



Isolation and Molecular Characterization of Single-Chain Fv Antibodies Raised against Pollen Allergens from Japanese Cedar (*Cryptomeria japonica* D. Don)

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We report here the isolation and molecular characterization of single-chain Fv (scFv) antibodies raised against two major allergens, Cryj1 and Cryj2, in the pollen of *Cryptomeria japonica* by the phage display method. Recombinant phages that produced scFv antibodies that bound to Cryj1 or Cryj2 were isolated by selection with immobilized antigens in microtiter plates. After selection of six Cryj1- and four Cryj2-specific scFv antibodies with strong binding activity, we performed pairwise interaction analysis of them by surface plasmon resonance. The analysis revealed that the scFv antibodies against Cryj1 bound to only four non-overlapping epitopes, with dissociation constants that ranged from 4.84×10^{-9} M to 1.62×10^{-7} M. By contrast, four Cryj2-specific scFv antibodies inhibited each other's binding to Cryj2, with dissociation constants from 1.11×10^{-7} M to 4.21×10^{-7} M. Our results indicate that recombinant technology provides a time-saving method for the production of antibodies against pollen allergens.

Key words: allergens; *Cryptomeria japonica* D. Don; Japanese cedar pollinosis; phage display; single-chain Fv

Japanese cedar, the sugi (*Cryptomeria japonica* D. Don), is one of the most important conifers in the commercial production of lumber in Japan. However, two major allergenic proteins, encoded by the Cryj1 and Cryj2 genes in sugi, are associated with the pollen derived from male flowers, and they cause serious pollinosis that has become a significant medical problem in Japan.¹⁻³⁾ The symptoms caused by these proteins have been investigated in depth, and the mechanisms responsible for the pollinosis and methods of prevention and treatment have also been reported.^{4,5)}

Yasueda *et al.*¹⁾ succeeded in purifying Cryj1 protein from pollen, and found that it is a basic glycoprotein of approximately 40 kDa with a pI that ranges from 8.9 to

9.2. The partial amino acid sequence of the purified Cryj1 has allowed the isolation of two cDNA clones.⁶⁾ Analysis of the amino acid sequences deduced from the cDNAs and enzymatic analysis of recombinant Cryj1 protein revealed that it belongs to the pectate lyase family.^{6,7)} Furthermore, Cryj1 transcripts are abundant in pollen but are not detectable in other tissues, such as the leaves, stems, roots, and male and female strobili.⁸⁻¹⁰⁾ It has also been reported that the Cryj1 protein is localized on the sexines, nexines, orbicles, and the cell walls of developing cells in the pollen.¹¹⁾ In addition to expression of the Cryj1 gene, specific and preferential expression of other genes for pectate lyase-like proteins in pollen has been reported for several other plant species.¹²⁻¹⁸⁾ Genes for polygalacturonase-like proteins, such as Cryj2, and the gene for a pectin methyl esterase are also expressed in pollen.¹⁹⁻²²⁾ These pectin-metabolizing proteins probably play important roles in the development of pollen and the growth of pollen tubes, which require a rapid turnover of pectin.¹⁴⁾

Monoclonal and polyclonal antibodies raised against two major pollen allergens were isolated from mice and rabbits.²³⁻²⁷⁾ Enzyme-linked immunosorbent assays (ELISAs) to determine the levels of Cryj1 and Cryj2 in pollen were developed using these antibodies.^{23,28,29)} Assays with the specific antibodies allowed comparison of the levels of the antigens in the pollen of various sugi clones cultivated under different environmental conditions.²⁹⁻³⁴⁾ However, in some cases, the precise quantification of Cryj1 failed because of variations in the amino acid sequences of the Cryj1 polypeptides among the clones that prevented detection by the available antibodies.³⁵⁾ Hence, additional antibodies with different immunological characteristics are required, and a convenient, cheap, and rapid method for the production of diverse collections of antibodies is needed.

In recent years, a genetic engineering technique known as phage display has been developed to generate the artificial antibodies.³⁶⁾ This technology allows the

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Abbreviations: scFv, single-chain Fv; V_H, heavy-chain variable region; V_L, light-chain variable region; HRP, horseradish peroxidase; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium; His-tag, polyhistidine tag; SPR, surface plasmon resonance; NHS, N-hydroxysuccinimide; RU, resonance units; K_d, dissociation constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; CDR, complementarity-determining region

production of functional recombinant antibodies on the surface of bacteriophages and their accumulation in the periplasm of *Escherichia coli* cells. In addition, antibody fragments, including single-chain Fv (scFv) fragments, have been successfully constructed by the use of cDNAs that encode light (V_L) and heavy (V_H) variable regions that are joined *via* a short spacer sequence. Preparation of antibodies by these recombinant technologies is relatively easy compared with the traditional hybridoma method. Specific scFv antibodies can be selected by linking phenotype (binding affinity) to genotype. Selected antibody fragments that do not have the required affinity can be subjected to cycles of genetic mutation and further selection to enhance their affinity.³⁷⁾ This method has the potential to generate different types of antibody with strong affinity for the antigen, high stability, and various other desirable immunological characteristics.

