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Molecular and Immunochemical Characteristics of Monoclonal and Recombinant Antibodies Selective for the Triazine Herbicide Simetryn and Application to Environmental Analysis

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A monoclonal antibody (mab) selective for the thiomethyl-*s*-triazine herbicide simetryn was obtained and characterized in enzyme-linked immunosorbent assay (ELISA). An IC_{50} value for simetryn was 8.5 ng/mL, and the detection range extended from 1.1 to 70 ng/mL in ELISA. The cDNAs encoding variable heavy chain (VH) and variable light chain (VL) of the mab were cloned to produce various recombinant antibodies. Single-chain variable fragment (scFv) antibodies derived from the mab were characterized in ELISA and showed similar reactivities and specificities to the parent mab. A urea denaturation test revealed that the scFv antibodies bound to simetryn were more stable than those in the absence of antigen. A sandwich ELISA based on VH and VL fragments of the mab was successfully developed and showed similar sensitivity to those based on the mab and scFv antibodies in ELISA. In the recovery experiments using spiked environmental samples, the results obtained in ELISA based on the mab were favorably correlated with those by HPLC.

KEYWORDS: ELISA; simetryn; triazine herbicide; monoclonal antibody; scFv

INTRODUCTION

The triazine group of herbicides is the most well-known and has been widely used for more than 40 years in the world. These chemicals inhibit photosynthesis in plants by blocking the electron transfer at the reducing site of chloroplast photosystem II electron transport (*1*). The thiomethyl-*s*-triazine herbicide simetryn introduced in 1969 in Japan is one of the principal herbicides used for control of broad-leaved weeds in paddy fields. Paddy fields for rice cultivation make up 55% of total farmland in Japan (*2*), which are some of the most important sources of environmental water pollution by pesticides because of easy runoff into rivers after the application (*3*). Simetryn has relatively high water solubility (450 mg/L) (*4*) and is of concern with respect to the influence to human health and aquatic organisms (*5*, *6*). Therefore, it is necessary to know the actual concentration levels of simetryn in the surface water.

Quantification of simetryn residues in environmental samples has been carried out by instrumental methods such as gas chromatography (*5*, *6*) or high-performance liquid chromatography (HPLC) (*7*). However, these methods require time-consuming cleanup steps and expensive equipments. On the other hand, immunoassay is a cost-effective and relatively simple method that can handle a lot of samples in a short time. The use of monoclonal antibody (mab) has some advantages such as the homogeneity of affinity and specificity to an antigen. Moreover, cloning and expression methods of antibody genes have opened the opportunities for antibody engineering (*8*). Production of recombinant antibodies in bacterial culture is even readily available and less expensive than that of mabs in animal cell culture. Gene manipulation is also available to alter the property of recombinant antibodies. Triazine herbicides such as atrazine are one of the most extensively investigated groups for environmental analysis by immunoassays and for development of recombinant antibodies (*9*−*13*).

In this paper, we attempted (1) to produce anti-simetryn monoclonal and recombinant antibodies, (2) to investigate the molecular and immunochemical characteristics of the antibodies in enzyme-linked immunosorbent assay (ELISA), and (3) to validate the ELISA system for the detection of simetryn residues in the environmental water samples.

MATERIALS AND METHODS

Chemicals and Biochemicals. Simetryn [2,4-bis(ethylamino)-6-methylthio-1,3,5-triazine] (**Figure 1**) and other triazine herbicides were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Simetryn haptens S1 and S2 (**Figure 1**) were synthesized in Otsuka Chemical Co., Ltd. (Tokushima, Japan). Rabbit serum albumin (RSA), bovine serum

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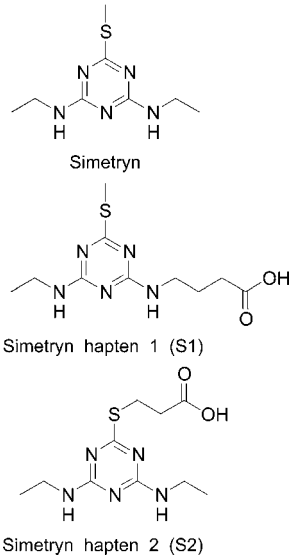

Figure 1. Chemical structures of simetryn and two haptens synthesized for this study.

albumin (BSA), and mouse anti-FLAG tag mab were purchased from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin (KLH) and anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Block Ace was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). DNA polymerase was purchased from BD Biosciences Clontech (Palo Alto, CA). DNA restriction endonucleases were purchased from Takara Bio, Inc. (Shiga, Japan). Mouse anti-HSV tag mab, pT7Blue T-vector, and pET-27b bacterial expression vector were obtained from Novagen, Inc. (Madison, WI). All other chemicals and organic solvents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of Hapten−Protein Conjugates. Simetryn haptens S1 and S2 were covalently attached to carrier proteins BSA, RSA, and KLH by the mixed anhydride method (*14*) with some modifications as described previously (*15*). The resulting conjugates were used as immunogen for mice, except for S1−RSA and S2−RSA used as coating antigen in ELISA.

Production of mabs. Five-week-old BALB/c female mice (Japan SLC Inc., Shizuoka, Japan) were immunized with S1−KLH, S1−BSA, S2−KLH, or S2−BSA as described previously (*15*). Their splenocytes were fused with P3-X63-Ag8.653 myeloma cells (*16*) according to the method by Köhler and Milstein (*17*) and screened for binding ability of their secreting antibodies toward simetryn as previously described (*15*, *18*). Culture supernatant of cloned hybridoma cells was used as an anti-simetryn mab in ELISA. Isotype of a cloned mab was determined with anti-mouse subclass specific antiserum (Bio-Rad Laboratories, Hercules, CA) according to the instructions of the manufacturer.

