes were detected by using polyclonal scFvs (antiND). However, scFvs of 2A3 did not react with denatured hew- and h-lysozymes. These observations indicate that 2A3 has binding activity toward only native hew- and h-lysozymes.

Analyses of the V_H-CDR3 Region—The sequence (RRYALDY) of the V_H-CDR3 region of the clone 2A3 was compared with the corresponding region from more than 100 other antibodies by using BLAST (*23*) and FASTA (*24*). However, this sequence was not found in the V_H-CDR3 region of any known antibodies. We observed that Arg does

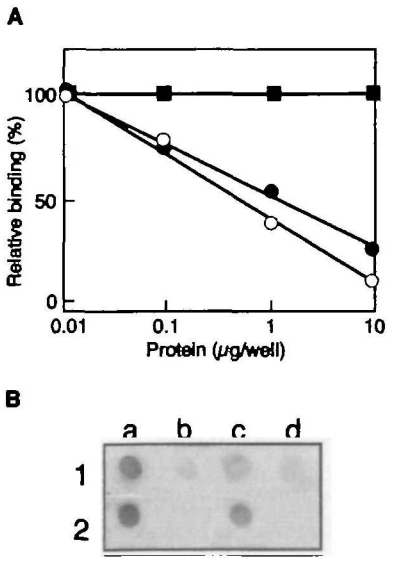

Fig. 2. **Characterization of interaction between lysozyme and scFvs of 2A3.** (A) scFvs of 2A3 were preincubated for 1 h with different concentrations of native hew-lysozyme (●), native h-lysozyme (○), or BSA (■), the mixtures were added to a microtiter plate, and their absorbance at 415 nm was determined. When proteins were not added to the microtiter plate, the signal was shown as 100%. (B) Dot blotting with scFvs of 2A3. Aliquots of 10 μg of native hew-lysozyme (a, b) and native h-lysozyme (c, d) were bound directly to the PVDF membrane, then lysozymes in lanes b and d were denatured by the method described. Native and denatured lysozymes were detected with either antiND (polyclonal scFvs) (1) or scFvs of 2A3 (2) as described in "MATERIALS AND METHODS."

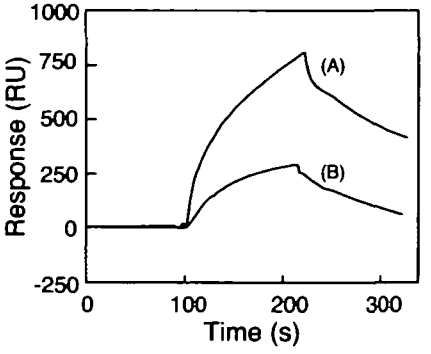

Fig. 3. **Sensorgrams of scFvs of 2A3 with native lysozymes.** Native hew- and h-lysozymes were immobilized on the sensorchip CM5 surface with a 1:1 mixture of *n*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pharmacia). Ten microliters of scFvs solution was injected at the concentration of 500 μg/ml over the surface at a flow rate of 5 μl/min at room temperature.

not appear at positions 1 and 2 of the V_H-CDR3 region in other antibodies, suggesting that these two Arg residues in 2A3 are important in the recognition of the native form of the lysozyme. In addition, 43% of other antibodies examined were surprisingly found to have a Met residue at position 5. Based on these findings, we carried out mutagenesis using the PCR method. In this experiment, R^1 and R^2 were both replaced with either the basic amino acid lysine or the hydrophobic amino acid leucine, and L^5 was replaced with methionine. The mutants were designated as R1K, R2K, R1L, R2L, and L5M. The mutated DNAs were inserted into the pHEN1 plasmid (*6*) and the mutations were confirmed by DNA sequencing. The binding activities to hew- and h-lysozymes were determined by ELISA using the scFvs (data not shown). All mutants showed the same binding specificity to native lysozyme as did wild-type 2A3. The binding activities of R1L and R2L to both types of native lysozyme decreased to about 30% of those of the wild type, that of R2K also decreased to about 50% of that of the wild type, and that of R1K decreased slightly. Interestingly, the binding activities of L5M increased slightly compared with those of the wild type. These results suggest that Arg residues at positions 1 and 2 of the V_H-CDR3 region are important for the recognition of native conformations of lysozymes, and that a Met residue at position 5 increases the strength of antigen binding.