In the present report, we describe the isolation of scFv antibodies against two major sugi allergens, Cryj1 and Cryj2, using phage display technology, and characterize the antibodies and their potential uses.

Materials and Methods

Construction of a phage library. Seven week-old BALB/cBy female mice were given four intraperitoneal injections of 25 µg of an extract of pollen from *Cryptomeria japonica* that contained Cryj1 and Cryj2 (Hayashibara Biochemical Laboratories, Okayama, Japan). The mice were immunized with the extract every 2 weeks. The first dose of pollen included Imject® Freund's adjuvant (Pierce, Rockford, UK) and the second through fourth doses included Ribi adjuvant (Ribi ImmunoChem Research, Hamilton, MT). Three d after the fourth injection, the spleens were harvested from the immunized mice. All animal experiments were performed under guidelines approved by the Japanese Society for Laboratory Animal Resources.

Total RNA was prepared from the spleen tissues of the mice using Isogen (Nippon Gene, Tokyo), and then first-strand cDNA was generated by the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Tokyo). The scFv antibodies were generated as described by Pope *et al.*,³⁸⁾ with minor modifications, as follows: Using the first-strand cDNA as template, we amplified the V_H and V_L genes separately and in combination by three subsequent PCRs. Amplification of each cDNA fragment by PCR was performed under the following conditions: incubation for 3 min at 96 °C; then 25 cycles of 30 s at 96 °C, 45 s at 55 °C, and 60 s at 72 °C; and final extension at 72 °C for 7 min. Samples were purified from agarose gels. After the first PCR, products corresponding to the V_H and V_L fragments were mixed, and assembly PCR was performed with a flexible linker (Gly₄Ser)₃. The conditions for PCR were 7 cycles of incubation at 94 °C for 60 s, and 63 °C for 4 min, without primers. The third-step PCR extended the products of the second PCR product by overlap PCR, as follows. A modified 5'-primer attached to a *Sfi*I site and a 3'-primer with a *Not*I site were used in the amplification of scFv fragments. PCR was performed under the same conditions as the first PCR, except that the annealing temperature was 65 °C.

Cloning of scFv cDNA into a second expression vector. The PCR-amplified scFv fragments and pCANTAB 5E phagemid vectors were digested with *Not*I and *Sfi*I, and the fragments were purified from agarose gels. The resulting sequences, which encoded scFv antibodies, were inserted into the pCANTAB 5E phagemid vector using T4 ligase (Takara Bio, Ohtsu, Japan) at 16 °C for 16 h. Populations of ligated DNA were introduced into *E. coli* XL-1 Blue with a Gene Pulser (BioRad Laboratories, Tokyo). The library was amplified by culture at 37 °C in 2 × YT medium (17 g/l of tryptone, 10 g/l of yeast extract, and 5 g/l of NaCl), supplemented with ampicillin (100 µg/ml) and glucose (20 g/l).

Selection of Cryj1- and Cryj2-specific antibodies. A library of phage-displayed scFv antibodies with 7×10^8 clones was used in the selection of recombinant antibodies. Phage-inoculated *E. coli* cells were cultivated until the logarithmic phase of growth. Phage rescue was performed with M13KO7 helper phage (Invitrogen) and overnight amplification in 2 × YT supplemented with 100 µg/ml of ampicillin and 50 µg/ml of kanamycin at 30 °C. Phages were precipitated with 20% polyethylene glycol plus 2.5 M NaCl, and were resuspended in phosphate-buffered saline (PBS). Then they were incubated with 2% skim milk at room temperature for 1 h to eliminate nonspecific binding. Microtiter plates were blocked with 2% skim milk for 1 h and then coated with 5 µg/ml of Cryj1 or the same amount of Cryj2 (Seikagaku, Tokyo) in PBS. The coated plates were incubated with the phage preparation for 1 h. After incubation, the plates were washed 10 times with PBS plus 0.05% Tween-20, and then with PBS. The bound phages were eluted by incubation for 20 min with 100 µl per well of freshly prepared 0.1 M glycine buffer (pH 2.2). Each preparation of eluted phages was neutralized with 1 M Tris-HCl (pH 6.8). The resulting phages were introduced into *E. coli* XL-1 Blue cells at 37 °C with shaking at 250 rpm for 20 min. For the next round of selection, 10 ml of the culture of infected *E. coli* cells was added to 200 ml of 2 × YT supplemented with 100 µg/ml of ampicillin and 20 g/l of glucose, and the cells were grown to logarithmic phase. Then rescue with M13KO7 helper phage, amplification, and selection were performed as described above.

ELISA for antibody screening. After the screening for selection of antibodies, the *E. coli* clones that produced an individual recombinant antibody were selected and grown at 30 °C in the well of a microtiter plate in 200 µl of 2 × YT, with rescue by M13KO7 helper phage as described above. The preparation of amplified phages was blocked with 2% skim milk for 1 h and then placed in the wells of microtiter plates that had been coated with Cryj1, Cryj2, or bovine serum albumin (BSA). After incubation for 1 h, the plates were washed 3 times with PBS plus Tween-20 and incubated with mouse antibodies against M13 conjugated with horseradish peroxidase (HRP) (Amersham Biosciences, Buckinghamshire, UK) for 1 h at room temperature. After three washes with PBS, a 2',2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS) solution was added to each well, and the absorbance of the reaction mixture was recorded at 405 nm.