Cloning of cDNAs Coding for Variable Domains of an Anti-Simetryn mab. Recombinant DNA techniques were done according to the standard procedures (*19*). A mRNA fraction was extracted from 3×10^7 hybridoma cells using a QuickPrep micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, U.K.) according to the instructions of the manufacturer and reverse-transcribed. Specific amplification of cDNA fragments coding for the variable heavy chain (VH) and variable light chain (VL) of an anti-simetryn mab was done by the 5′-rapid amplification of cDNA end (RACE) method using a SMART-RACE cDNA Amplification Kit (Clontech) with two gene-specific primers (GSPs) for IgG_{2b} (5′-GGAGGAACCAGTTGTATCTC-CACACC-3′) and κ chain (5′-AGATGGATACAGTTGGTGCAGCAT-CAGC-3′). Amplified cDNA fragments were cloned into pT7Blue T-vector and transformed into competent *Escherichia coli* JM109 cells for DNA sequence analysis.

DNA Sequence Analysis. Inserts of recombinant pT7Blue plasmids were sequenced by the dideoxy method (*20*) in an automated fluorescence-based DNA sequencer 5500-L (Hitachi, Ltd., Tokyo, Japan). DNA and deduced amino acid sequences were analyzed using a DNA sequence analysis software, Genetyx-Mac 7.3 (Software Development Co., Tokyo, Japan).

Constructions of Expression Plasmids for Various Recombinant Antibody Genes. On the basis of the nucleotide sequences of cDNA clones coding for VH and VL domains of an anti-simetryn mab, synthetic oligonucleotides were designed to construct various recombinant antibody genes (**Table 1**). Two sorts of single-chain variable fragment (scFv) genes (HL and LH types, see **Figure 2**) with a flexible peptide linker $(Gly_4Ser)_3$ (*21*, *22*) were constructed by PCR as previously described (*18*). In addition, three kinds of LH type of scFv genes containing a peptide linker consisting of Gly_4Ser, $(Gly_4Ser)_2$, and $(Gly_4Ser)_4$ different in the length were constructed with the primers listed in **Table 1**. The VH and VL fragment genes (**Figure 2**) were also constructed with the primers (**Table 1**) as described previously (*23*). These constructed antibody genes were ligated into pT7Blue T-vector and transformed into *E. coli* JM109 cells for DNA sequencing. The scFv antibody and VH fragment genes were recovered by *Nhe*I/*Nco*I digestion from recombinant plasmids containing no deletion or substitution of nucleotides and ligated into a similarly restricted expression vector pET-27b. The VL fragment gene was recovered by *Bpu1102*I/*Nco*I digestion from plasmids as well and ligated into a similarly restricted vector pET-27b. These expression plasmids constructed (**Figure 2**) were each transformed into host strain *E. coli* BL21(DE3)pLysS cells for isopropyl-β-D-thiogalactopyranoside (IPTG)-induced expression. The preparation of recombinant antibodies was done as described previously (*23*). Periplasmic fractions of recombinant *E. coli* cells were directly used as a scFv antibody, VH fragment, or VL fragment in ELISA.

Immunoblot Analysis. A total of 10 μg of periplasmic proteins was separated in a 12% SDS−polyacrylamide gel (*24*). After transfer to a polyvinylidene fluoride membrane (GE Osmonics Labstore, Minnetonka, MN), the membrane was probed with an anti-HSV mab (0.2 μg/mL). The anti-HSV mab specifically binds to a HSV-tag peptide sequence (SQPELAPEDPED) at the C-terminal region of scFv antibody or VH fragment. Immunoreactive proteins were visualized using nitroblue tetrazolium and 5-bromo-4-choloro-3-indolyl phosphate as a substrate.

ELISA Based on mab. Each well of microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) was coated with 100 μL of S1−RSA (2 μg/mL) in phosphate-buffered saline (PBS: 10 mM phosphate, and 0.9% (w/v) NaCl at pH 7.2) overnight at 4 °C and blocked with 25% Block Ace in PBS overnight at 4 °C. After the plates were washed with PBS, 50 μL of standard solutions or samples and 50 μL of a mab were added to each well, followed by an incubation for 1 h at 25 °C. After washing, 100 μL of anti-mouse IgG conjugated to HRP antibody diluted 1:2000 in PBS containing 10% Block Ace (PBS-B) was added and incubated for 1 h at 25 °C. After washing, 100 μL of enzyme substrate (2 mg/mL *o*-phenylenediamine in 0.1 M phosphate-citrate buffer at pH 5.0, containing 0.03% H_2O_2) was added and incubated for 10 min at room temperature. The enzyme reaction was stopped by the addition of 50 μL of 4 N sulfuric acid, and the absorbance was read in a dual-wavelength mode (492−630 nm) in a microplate reader MTP-120 (Corona Electric Co., Ltd., Ibaraki, Japan).

ELISA Based on scFv Antibody (Figure 3A). The ELISA assay was performed as described above, except for the addition of 50 μL of a scFv antibody in place of a mab with an incubation for 1 h at 25 °C, followed by washing and adding 100 μL of an anti-HSV mab (0.1 μg/mL) in 2× PBS containing 10% Block Ace with another incubation for 1 h at 25 °C.

ELISA Based on VH and VL Fragments (Figure 3B). The ELISA assay was performed in the same method as that used for the assay based on scFv antibody, except for the addition of 25 μL of VL fragment and 25 μL of VH fragment in place of a scFv antibody with an incubation for 1 h at 25 °C, followed by washing and adding 100 μL of an anti-FLAG mab (1 μg/mL) in PBS-B with an incubation for 1 h at 25 °C. The anti-FLAG mab specifically binds to a FLAG-tag peptide sequence (DYKDDDDK) at the C-terminal region of the VL fragment.