Determination of Kinetic Parameters by BIACORE System—To substantiate the above results, we confirmed the binding of 2A3 scFv and its mutants to both hew- and h-lysozymes using the BIACORE system. In this experiment, scFvs with a His-tag were expressed as described in "MATERIALS AND METHODS," extracted scFvs were purified to a single band by SDS-PAGE and confirmed to have the same activities (data not shown). Native hew- and h-lysozymes were immobilized in the BIACORE 1000 on Sensor Chip CM5, and the binding of 2A3 scFv and its mutants to the two types of lysozyme was monitored. The sensorgram of binding of 2A3 to immobilized native hew- and h-lysozymes demonstrates that 2A3 binds more strongly to native hew-lysozyme than to native h-lysozyme (Fig. 3). Association and dissociation rate constants were determined from the curve. As shown in Table II, the K_d values for native hew- and h-lysozymes were 3.78×10^{-9} M and 9.31×10^{-9} M, respectively, indicating that 2A3 has stronger binding affinity for native hew-lysozyme than for native h-lysozyme. This result is understandable from the observations on k_{ass} and k_{diss} (Table II), indicating that 2A3 associated more easily with native hew-lysozyme and dissociated less easily from it compared with native h-lysozyme. As for the mutant L5M, the K_d values for native hew- and h-lysozymes were 3.04×10^{-9} and 2.99×10^{-9} M, respectively, demonstrating that the binding of L5M to both lysozymes increased, especially to h-lysozyme. These results are supported by the values of k_{ass} and k_{diss} shown in Table II, demonstrating that the association of L5M with native h-lysozyme is faster, but the dissociation is slower than that of 2A3. The binding of R1L, R2L, R1K, and R2K to both lysozymes decreased, especially for R1L and R2L. Their k_{ass} and k_{diss} indicate that these mutant antibodies associated less easily with both types of native lysozymes. These results support the observation that Arg residues at positions 1 and 2 are important for binding to native form of lysozyme.

TABLE II. **Kinetic parameters of scFvs of 2A3 and its mutants for native hew- and h-lysozymes.**

		k_{ass} (10^6 M^{-1} s^{-1})	k_{diss} (10^{-2} s^{-1})	K_d (10^{-8} M)
Wild	hew-L	3.54±0.27	1.34±0.06	0.38±0.05
	h-L	2.79±0.22	2.60±0.12	0.93±0.12
R1L	hew-L	0.17±0.02	1.99±0.13	11.36±2.01
	h-L	0.07±0.00	1.21±0.08	18.23±1.44
R2L	hew-L	0.21±0.02	1.80±0.11	8.63±1.15
	h-L	0.05±0.00	1.78±0.10	38.67±3.04
R1K	hew-L	1.42±0.12	1.75±0.07	1.23±0.15
	h-L	1.38±0.06	2.50±0.06	1.81±0.12
R2K	hew-L	1.72±0.14	2.05±0.05	1.19±0.13
	h-L	1.91±0.08	2.24±0.09	1.18±0.10
L5M	hew-L	2.05±0.00	0.63±0.05	0.30±0.02
	h-L	4.92±0.24	1.47±0.06	0.30±0.03

Results are means ± SD ($n \geq 3$).

DISCUSSION

We have shown that scFvs with binding activities toward native lysozymes but not denatured forms of protein can be isolated from a human synthetic phage display antibody library. The clone 2A3 passed through the denatured lysozyme column without binding, and the 2A3 scFv did not react by dot blotting with lysozyme which had been denatured on a PVDF membrane. These results indicate that the 2A3 scFv has no binding activity toward denatured lysozyme. We tried to confirm these results by using the BIACORE system, but experiments using denatured proteins were technically difficult, and such results using the BIACORE system have never been reported. On the other hand, the clone 2A3 bound to the native lysozyme column, and the 2A3 scFv was found to react with native lysozyme by ELISA and BIACORE monitoring. Based on these observations, it seems reasonable to conclude that these scFvs recognize native lysozyme but not denatured forms.