After phage ELISA, the plasmids in the individual positive clones were isolated, and then these plasmids were introduced to *E. coli* HB2151, which can produce intact scFv protein in the absence of phage particles. After transformation of the *E. coli* cells, individual clones were grown at 30 °C in the wells of microtiter plates in 400 µl of 2 × YT supplemented with isopropyl-β-D-thiogalactopyranoside (1 mM) and ampicillin. The periplasmic scFv extracted from each clone of the transformed *E. coli* cells was blocked with 2% skim milk for 1 h and then placed in the well of microtiter plates that had been coated with Cryj1, Cryj2, or BSA. After incubation for 1 h, the plates were washed 3 times with PBS plus Tween-20 and incubated with mouse Etag-specific antibody conjugated with HRP (Amersham Biosciences) for 1 h at room temperature. Then the plates were washed 3 times and ABTS solution was added to each well. Finally, the absorbance was recorded at 405 nm.

Sequence analysis of selected clones. The coding sequences for the scFv antibody in the various clones were sequenced by ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Tokyo) using the pCANTAB 5E set of sequencing primers (Amersham Biosciences). Sequence comparisons were performed by searching the IMTG Database on the internet (<http://imtg.cines.fr/>).

Construction of plasmids for the expression of scFv genes. For the construction of expression vectors that encoded truncated forms of the Cryj1- and Cryj2-specific scFv antibodies with a polyhistidine tag, we amplified partial sequences of the genes for Cryj1- and Cryj2-specific scFv (Cj1-56, -65, -67, -69, -73, and -84; and Cj2-54, -80, -90, and -103) by PCR with the pCANTAB 5E phagemid vector and upstream primer 5'-CAACGTGAAAAAATTATTATTCGC-3' plus the following downstream primers with the coding sequence for the polyhistidine tag (His-tag): 5'-AgcggccgcttaGTGGTGGTGGTGGTGGTGGCG-TTTTATTTCAGCTTGGTCCCCC-3' for Cj1-56, Cj2-90, and

Surface plasmon resonance (SPR) analysis. Experiments were performed with a BIAcore X instrument (Biosensor, Uppsala, Sweden)

To isolate phage antibodies that would bind to Cryj1 or to Cryj2 from the library, phages were rescued from the transformed *E. coli* cells and then selected on microtiter plates coated with either purified Cryj1 or purified Cryj2. In the first selection, we screened 4.0×10^{12} plaque-forming units (pfu) of the phage for binding to the various allergenic proteins. The first screening yielded 893 and 1,103 pfu of phage that bound to Cryj1 and Cryj2 respectively. The second round of screening resulted in 0.8 million- and 0.6 million-fold enrichment of phages that produced Cryj1- and Cryj2-specific antibodies respectively. In the final round of screening, further enrichment was achieved (2.3-fold and 1.3-fold, as compared to the results after the second round respectively). Thus overall enrichment during the three rounds of screening was approximately 1.9 million- and 0.9 million-fold respectively as compared to the results prior to screening. To avoid over-selection,⁴⁰⁾ we selected 96 clones for each antigen at random after each round of screening and tested them for their ability to bind to Cryj1 or Cryj2 by phage ELISA. We identified 86 and 105 phage clones that were apparently able to

Table 1. Categorization of Cryj1-Specific scFv Antibodies in Terms of the Amino Acid Sequences of Their CDR3-H Regions

Group	Subgroup	Clone	CDR3 sequence	Phage ELISA	ELISA
1	1-1	Cj1-2	ARPLYGSRYYYAMDY	+++	++++
		Cj1-3		+	+
		Cj1-4		++	+
		Cj1-6		++++	++
		Cj1-63		+	++++
		Cj1-67*		++	+++++
	1-2	Cj1-27	T-----	+	+
	1-3	Cj1-10	-----G---	++	++++
		Cj1-51		++++	++++
		Cj1-68		++	++++
	1-4	Cj1-31	-----S-----	+	++++
	1-5	Cj1-17	T-----G---	+	+++
		Cj1-47		+	+++
		Cj1-49		+	++++
	1-6	Cj1-70	-----N-----G---	++++	++++
	1-7	Cj1-5	-----K---GL--	+	++
2	1-8	Cj1-29	--GGLRLQTG----	+	+
		Cj1-30		+	+
		Cj1-69*		+	++++
	1-9	Cj1-65*	--GYGSRYG-----F	+	++++
2	2-1	Cj1-84*	ARRDMIATDAMDY	++	++++
	2-2	Cj1-50	-----F	++	++++
3	3-1	Cj1-19	NAGGNSEFAY	+	+++
		Cj1-22		+++	++++
		Cj1-25		+	+
		Cj1-46		++	++++
		Cj1-52		+	+++
		Cj1-53		+++	++++
		Cj1-54		+	++
		Cj1-55		+++	++++
		Cj1-56*		+	++++
		Cj1-79		++	++++
	3-2	Cj1-83	-----A-T-	++	++++
	3-3	Cj1-13	----S----	+	+
	3-4	Cj1-78	----Y----	+	++++
4	4-1	Cj1-16	ARDGNFAY	+	+
		Cj1-73*		+	++++