Sandwich ELISA with VH and VL Fragments (Figure 3C). A total of 100 μL of the VH fragment was immobilized via His tag on

Table 1. Primers Used in This Study[a]

primer	nucleotide sequence (5′–3′)
primer 1	CCATGGATGATGTACAGCTTCAGGAGTCAGGAC
primer 2	GCTAGCCCGTTTGATTTCCAGCTTGGT
primer 3	AGAGCCACCTCCGCCTGAACCGCCTCCACCTGCAGAGACAGTGACCAGAGTCC
primer 4	GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGATGTTGTGATGACCCAAAGTCC
primer 5	CCATGGATGATGTTGTGATGACCCAAAGTCC
primer 6	GCTAGCTGCAGAGACAGTGACCAGAGT
primer 7	AGAGCCACCTCCGCCTGAACCGCCTCCACCCCGTTTGATTTCCAGCTTGGT
primer 8	GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGATGTACAGCTTCAGGAGTCAGGA
primer 9	GCTCAGCTTTATCATCATCATCTTTATAATCCCGTTTGATTTCCAGCTTGGT
primer 10	AGAGCCACCTCCGCCCCGTTTGATTTCCAGCTTGGT
primer 11	GGCGGAGGTGGCTCTGATGTACAGCTTCAGGAGTCAGGA
primer 12	AGAGCCACCTCCGCCTGAACCGCCTCCACCTGAACCGCCTCCACCTGAACCGCCTCCACCCCGTTTGATTTCCAGCTTGGT
primer 13	GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGGCGGTGGCGGATCGGATGTACAGCTTCAGGAGTCAGGA

[a] SMT3110scFv/HL gene was constructed with primers 1–4. SMT3110scFv/LH gene was constructed with primers 5–8. The VH fragment gene was constructed with primers 1 and 6. The VL fragment gene was constructed with primers 5 and 9. SMT3110scFv/L5H gene was constructed with primers 5, 6, 10, and 11. SMT3110scFv/L10H gene was constructed with primers 5, 6, 8, and 10. SMT3110scFv/L20H gene was constructed with primers 5, 6, 12, and 13.

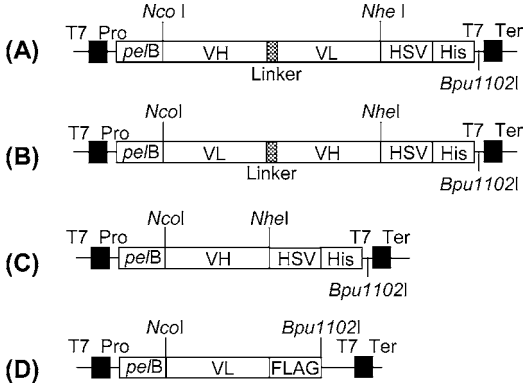

Figure 2. Construction of the recombinant antibodies SMT3110scFv/HL, SMT3110scFv/LH, and VH and VL fragments. T7 pro, T7 promoter; T7 ter, T7 terminator; pelB, *pel*B leader; HSV, HSV tag; His, 6× histidine tag; FLAG, FLAG tag. *Nco*I, *Nhe*I, and *Bpu1102*I are restriction sites. (A) SMT3110scFv/HL. (B) SMT3110scFv/LH. (C) VH fragment. (D) VL fragment.

nickel-nitrilotriacetic acid (Ni-NTA)-coated microtiter plates (Qiagen GmbH, Hilden, Germany) and blocked with 3% (w/v) skim milk in PBS. After washing, 50 μL of simetryn standard solutions and 50 μL of VL fragment were added and incubated for 1 h at 25 °C. The continuation was performed in the same method as that used for the ELISA based on VH and VL fragments.

Comparison of Intrinsic Stability of scFv Antibodies by Urea Denaturation. For the investigation of stability, scFv antibodies were purified from periplasmic extracts with Ni-NTA resin (Qiagen GmbH, Hilden, Germany) according to the instruction of the manufacturer and dialyzed against 50 mM phosphate buffer containing 2 M ammonium sulfate (pH 7.4) for 3 h at 4 °C. Then, samples were applied to hydrophobic chromatography using a TSK gel Ether-5PW column (Tosoh Co., Tokyo, Japan) as described by Morimoto and Inouye (*25*). The scFv antibodies were eluted with a linear gradient of ammonium sulfate from 2 to 0 M in 50 mM phosphate buffer at a flow-rate of 0.5 mL/min.

Urea-induced denaturation was analyzed by measuring the intrinsic fluorescence emission spectra of scFv antibodies because of the shift of maximum fluorescence emission from proteins (*26*, *27*). After an overnight incubation of the scFv antibodies in 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 0−8 M urea in the presence or absence of the antigen (1 μg/mL simetryn) at 4 °C, a fluorescence emission spectrum was scanned from 320 to 380 nm at 280 nm of an excitation wavelength in a fluorescence spectrophotometer F-2500 (Hitachi, Ltd., Tokyo, Japan). Denaturation curves were obtained by plotting the normalized wavelength shift of maximal fluorescent intensity versus the urea concentration.

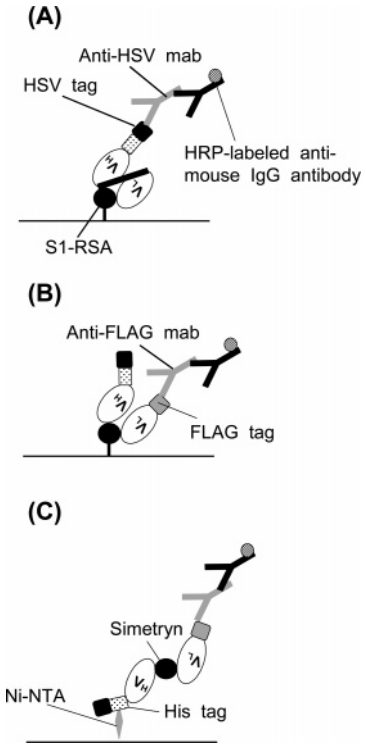

Figure 3. Conceptual representation of the three ELISA formats utilized in this study. (A) ELISA based on scFv antibodies. (B) ELISA based on Fv fragments. (C) Sandwich ELISA based on the VH and VL fragments.