The clone 2A3 was found to bind to both native hew- and h-lysozymes, although most monoclonal antibodies do not show cross-reactivity between human and hew lysozymes. The results may characterize synthetic phage display antibody fragments. From these results, the possibility exists

that antibody fragments of 2A3 recognize some common specific site on the structures of two lysozymes, which resemble each other.

Recently, phages with a range of binding specificities against either self or foreign antigens were isolated from a single phage antibody library (*4*, *7*, *9*, *11*, *16*, *25*), and phage antibodies have become a resource for the development of therapeutic antibodies (*26*). Nevertheless, there have been no reports of the isolation of phage library-derived scFvs which distinguish among protein conformations. There have been several reports of monoclonal antibodies which preferentially bind to native forms of proteins, though these were isolated by chance (*27–29*). Our success highlights the possibility of selecting such a scFv from a synthetic phage display library not by chance but by design. In this study, the number of clones that bound to native lysozyme but not to denatured forms was highly limited. We used the secondary stock of the library in our experiment, suggesting that the number of positive clones with strong binding within it was limited. The first stock of the library contains a repertoire of $>10^8$ clones (*9*), and mutant phage antibodies obtained in our experiment must also be contained in the original library. The fact that we could not obtain such mutant clones from the secondary stock suggests that the repertoire of clones in the secondary stock is diminished.

Mutational and biomolecular analyses have revealed that Arg residues at positions 1 and 2 of the V_H-CDR3 region are highly important. In the library we used, only the amino acid sequence of V_H-CDR3 region is randomly synthesized, and those of other regions are derived from known sequences (*9*). Thus the V_H-CDR3 region is considered to be highly specific, although all CDR regions are necessary for antigen binding. The Arg-Arg at these positions was found to be unique among antibody sequences known so far by using BLAST (*23*) and FASTA (*24*). This may be partly because the V_H-CDR3 sequence is randomly synthesized. After this manuscript was completed, a human antibody-antigen binding domain specific for human carcinoembryonic antigen was found to have the sequence (RRYALDY) (a patent, Accession AR036439). It was shown that the k_{ass} and the k_{diss} of R1K, R2K, R1L, and R2L for native hew-lysozyme were smaller than those of 2A3, indicating that association of the mutant scFvs with native hew-lysozyme became more difficult and their dissociation became easier. The K_ds of R1L and R2L decreased dramatically, but such a marked decrease of K_ds was not observed for R1K and R2K, suggesting that a positive charge is necessary at these positions. The mutant L5M displayed a lower k_{diss} for both types of native lysozyme, indicating that the mutant protein dissociates less easily. This lower k_{diss} might be responsible for the stronger binding of L5M compared with wild-type 2A3. These observations suggest that a Met residue at position 5 increases binding to native lysozyme. The fact that 43% of the antibodies examined by BLAST (*23*) and FASTA (*24*) have a Met residue at this position suggests that Met plays an important role in antigen binding. In the course of our study, we found that even the wild-type fragment is not so stable, and we used the sample as soon as possible after preparation. It is possible that some mutant fragments are more unstable than the wild type, although we did not check the stability of the mutant fragments.

Recent studies have shown that conformational changes in amyloidogenic h-lysozyme lead to amyloid fibril formation and cause amyloidosis (*13*). We have isolated from the phage display library a scFv that distinguishes between native and denatured forms of lysozyme. In our studies, the native lysozyme-specific scFv was successfully obtained in a short time with a combination of panning and affinity columns. These procedures are available for selecting phage antibodies, but not for obtaining monoclonal antibodies in the usual way. Thus the method is expected to prove useful for producing scFv reagents needed for such studies. These observations demonstrate that a combination of the design and phage display provides a simple and effective tool for obtaining tailor-made antibodies of interest.

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