Thirty-seven clones were divided into four groups and 16 subgroups according to the lengths and amino acid sequences of their CDR3-H regions respectively. Identical amino acid residues are indicated by dashes. The binding activity of phage-displayed scFv and soluble scFv to purified Cryj1 is expressed in terms of + to +++++, from weak to strong. In-depth analysis was performed using the clones indicated by asterisks.

bind to Cryj1 and Cryj2 respectively. In contrast to the selected clones, no clones of phage rescued from the library before selection bound to any significant extent to Cryj1 or Cryj2 (data not shown). Our results suggest that scFv-displaying phages with high affinity for Cryj1 or Cryj2 had been selected from our library by affinity screening.

DNA sequencing analysis of selected clones

To characterize the selected clones (86 and 105 clones respectively for Cryj1 and Cryj2) in terms of their nucleotide sequences, we performed sequencing analysis of the scFv-coding region in each clone. We found that 37 and 39 clones specific for Cryj1 and Cryj2 respectively had sequence characteristics of typical scFv fragments. Although all the clones tested were unique in terms of the nucleotide sequences of their variable

regions, some of them could be classified in terms of the characteristics of the sequences in complementarity-determining region 3 of the V_H gene (CDR3-H). Tables 1 and 2 show the classification of deduced amino acid sequences of CDR3-H, based on an analysis supported by the IMGT/V-QUEST program at the IMGT database (<http://imgt.cines.fr/>). As indicated in Table 1, our Cryj1-specific scFv antibodies could be classified mainly into four groups on the basis of the number of amino acid residues in CDR3-H, which ranged from 8 to 15. Cryj1-specific scFv antibodies with CDR3-H regions of 15 amino acid residues (group 1) could be classified into nine subgroups on the basis of differences among their amino acid sequences (Cj1-1 to Cj1-9; Table 1). Similarly, CDR3-H regions with 13 (group 2) and nine (group 3) amino acids could be categorized into two and four subgroups respectively. As

Table 2. Categorization of Cryj2-Specific scFv Antibodies in Terms of the Amino Acid Sequences of Their CDR3-H Regions

Group	Subgroup	Clone	CDR3 sequence	Phage ELISA	ELISA
1	1-1	Cj2-24	ARHEGNMVTAMDY	+	++
		Cj2-42		++	++++
		Cj2-46		+	+
		Cj2-47		+	+++
		Cj2-59		++++	++++
		Cj2-61		+++++	++++
	1-2	Cj2-48	-----L--	+	++++
		Cj2-75		+	+++
		Cj2-76		++	+++
		Cj2-77		+++	++++
		Cj2-80*		+	++++
		Cj2-92		+	++++
	1-3	Cj2-40	--DY-STYGYWF-V	+	+
2	2-1	Cj2-33	ARNHGYDGTWFAY	+	+
	3-1	Cj2-64 Cj2-88	ARSGGHDYFFDY	+++++	++++ +
3	3-2	Cj2-85	----R--Y---	+	+++
	3-3	Cj2-43	---D---Y---	++	++++
		Cj2-44		+++	++++
		Cj2-63		+++++	++++
		Cj2-65		+++++	++++
		Cj2-66		+++++	++++
		Cj2-101		+++	++++
		Cj2-103*		++	+++++
	3-4	Cj2-87	---D---YL---	+++	++++
	3-5	Cj2-30	---A---Y---	+	++++
	3-6	Cj2-10	---A---Y-E-	++++	++++
		Cj2-50		+	++++
		Cj2-67		+++++	++++
	3-7	Cj2-68	-K-----Y---	+	+++
	3-8	Cj2-9	-K---R--Y---	++++	++++
		Cj2-84		+++	++++
	3-9	Cj2-54*	---N-Y-TR-A-	+++++	++++
		Cj2-71		++	++++
	3-10	Cj2-52	---N-Y-TW-A-	+++++	+
		Cj2-74		+	+++
	3-11	Cj2-105	---NNR--V---	+	++++
	3-12	Cj2-90*	---NYK-S--E-	+	++++
4	4-1	Cj2-51	AGGAY	+	+

Thirty-nine clones were divided into four groups and 17 subgroups according to the lengths and amino acid sequences of their CDR3-H regions respectively. Identical amino acid residues are indicated by dashes. Binding of phage-displayed scFv and soluble scFv to purified Cryj2 is expressed as in Table 1. In-depth analysis was performed using the clones indicated by asterisks.

in the case of Cryj1-specific scFv, the antibodies raised against Cryj2 could be also classified into four groups in terms of the length of the CDR3-H region (Table 2). Most of the Cryj2-specific scFv clones belonged to two groups with CDR3-H regions of 14 (group 1) and 12 (group 2) amino acids respectively. The sequence variability of CDR3-H in the latter group was larger than that in the former (Table 2).