Environmental Samples. River water samples (Kanzaki River, Hyogo, Japan), rice paddy water samples (experimental paddy field in Kobe University, Hyogo, Japan), and tap water samples (city water, Hyogo, Japan) were spiked with simetryn at 5, 10, and 50 ng/mL. For ELISA determination, spiked water samples were directly determined without any preparation. For HPLC analysis, the solid-phase extraction (SPE) method was employed. Spiked water samples (250 mL) were passed through a Sep-Pak C_{18} cartridge (Waters Co., Milford, MA) conditioned with acetonitrile, deionized water, and acetonitrile/water (3:7 by volume). Elution was performed with 7 mL of acetonitrile, and eluates were evaporated to dryness. Finally, the residues were reconstituted in 2 mL of methanol and analyzed by HPLC.

Soil samples (experimental paddy field in Kobe University, Hyogo, Japan) were fortified with simetryn at 500, 1000, and 5000 ng/g. Spiked soil samples (10 g) were shaken for 30 min with 20 mL of methanol, followed by centrifugation for 10 min at 1500*g*, and this extraction step was repeated again. For ELISA, the methanol extracts were subsequently diluted to 1:10 with distilled water and the diluted solution was used for the analysis. For HPLC analysis, methanol extracts were

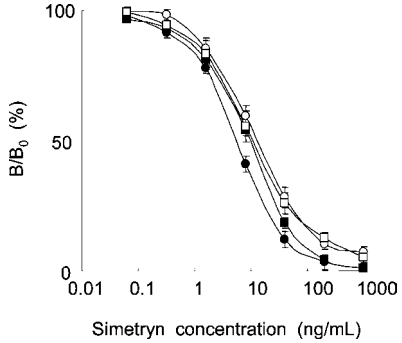

Figure 4. Inhibition curves of mab, scFv antibodies, and Fv fragments for simetryn in ELISA. The assay was performed as described in the Materials and Methods. SMT3110 (■), SMT3110scFv/HL (○), SMTs3110scFv/LH (●), and Fv fragments of SMT3110 (□).

evaporated and redissolved in 10 mL of methanol/water (1:1 by volume). Then, the purification using SPE method was performed as described above.

HPLC Analysis. An HPLC system with an UV detector (Hitachi) equipped with a Cosmosil C_{18} column (4.5 × 150 mm, Nacalai Tesque, Inc., Kyoto, Japan) was used for the analysis of simetryn. The flow rate of the mobile phase was 1.0 mL/min with a sample injection volume of 10 μL. HPLC conditions were as follows: column temperature, 40°C; mobile phase, acetonitrile/water 75:25 to 20:80 for 30 min. Simetryn was detected at a wavelength of 230 nm.

RESULTS

Production and Characterization of Anti-Simetryn mab. Six female mice were immunized with S1−BSA or S1−KLH, and four mice were immunized with S2−BSA or S2−KLH. After a series of boosts, all mice immunized with S1 conjugates showed a good antibody titer and reactivity to simetryn. However, mice immunized with S2 conjugates exhibited an insufficient antibody titer, and three of four antisera raised against S2 conjugates did not react to simetryn. Cell fusion was performed between myeloma cells and spleen cells from the immunized mice, and several hybridoma cells were cloned by the limiting dilution method. The most reactive mab with simetryn was named SMT3110 that was derived from the mouse immunized with S1−KLH. An isotyping experiment revealed that the isotype of SMT3110 was IgG_{2b} and κ chains.

The anti-simetryn mab SMT3110 was characterized in ELISA. The assay conditions of ELISA were optimized as described in the Materials and Methods. The values of absorbance were converted to B/B_0 (%) values according to the following formula:

$$B/B_0\,(\%) = (A - A_{\text{excess}}/A_0 - A_{\text{excess}}) \times 100$$

where A is a value of absorbance for each sample or standard, A_0 is a value of absorbance for the zero standard, and A_{excess} is a value of absorbance for an excess of analyte. A standard curve of SMT3110 toward simetryn in ELISA is shown in **Figure 4**. An IC_{50} (concentration of analyte giving 50% inhibition) value for simetryn was 8.5 ng/mL, and the detection range was from 1 to 70 ng/mL. When the competitive reaction was performed at a low temperature of 4 °C, an increased sensitivity of the assay was found (data not shown). However, the assay procedure became complicated, and the reproducibility was lower than that obtained at 25 °C. Therefore, the competitive reaction was performed at 25 °C. The immunoassay method using S1 hapten and the mab SMT3110 is now patented in Japan (*28*).

Cloning and Sequence Analysis of cDNA Clones Coding for the Variable Domains of SMT3110. To clone the genes coding for VH and VL domains of SMT3110, cDNAs were synthesized from mRNA isolated from hybridoma cells. The 5′-RACE method was performed with GSPs for IgG_{2a} or κ chain. The amplified cDNA fragments with an expected length of about 500 bp were confirmed by an agarose gel electrophoretic analysis. Nucleotide and deduced amino acid sequences of the VH and VL domains of SMT3110 were deposited in the GenBank/EMBL/DDBJ databases under the accession number AB100168 (VH of SMT3110) and number AB100169 (VL of SMT3110). The cDNA coding for the VH domain of SMT3110 comprises 348 bp encoding 116 amino acid residues and is a member of mouse subgroup IA according to the classification of Kabat (*29*). The VL gene comprises 339 bp encoding 113 amino acid residues, belonging to subgroup II.