ELISA with soluble antibodies

To confirm the affinities of the antibodies selected by phage ELISA, we also performed ELISAs with soluble scFv antibodies after separation of the phage coat proteins from the antibody molecules. Using isolated plasmids from each of the clones listed in Tables 1 and

2, we produced soluble scFv antibodies in another strain of *E. coli* (HB2151), which were able to produce antibodies in the absence of phage coat protein. After preparation of the various soluble scFv antibodies, we performed ELISAs with Cryj1 and Cryj2 proteins. As indicated in Tables 1 and 2 (see ELISA column in each Table), the binding affinity of the soluble antibodies to each antigen varied, as observed in the case of ELISAs with the phage-displayed antibodies. In the ELISAs with the soluble antibodies from most of the clones, the affinities were higher than those in the ELISAs with phage antibodies. The enhanced binding activity in the soluble antibodies might have been due to the release of scFv antibodies from phage particles, which might have contributed to appropriate folding of antibody peptides.

Purification and characterization of His-tagged antibodies

For further analysis, we selected six Cryj1-specific (Cj1-56, -65, -67, -69, -73, and -84) and four Cryj2-specific (Cj2-54, -80, -90, and -103) clones, all with strong binding activity and with differences in amino acid sequences in CDR3-H. To produce efficiently soluble scFv antibodies from the selected clones, we changed the expression vector from pCANTAB 5E to pET20 with a His-tagged sequence. After the coding sequences of scFv in the clone had been amplified by PCR, we cloned the result DNA into pET20. Soluble scFv antibodies with a His-tag were produced in *E. coli* BL21 (DE3) cells that had been transformed with the various plasmids, and then the antibodies were purified by affinity and subsequent size-exclusion chromatography. As indicated in Fig. 1, the sizes of all the purified antibodies as estimated by SDS-PAGE were consistent with those predicted from the respective nucleotide sequences.

In addition to an extract of pollen (Cry) that was used in the immunization of the mice, we compared the binding of the purified antibodies to five different proteins, including Cryj1 and Cryj2, with the binding to the same protein of commercially purchased monoclonal antibodies raised against Cryj1 and against Cryj2. Cry includes both Cryj1 and Cryj2 proteins. In the case of Cryj1-specific scFv antibodies, all of the clones tested bound to purified Cryj1 protein and Cry (Fig. 2A). The binding activities of five of them (Cj1-65, -67, -69, -73, and -84) to the purified Cryj1 were significantly greater than that of one of the purchased monoclonal antibodies (Cj1mAb26), and almost the same as that of another (Cj1mAb13). In contrast, the binding of Cryj1-specific scFv antibodies to Cryj2 protein was minimal. In the case of the Cryj2-specific scFv antibodies, all of the purified antibodies (Cj2-54, -80, -90, and -103) bound to purified Cryj2 (Fig. 2B) to the same extent as the purchased Cj2mAbT27. They also bound to Cry as Cryj1-specific scFv antibodies. Although all of the scFv antibodies raised against Cryj2 reacted weakly and non-specifically with Cryj1, the extent of binding of Cj2-80, Cj2-90, and Cj2-103 to Cryj1 was significantly lower than that of Cj2mAbT27. In addition to binding to the antigens described above, the extent of non-specific binding of our scFv antibodies to BSA was significantly lower than that of the three purchased monoclonal antibodies (Fig. 2A and B). These results indicate that our scFv antibodies had greater specificity for the respective antigens than the purchased antibodies did.

Determination of the affinity of binding of each scFv to Cryj1 and to Cryj2 by SPR

We also analyzed the affinity of the scFv antibodies for the respective antigens by SPR with immobilized Cryj1 and Cryj2. We determined the association (k_{on}) and dissociation (k_{off}) rate constants, as well as the association constant (K_d), for each scFv antibody. k_{on} indicates the number of complexes formed in 1 s in a solution in which each component is present at 1 M; k_{off} indicates the ratio of unbound antibodies to the complexes during dissociation for 1 s; and K_d expresses the strength of binding. For Cryj1-specific scFv, k_{on} and k_{off} ranged from 3.35×10^4 to 2.03×10^5 ($M^{-1} s^{-1}$) and

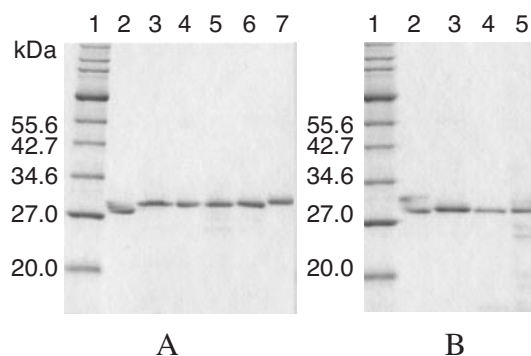


Fig. 1. SDS-PAGE of Purified scFv Antibodies.