(A)

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SMT3110   1 DVQLQESGPGLVKPSHSLSLTCSVTGYSITSGYYWNWIRQFPGNKLEWMG  50
IPR-7     1 E----Q-PGE----GA-MKIS-KAS---F-.--TM--VK-SH-KN---I-  49
K411B     1 Q-K--Q--G---Q..A........S-FT.SD.VWMD-V--S-EKG---VA  38
ATR35     1 QQ--V---GR--T-GG--T---T-S-IDLSRNA.MG-V--A--EG---I-  49
G3        1 QE--V---GR--T-GTP-T---T-S-F-LS-.--MS-V--A--KG---I-  49
4063      1 Q----------A--Q---I--T-S-F-L-N.-NLQ-V--P--KG---L-  49

SMT3110  51 YII..YDGSNNY.NPSLKNRISITRDTSKNQFFLNLNSVTTEDTATYYCT  97
IPR-7    50 L-NP.-N-GTS-.-QKF-GKATL-V-K-SSTAYME-S-L-F--S-V-F-A  97
K411B    39 E-RNKANNHAA-YAE-V-G-FTVS--D--SNVY-HM--LRP-D-GI----  88
ATR35    50 T--VGGST...-YA.-W.AKGRFSISKTSTTVD-KIT-P--------F-A  94
G3       50 T-SSGGGNK..WYA.-W.AKGRF-ISKTSTTVD-KIT-P--------F-A  95
4063     50 I-WPGGTTN..-.-SA-MS-LT--K-N-LS-V--KM--LRSD---M---A  96

SMT3110  98 IFSTTSAY........WGQGTLVTVSA                        116
IPR-7    98 RGNNPYY-AMD.Y...-----T----.                        119
K411B    89 RH-..YR-DGFAY...---........                        102
ATR35    95 SYASGFGQYKINYFYM----------.                        120
G3       96 RVGG.YNTVD.YYANI--P-------.                        119
4063     97 SYH..YGVA...Y...-----T----S                        115
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(B)

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SMT3110   1 DVVMTQSPLSLSVGLGDQASISCTASQSLVHSNGNTYLHWYLQKPGQSPK  50
IPR-7     1 -IEL-------P-S---------RS----L--S-Y-F------------  50
K411B     1 ........A--A-S--QR-T---R--E-.-DIY--SFM---Q-----P--  41
ATR35     1 .Q-L--T-S-V-AAV-GTVT---QS----FMN-....-A--Q-----P--  46
G3        1 EL----T-A-VE-AV-GTVT-K-Q----IG.-Q....-S--Q--S--R--  45
4063      1 -IEL----AIMFASP-EKVTMT-S--S-VT-......MN--Q--SRT---  44

SMT3110  51 LLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCAQSTHF.  99
IPR-7    51 ----T----F-------------------------------S----V.  99
K411B    42 ----RA--LET-I-A-------R-----T-NP---D-VAT---Q--KSA.  90
ATR35    47 ----GA-TLA----S--K------Q---T--D-QCD-AAT-Y--GG..YR  94
G3       46 ----RA-TLA---SS--K----------T--G-QCD-AAT-Y-Q-GFTSN  95
4063     45 -W--DT-KLP----G--------NS-S-T--SM----VAT-Y-F-GSGY.  93

SMT3110 100 P..WTFGGGTKLEIKR                                   113
IPR-7   100 -.AL---A--------                                   114
K411B    91 -..Y------------                                   104
ATR35    95 -HT-A-----DV--R-                                   110
G3       96 NIENP-----E-V--.                                   111
4063     94 -..F------E-----                                   107
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Figure 5. Comparison of the deduced amino acid sequences of variable regions of six anti-triazine antibodies. (A) VH domain. (B) VL domain. Bars indicate the same residues with SMT3110, and dots were inserted to maximize the alignment.

Amino acid sequences coding for both VH and VL domains of SMT3110 were compared with other recombinant antibodies specific for *s*-triazine herbicides previously reported (*9*−*13*) as shown in **Figure 5**. The clones G3 (*9*) and ATR35 (*12*) were isolated from rabbits, whereas the clones 4063 (*10*), K411B (*11*), and IPR-7 (*13*) were derived from mice. These antibodies are mainly targeting atrazine. The identities of amino acid sequences in the VH domain between SMT3110 and each of the others were 41−56%. The VL domain of SMT3110 shared 48−64% of the identities with each of the other anti-*s*-triazine herbicide antibodies, except for IPR-7 with a high identity of 85%. The amino acid sequences of framework region (FR) 2 and FR3 in VL domains between both SMT3110 and IPR-7 were completely identical to each other. There was only one amino acid difference between SMT3110 and IPR-7 in FR4 and complementarity-determining region (CDR) 2 of VL domains.

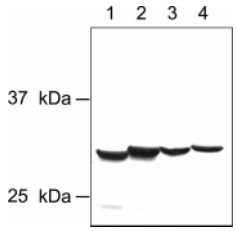


Figure 6. Immunoblot analysis of periplasmic production of scFv antibodies with various lengths of a linker in *E. coli* cells. Proteins were separated by SDS–PAGE and electrotransferred to a membrane. Lane 1, SMT3110scFv/L5H; lane 2, SMT3110scFv/L10H; lane 3, SMT3110scFv/LH; and lane 4, SMT3110scFv/L20H.

Production and Characterization of Anti-Simetryn scFv Antibodies. The cDNAs coding for VH and VL domains of SMT3110 were allowed to self-prime through the complementary region of a peptide linker sequence in the PCR reaction using the primers designed to contain a restriction enzyme site (*Nco*I or *Nhe*I) and a part of the peptide linker. The VH and VL domains were connected as VH−linker−VL (SMT3110scFv/HL) and VL−linker−VH (SMT3110scFv/LH) configurations. Construction of scFv genes expected to be 750 bp was confirmed by the agarose gel electrophoretic analysis. The scFv genes confirmed to include no errors during PCR amplifications were inserted into pET-27b expression vector. To obtain scFv protein, the constructed expression plasmids were transformed into *E. coli* BL21(DE3)pLysS strain cells. The scFv gene expression was induced by the addition of 1 mm IPTG, followed by extraction from recombinant *E. coli* periplasm by osmotic shock. The binding activity of scFv antibodies to simetryn was determined in ELISA as shown in **Figure 3A**. Standard curves of the parent mab, SMT3110scFv/HL, and SMT3110scFv/LH were shown in **Figure 4**. The IC_{50} values of SMT3110scFv/HL and SMT3110scFv/LH for simetryn were 12 and 5.8 ng/mL, respectively. The result showed that both scFv antibodies have similar reactivity to the parent mab (IC_{50} value = 8.5 ng/mL) toward simetryn. The order of linkage of variable domains seemed not to affect the reactivity of SMT3110scFv antibodies. The gene encoding SMT3110 and the immunoassay method using SMT3110scFv are now patented in Japan (*30*).

Investigation of the Length of a Peptide Linker of SMT3110scFv Antibodies. To investigate the effects of a peptide linker covalently connecting both variable domains on antigen binding, three different variants containing a linker different in the length (SMT3110scFv/L5H, SMT3110scFv/L10H, and SMT3110scFv/L20H) were additionally produced. The expression of these scFv genes in *E. coli* cells was confirmed by an immunoblot analysis with periplasmic extracts as shown in **Figure 6**. Each band was observed at the expected position of the size. These scFv antibodies were compared with respect to the reactivity toward simetryn in ELISA (**Figure 7**). The IC_{50} values of SMT3110scFv/L5H, SMT3110scFv/L10H, and SMT3110scFv/L20H were 13, 11, and 9.4 ng/mL, respectively, showing that SMT3110scFv/LH (5.8 ng/mL) is the most sensitive among all variants tested in ELISA.

Cross-Reactivity. Cross-reactivity of SMT3110 and SMT3110scFv/LH was determined by comparing IC_{50} values for various triazine compounds in ELISA. Calculation was done by the below formula:

$$\text{cross-reactivity (\%)} = (IC_{50} \text{ for simetryn}/IC_{50} \text{ for a tested compound}) \times 100$$

The result of cross-reactivity tests with the triazine compounds is shown in **Table 2**. Both antibodies showed over 30% cross-reactivity with irgarol, which is an antifouling agent used as an additive in boat paints. More than 5% cross-reactivity was observed with the other thiomethyl-*s*-triazine herbicides, except for prometryn. Both antibodies did not exhibit significant cross-reactivity with chloro-*s*-triazine herbicides and simetryn metabolites. The SMT3110scFv/HL also showed similar cross-reactivity profiles (data not shown).

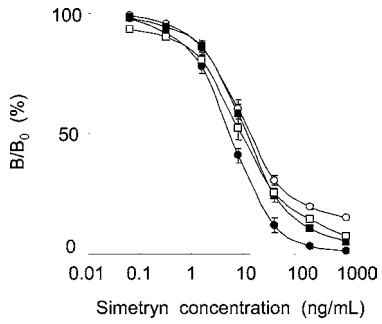
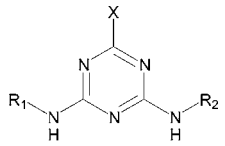


Figure 7. Inhibition curves of scFv antibodies with various lengths of a linker for simetryn in ELISA. The assay was performed as described in the Materials and Methods. SMT3110scFv/L5H (○), SMT3110scFv/L10H (■), SMT3110scFv/LH (●), and SMT3110scFv/L20H (□).

Table 2. Cross-Reactivity of the mab SMT3110 and SMT3110scFv/LH with Various *s*-Triazine Compounds in ELISA



				Mab SMT3110		SMT3110scFv /LH	
-R_1	-R_2	-X	compound	IC_{50} (ng/mL)	CR[a] (%)	IC_{50} (ng/mL)	CR[a] (%)
CH_2CH_3	CH_2CH_3	SCH_3	simetryn	8.5	100	5.8	100
CH_2CH_3	$CH(CH_3)_2$	SCH_3	ametryn	76	12	85	6.8
CH_2CH_3	$CH(CH_3)CH(CH_3)_2$	SCH_3	dimethametryn	61	14	110	5.3
$CH(CH_3)_2$	$CH(CH_3)_2$	SCH_3	prometryn	850	1.0	760	0.8
CH_2CH_3	$C(CH_3)_3$	SCH_3	terbutryn	66	13	37	16
	$C(CH_3)_3$	SCH_3	irgarol	27	31	16	36
CH_2CH_3	CH_2CH_3	OCH_3	simeton	270	3.0	160	3.6
CH_2CH_3	CH_2CH_3	Cl	simazine	3900	0.2	4500	0.1
CH_2CH_3	$CH(CH_3)_2$	Cl	atrazine	8400	0.1	>5800	<0.1
CH_2CH_3	CH_2CH_3	OH	2-hydroxysimetryn	>10000	<0.1		
CH_2CH_3	H	OH	2-hydroxy-4-desethylsimetryn	>10000	<0.1		
H	H	OH	2-hydroxy-4,6-didesethylsimetryn	>10000	<0.1		

[a] Cross-reactivity.

Reactivity of Fv Fragments with Simetryn in ELISA. To analyze the interaction between both variable domains, VH and VL fragments of SMT3110 were separately prepared. Expression of the genes in recombinant *E. coli* cells was confirmed by immunoblot analysis (data not shown). Fv fragments of SMT3110 were characterized in ELISA. Binding activity of Fv fragments toward simetryn was observed in the case of co-incubation of both VH and VL fragments as shown in **Figure 3B**. A standard curve is shown in **Figure 4**, and the IC_{50} value was 11 ng/mL. When either VH or VL fragment was absent, the other fragment did not bind to the immobilized antigen by

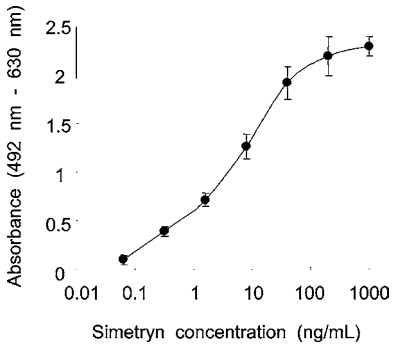


Figure 8. Standard curve of the VH and VL fragments for simetryn in sandwich ELISA. The assay was performed as described in the Materials and Methods.