A, Cryj1-specific scFv antibodies. Lane 1, Molecular mass markers; lanes 2 through 7, Cj1-56, Cj1-65, Cj1-67, Cj1-69, Cj1-73, and Cj1-84. B, Cryj2-specific scFv antibodies. Lane 1, Molecular mass markers; lanes 2 through 5, Cj2-54, Cj2-80, Cj2-90, and Cj2-103.

from 7.08×10^{-4} to 8.37×10^{-3} (s^{-1}) respectively, with consequent values of K_d of 4.84×10^{-9} to 1.62×10^{-7} M (Table 3). The k_{on} values for Cryj1-specific scFv antibodies were 10^2 - to 10^3 -fold lower than those for Cj1mAb13. The k_{off} of these antibodies were 10^2 - to 10^3 -fold lower than that for Cj1mAb13. Cj1-84 had the highest K_d value and the highest affinity for Cryj1 among the isolated Cryj1-specific scFv antibodies.

In the case of the Cryj2-specific scFv, the k_{on} and k_{off} values ranged from 5.67×10^3 to 6.74×10^4 ($M^{-1} s^{-1}$) and from 1.13×10^{-3} to 1.72×10^{-2} (s^{-1}) respectively, with resulting values of K_d of 1.11×10^{-7} to 4.21×10^{-7} M (Table 3). The association rate constants of the Cryj2-specific scFv antibodies for Cryj2 were 0.82- to 10-fold higher than that of Cj2mAbT27. The k_{off} values of the Cryj2-specific scFv antibodies were 10- to 10^2 -fold lower than that of Cj2mAbT27.

Characterization of binding epitopes on Cryj1 and Cryj2 by SPR

We performed pairwise interaction analysis of His-tagged Cryj1- and Cryj2-specific scFv antibodies by SPR using pairs of scFv at concentrations that resulted in near saturation of the chip's surface. The amount of scFv bound was determined in resonance units (RUs) for each member of a pair, and then the two members of the pair were mixed together at a final concentration equivalent to that used in measurements with the individual scFv antibodies.⁴¹⁾ When a significant increase in RUs was achieved by injection of the mixture of scFv antibodies as compared to the result for each member of the pair, we postulated that these scFv antibodies recognized different epitopes on the antigen. In other words, the absence of an increase in RUs indicated that each scFv in the pair recognized the same or an overlapped epitope on the antigen.

Table 4 shows the data for the pairwise interaction analysis of scFv antibodies by SPR. In the case of the Cryj1-specific scFv antibodies, interfering interactions were observed with only two pairs of antibodies, Cj1-65 plus Cj1-67 and Cj1-73 plus Cj1-84 (gray background in the Table). The results for the other pairs were almost equal to the sum of the values from each member of the pair, indicating that the members of these pairs were

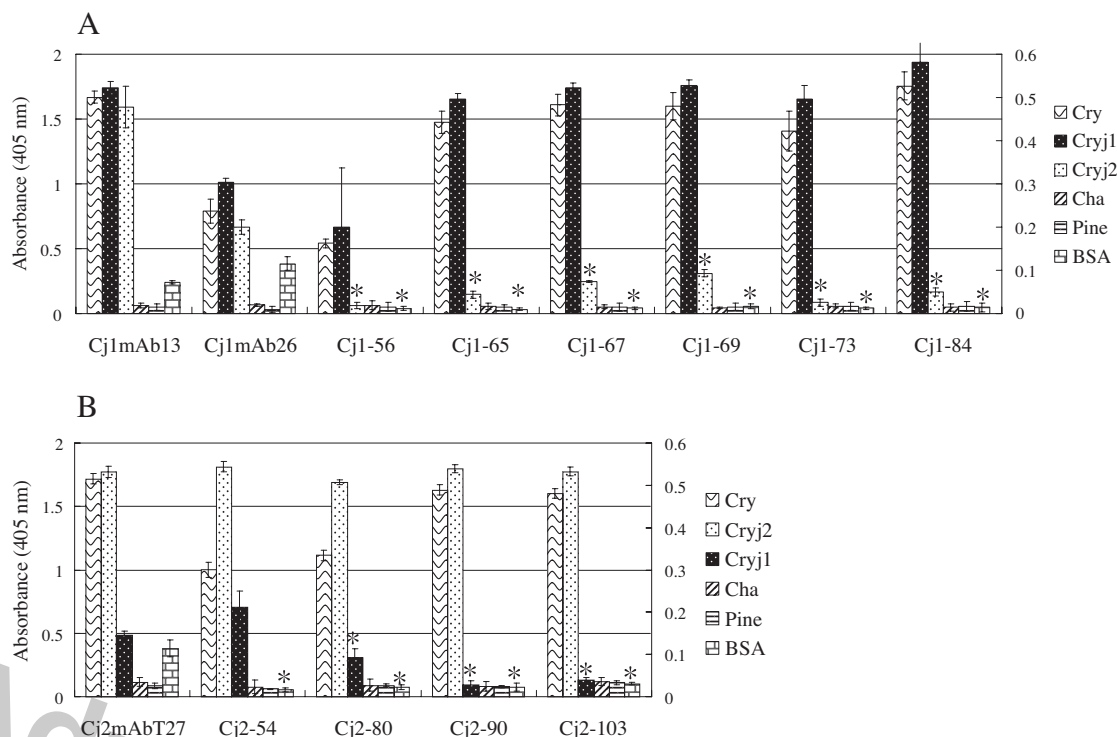


Fig. 2. ELISAs with Purified scFv Antibodies.