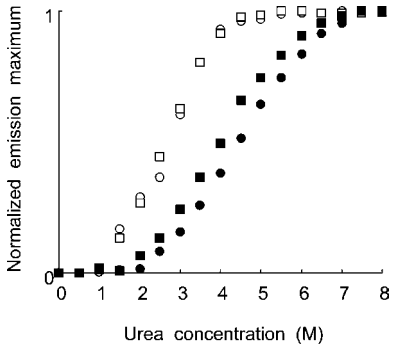


Figure 9. Comparison of intrinsic stabilities of SMT3110scFv/HL and SMT3110scFv/LH by urea denaturation. SMT3110scFv/LH without simetryn (○), SMT3110scFv/LH with simetryn (●), SMT3110scFv/HL without simetryn (□), and SMT3110scFv/HL with simetryn (■).

itself. The results showed that the ELISA based on Fv fragments had a similar sensitivity to that based on scFv antibodies toward simetryn.

Noncompetitive Sandwich ELISA with the VH and VL Fragments. We attempted to develop a sandwich ELISA with VH and VL fragments without any competition to haptens. After the VH fragment was immobilized onto microtiter plates via Ni-NTA, the VL fragment was co-incubated with simetryn (**Figure 3C**). The assay result revealed that the VH fragment interacts with the VL fragment via simetryn. A standard curve for simetryn is shown in **Figure 8**, and the midpoint value of the curve was 10 ng/mL. This value was comparable to the other ELISA assays developed in this study.

Intrinsic Stabilities of SMT3110scFv Antibodies. Thermodynamic stability of the SMT3110scFv antibodies was determined by urea denaturation. Stability of the purified SMT3110scFv/HL and SMT3110scFv/LH was compared with each other in the presence and absence of the antigen simetryn (**Figure 9**). In the absence of simetryn, the midpoints of the resulting curves for SMT3110scFv/HL and SMT3110scFv/LH were 2.6 and 2.8 M urea, respectively. In the presence of simetryn, the midpoints for SMT3110scFv/HL and SMT3110scFv/LH were increased to 4.0 and 4.5 M, respectively. This result suggested that SMT3110scFv antibodies become more stable by binding to simetryn.

Recovery Analysis of Spiked Environmental Samples by ELISA and HPLC. Environmental samples (river water, rice paddy water, tap water, and soil) were examined in ELISA based on SMT3110 to measure simetryn content. Prior to recovery tests, matrix effects on the assay were estimated. Simetryn standard solutions were prepared with river water, rice paddy water, tap water, and a methanol extract of soil diluted to 1:10 in distilled water. Each standard curve was compared to that in PBS or PBS containing 10% methanol as a control (**Figure 10**). In the case of water samples, standard curves obtained with samples agreed with the PBS control. In the case of soil samples, a standard curve obtained with samples agreed with a control prepared with PBS containing 10% methanol. Therefore, the ELISA could be used for river water, rice paddy water, tap water, and soil samples without any sample cleanup.

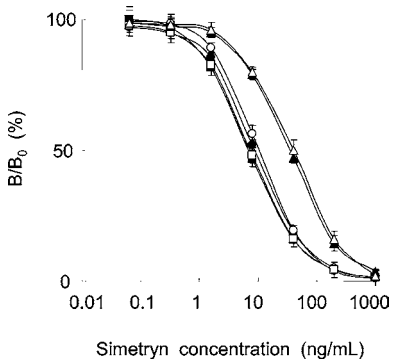


Figure 10. Matrix effects on the reactivity of SMT3110 with simetryn in ELISA. Standard solutions were prepared in PBS (●), tap water (○), river water (■), paddy water (□), PBS containing 10% methanol (▲), soil extracts diluted with water in 1:10 (△).

Table 3. Results from the Determination of Simetryn in Spiked Environmental Samples

sample	spiked concentration (ng/mL)	mean ± SD (ng/mL)	recovery (%)	CV (%)
river water	5	5.5 ± 1.0	110	12
(n = 9)	10	9.8 ± 1.0	98	10
	50	50 ± 4	101	8
rice paddy water	5	5.1 ± 0.6	101	11
(n = 9)	10	9.5 ± 1.0	95	11
	50	51 ± 4	102	8
tap water	5	4.5 ± 0.5	90	11
(n = 9)	10	11 ± 1	109	10
	50	49 ± 4	97	9
soil	500	480 ± 50	95	11
(n = 9)	1000	980 ± 40	98	4
	5000	4800 ± 200	96	4

Spiked simetryn concentrations covered a detection range from 5 to 50 ng/mL. **Table 3** summarizes the results of the recovery analysis. Mean recoveries at three spiked levels were 103% (varying from 98 to 110%) for river water, 99% (from 95 to 102%) for rice paddy water, 99% (from 90 to 109%) for tap water, and 96% (from 95 to 98%) for soil samples. Concerning the reproducibility, the average of the CV values was 9%. The results of recovery tests in ELISA were compared to the data obtained from HPLC analysis, and the desirable results were obtained. The correlation of the results between ELISA and HPLC was favorable as shown in **Figure 11** in the case of soil samples with a slope of 0.98 (r^2 = 0.99, n = 9).

DISCUSSION

To prepare anti-simetryn mabs, two kinds of simetryn haptens S1 and S2 were synthesized. After several immunizations with each hapten conjugated to carrier proteins, all mice immunized with S1 conjugates produced anti-simetryn antibodies, although S2 conjugates could not raise anti-simetryn antibodies in all immunized mice except for only one. It was reported that polyclonal antibodies showing an excellent reactivity toward

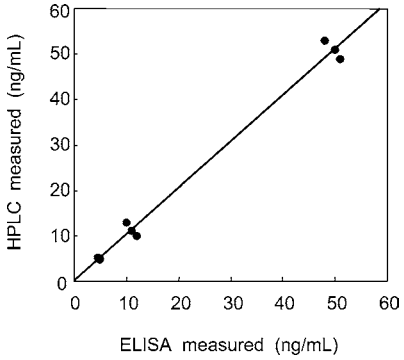


Figure 11. Correlation between HPLC and ELISA measurements of simetryn in spiked soil samples. $n = 9$, $r^2 = 0.99$, and $y = 1.03x + 0.13$.

triazine compounds were raised against the haptens with *tert*-butylamino, cyclopropylamino, or isopropylamino groups in place of the ethylamino groups of S2 hapten (*31*, *32*). Considering that these polyclonal antibodies were derived from rabbits, S2 hapten might be much less immunogenic for mice. Otherwise, further efforts may allow isolation of anti-simetryn mabs that recognize two ethylamino groups of simetryn by using S2 immunogen.