ELISA plates were coated with 1 μ g of Cryj1, Cryj2, an extract of the pollen of *Cryptomeria japonica* (Cry), an extract of the pollen of *Chamaecyparis obtuse* (Cha), an extract of the pollen of pine (Pine), and BSA respectively. After incubation of the individual wells with purified scFv antibodies, binding activity was measured as described in "Materials and Methods." A, Results for purified Cryj1-specific scFv antibodies. B, Results for purified Cryj2-specific scFv antibodies. The X-axis at the left corresponds to Cry, Cryj1 (A), or Cryj2 (B), and at the right to the other antigens. * $p < 0.05$ compared with values for Cj1mAb13 (A) and Cj2mAbT27 (B) respectively (Student's *t*-test).

Table 3. Binding Properties of Purified scFv Antibodies

Antibody	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_d (M)
Cj1-56	1.12×10^5	1.67×10^{-3}	1.49×10^{-8}
Cj1-65	6.72×10^4	1.39×10^{-3}	2.07×10^{-8}
Cj1-67	2.03×10^5	1.31×10^{-3}	6.46×10^{-9}
Cj1-69	3.35×10^4	9.01×10^{-4}	2.69×10^{-8}
Cj1-84	1.46×10^5	7.08×10^{-4}	4.84×10^{-9}
Cj1-73	5.16×10^4	8.37×10^{-3}	1.62×10^{-7}
Cj1mAb13	4.06×10^7	2.04×10^{-1}	5.02×10^{-9}
Cj1mAb26	4.51×10^5	1.41×10^{-1}	3.12×10^{-7}
Cj2-54	6.74×10^4	1.72×10^{-2}	2.55×10^{-7}
Cj2-80	5.67×10^3	1.13×10^{-3}	1.99×10^{-7}
Cj2-90	9.86×10^3	4.15×10^{-3}	4.21×10^{-7}
Cj2-103	3.94×10^4	4.39×10^{-3}	1.11×10^{-7}
Cj2mAbT27	6.93×10^3	2.19×10^{-4}	3.16×10^{-8}

Association (k_{on}) and dissociation (k_{off}) rate constants were determined with BIAcore evaluation software. The dissociation constant (K_d) was calculated from the following equation: $K_d = k_{off}/k_{on}$.

involved in non-interfering interactions with the antigen. By contrast, the epitope recognized by all of the Cryj2-specific scFv antibodies was either a single epitope or a set of overlapping ones. The data shown in Table 4 are summarized schematically in Fig. 3. In summary, six Cryj1-specific scFv antibodies were categorized into four independent groups on the basis of their different modes of epitope recognition. Although we also analyzed the pairwise interaction between our scFv antibodies and purchased antibodies, we failed to detect any correct interaction due to aggregation when they were mixed.

Discussion

Antibodies are produced by the immune system in response to infecting microorganisms and other foreign materials. In monoclonal-antibody technology, tumor cells that can proliferate indefinitely are fused with mammalian cells that produce an antibody. The result of this cell fusion is a hybridoma, which produces antibodies continuously. Since the production of antibodies by hybridomas is generally expensive, application remains restricted. Phage display provides a method for the time-saving production of antibodies without the use of hybridomas, and it can contribute to reducing the cost of antibody production.

Most commercially available antibodies raised against Japanese cedar allergens are produced by hybridomas. These antibodies are used as research tools, but there are limits to their application because the prices are relatively high (more than 200 JPY/ μ g). If antibodies against Japanese cedar allergens could be mass-produced cheaply, they could be exploited in many other ways, including, for example, forest-tree breeding. Efficient selection of plus trees with minimally allergenic pollen might be facilitated by the availability of antibodies with higher specificity to the antigens.

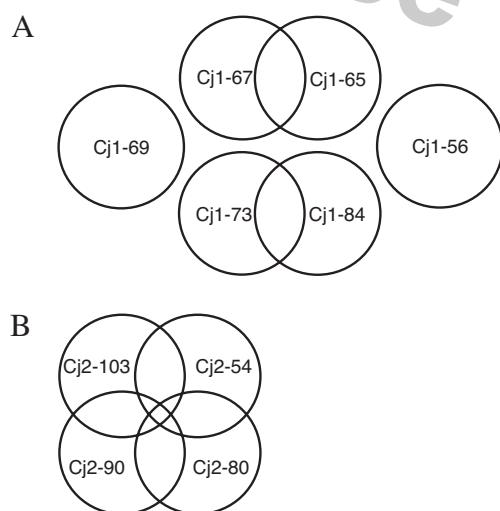
In the present report, we describe first the production of scFv antibodies against two Japanese cedar allergens, Cryj1 and Cryj2, by the phage display method. Our phage library contained more than 10^8 independent clones and was of a size similar to libraries reported by others.⁴²⁾ After screening of the library and the selection of clones on the basis of binding to Cryj1 or Cryj2 and