The cDNA clones coding for VH and VL domains of SMT3110 were isolated to produce various recombinant antibodies. A bacterial leader peptide *pel*B (*33*) was attached in-frame to the N terminus of recombinant antibodies for periplasmic exportation from a reducing environment of cytoplasm, because each variable domain contains a disulfide bond and the oxidation of cysteine thiols into disulfides normally occurs in an oxidizing compartment such as periplasm to form a stable folding in *E. coli* cells. To prepare SMT3110scFv antibodies, VH and VL domains were connected with a linker $(Gly_4Ser)_3$ that was designed so that Gly residues would confer the flexibility, while Ser would provide some solubility (*22*, *34*). The order of VH and VL domains did not affect the reactivity of SMT3110scFv antibodies with simetryn. Effects of length of the linker were further investigated because the linker length often affects flexibility and stability of scFv antibodies (*35*). The results showed that 15 amino acid residues $(Gly_4Ser)_3$ were the best length for the distance between VL and VH domains of SMT3110. A linker of an scFv antibody usually requires more than 12 amino acid residues in length to exhibit similar reactivity to the parent antibody (*36*, *37*). Therefore, the results obtained in this study agreed with their reports. It seems interesting to investigate their cross-reactivity of the variants, because different lengths of the linker might affect the antigen recognition.

Cross-reactivity of both SMT3110 and SMT3110scFv antibodies was determined for various triazine compounds in ELISA. These antibodies showed similar specificities among each other, implying SMT3110scFv antibodies as functional minimum units of the parent mab SMT3110. It was expected that both an ethylamino group and a methylthio group on the *s*-triazine ring were detrimental to antibody binding based on the structure of S1 hapten. The thiomethyl-*s*-triazine herbicide prometryn was hardly cross-reacted, because the herbicide has a methylthio group but not any ethylamino group. The triazine compounds in which a methylthio group was replaced with a chloro, a hydroxy, or a methoxy group at the 2 position were hardly cross-reacted with SMT3110. Similar results were observed with anti-atrazine and anti-hydroxyatrazine antibodies (*38*, *39*). It was surprising that irgarol that is substituted by a cyclopropylamino group instead of the ethylamino group of simetryn produced over 30% cross-reactivity. It might be considered that the cyclopropylamino group would allow the compound to keep the binding stable to the antibodies because of a rigid structure. On the other hand, a heterologous ELISA system was attempted to develop using S2 conjugates. However, it was failed because SMT3110 did not bind to S2 hapten. It was reported that polyclonal antibodies raised against a hapten similar to S1 reacted to another hapten similar to S2 (*31*, *32*). A heterologous ELISA would be available with other anti-simetryn mabs, if the hybridoma screening was performed using S2 conjugates.

The alignment of amino acid sequences between SMT3110 and other anti-triazine herbicide antibodies showed that the VL domain of SMT3110 has an identity of 85% with that of the anti-atrazine antibody IPR-7 (*13*). Both sequences were similar in both CDR and FR regions. It is critically interesting that the cross-reactivity of SMT3110 with atrazine was quite low (0.1%). Similar sequences between SMT3110 and IPR-7 would be involved in binding to the *s*-triazine ring. Antigen-binding activities of some anti-hapten antibodies depend on the VH domain rather than the VL domain. Therefore, the VL domain of SMT3110 would play a supplementary role with respect to antigen binding, and the VH domain would define the specificity.

The interaction between VH and VL domains of SMT3110 was investigated with separately produced VH and VL fragments in ELISA. The Fv fragments of SMT3110 showed a similar reactivity to SMT3110scFv antibodies and were bound to the antigen simetryn with a cooperative association of VH and VL fragments. Binding of separated VH and VL fragments via hapten antigen was reported with other anti-hapten antibodies (*23*, *40*, *41*). A urea denaturation experiment is a good way to test the effects of antigen on Fv fragments, because the fluorescence from indole rings of tryptophan residues in protein is highly sensitive to its environment (*42*, *43*). It revealed that the binding of SMT3110scFv antibodies to simetryn improved the stability of the protein folding, indicating that the antigen interacts between VH and VL fragments of SMT3110 to support the protein conformation. The similar results were reported previously (*26*, *44*), although differences in stability between HL and LH types of scFv antibodies has not been reported. The interaction between VH and VL fragments was employed to develop a noncompetitive sandwich ELISA. The standard curve was proportional to the analyte concentration in the sandwich ELISA and as sensitive as those based on mabs and scFv antibodies comparing the middle points of standard curves. The noncompetitive ELISA would be a more powerful immunoassay method, because the ELISA does not require any hapten compounds for assays and would be more sensitive in combination with fluorescent or luminescent enzyme substrates.

The recovery tests were performed to evaluate the ELISA reliability for the analysis of environmental samples. The results showed that the ELISA was not significantly affected by matrixes in surface water or soil. A correlation coefficient of 0.995 with a slope of 1.03 was obtained in the recovery tests with soils by HPLC and ELISA. This result showed that the ELISA is a promising tool for monitoring simetryn residues in the environment. In addition, the ELISA based on scFv antibodies and noncompetitive sandwich ELISA will give another methodology for environmental monitoring, leading to more cost-effective and sensitive assays.

ABBREVIATIONS USED

BSA, bovine serum albumin; CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent

assay; FR, framework region; GSP, gene-specific primer; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition; IPTG, isopropyl-β-D-thiogalactopyranoside; KLH, keyhole limpet hemocyanin; mab, monoclonal antibody; Ni-NTA, nickel-nitrilotriacetic acid; PBS, phosphate-buffered saline; PBS-B, PBS containing 10% Block Ace; RACE, rapid amplification of cDNA end; RSA, rabbit serum albumin; scFv, single-chain variable fragment; SPE, solid-phase extraction; VH, variable heavy chain; VL, variable light chain.

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