Table 4. Pairwise Interaction Analysis of scFv Antibodies by SPR

A						
Co-injected scFv	Injected scFv					
	Cj1-56	Cj1-65	Cj1-67	Cj1-69	Cj1-73	Cj1-84
None	55.7	134.3	219.3	152.6	137.3	238.1
Cj1-56	—	183.6	266.7	215.3	195.2	292.5
Cj1-65	—	—	214.6	245.1	254	330.9
Cj1-67	—	—	—	262.7	356.6	388
Cj1-69	—	—	—	—	203.6	390.7
Cj1-73	—	—	—	—	—	226
Cj1-84	—	—	—	—	—	—

B				
Co-injected scFv	Injected scFv			
	Cj2-54	Cj2-80	Cj2-90	Cj2-103
None	33.5	99.5	43.3	46.7
Cj2-54	—	73.6	47.4	47.4
Cj2-80	—	—	88.8	92.7
Cj2-90	—	—	—	46.4
Cj2-103	—	—	—	—

Results are shown for pairwise-interaction analyses of the interaction of Cryj1- and Cryj2-specific scFv antibodies with the corresponding antigens. The values are expressed in resonance units (RUs). The values for the binding of a scFv antibody to a Cryj1- or Cryj2-coated surface are shown at the top of column of each matrix. The values for the binding of co-injected scFv antibodies to Cryj1 or Cryj2 are shown in the matrix. Interfering and non-interfering interactions are shown on gray and white backgrounds respectively.

**Fig. 3.** Schematic Representation of Epitope Recognition by Purified scFv Antibodies.

A, Schematic overview of the differences among epitopes recognized in Cryj1 (A) and in Cryj2 (B). Overlapping circles denote competing scFv antibodies.

of DNA sequencing analysis, we finally selected six and four clones as sources of Cryj1- and Cryj2-specific antibodies respectively (Table 3). Compared to commercially purchased antibodies, such as Cj1mAb13, Cj1mAb26, and Cj2mAb27, our scFv antibodies bound to the corresponding antigens more specifically, with the exception of the Cj2-54 clone, whose cross-reactivity with Cryj1 was apparently higher than that of the purchased Cryj2-specific antibody (Fig. 2A and B). Our results indicate the utility of our method for the production of highly specific antibodies directed against pollen allergens.

In general, a larger association (k_{on}) and a smaller dissociation (k_{off}) rate constant indicate faster binding of an antibody to and slower detachment of the antibody from the antigen, respectively. The dissociation constant (K_d) can be calculated from k_{on} and k_{off} , and it is a useful measure of the strength of the binding (or affinity) of the antibody for its antigen. A smaller K_d value indicates strong binding strength of the antibody to the antigen and a low dissociation rate. As indicated in Table 3, the k_{on} and k_{off} values of our Cryj1-specific scFv antibodies were smaller than those of the purchased antibodies. In addition, the K_d values of Cj1-67 and Cj1-84 were almost the same as that of Cj1mAb13, and were lower than that of Cj1mAb26. These results suggest that these two scFv antibodies can be used at least in ELISA in the same way as the purchased antibodies, even though their binding to Cryj1 occurred more slowly than that of Cj1mAb13 and Cj1mAb26. In contrast to those of Cryj1-specific scFv, the k_{on} values of Cryj2-specific scFv antibodies were somewhat higher than that of purchased antibody Cj2mAb27, but no Cryj2-specific scFv antibodies had lower k_{off} values than Cj2mAb27. The k_{off} and K_d values of the Cryj2-specific scFv antibodies indicate their unsuitability for ELISA, as compared to Cj2mAb27.

The kinetic parameters of the selected scFv antibodies listed in Table 3 were not as disparate as we predicted. Katakura *et al.*⁴³⁾ reported that differences among screening conditions have a major effect on the rate of association and dissociation of selected antibodies. The limited diversity in terms of epitope recognition of our selected scFv antibodies might have been due to our use of the same conditions for all three rounds of screening. Changes in screening conditions might make it possible to isolate different types of antibody, even if one starts with the same library.

An antibody has six complementarity-determining regions (CDRs), CDR1-H, CDR2-H, CDR3-H, CDR1-L, CDR2-L, and CDR3-L. The active conformations of these regions are limited to specific canonical structures, except in the case of CDR3-H. It has been proposed that CDR3-H acts as a linker in the V, D, and J domains, playing an important role in antigen recognition.⁴⁴⁾ As shown in Tables 1 and 2, the diversity of amino acid sequences in the CDR3-H region of our scFv antibodies was relatively large, but the results of the epitope mapping by SPR analysis suggest that our antibodies recognized Cryj1 and Cryj2 via only four and a single epitope respectively (Table 4). Unfortunately, these results indicate that characterization of scFv antibodies in terms of sequence differences in the CDR3-H region was not helpful in the classification of the antibodies, which can react with different epitopes. Although we also analyzed the nucleotide sequences of the CDR3 region in the light chain of the antibodies, we failed to obtain any useful information on the relationship between the sequence differences and binding specificities of the antibodies (data not shown).

In this study, we produced scFv antibodies with strong specificity for Cryj1 and Cryj2 and we determined some of their kinetic parameters. Large-scale preparation of an scFv antibody is easier than that of a monoclonal antibody from a hybridoma. Future mutation of the nucleotide sequences of our clones might improve the

characteristics of the antibodies and lead eventually to a system for selection of plus trees that produce low-allergen pollen.

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