JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Enzyme-Linked Immunosorbent Assay with Monoclonal and Single-Chain Variable Fragment Antibodies Selective to Coplanar Polychlorinated Biphenyls

Hideyuki Inui,[†,‡,∥] Tetsuya Takeuchi,[‡,∥] Akari Uesugi,[‡,∥] Fumito Doi,[‡,∥] Mikio Takai,[†,‡,§,∥] Kosuke Nishi,[†,‡,∥,△] Shiro Miyake,[§,◇] and Hideo Ohkawa*[,†,‡]

[†]Research Center for Environmental Genomics and [‡]Graduate School of Science and Technology, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan,

[§]Horiba Biotechnology Company, Ltd., 48 Kishoin Shadocho, Minami-ku, Kyoto, Kyoto 601-8315, Japan

Supporting Information

ABSTRACT: Coplanar polychlorinated biphenyls (Co-PCBs) consisting of non-*ortho* and mono-*ortho*-chlorinated PCBs are dioxin-like compounds and cause wide contamination in the environment. To monitor Co-PCB residues, it was attempted to establish an enzyme-linked immunosorbent assay (ELISA) with monoclonal and recombinant antibodies selective to Co-PCBs. When 3,3′,5,5′-tetrachlorobiphenoxybutyric acid (PCBH)−keyhole limpet hemocyanin conjugate was immunized into mice, two monoclonal antibodies, Mab-0217 and Mab-4444, were obtained. 3,3′,5,5′-Tetrachlorobiphenyl (PCB80) was determined with an IC_{50} value of 2.6 and 0.46 ng mL^{-1} in ELISA based on Mab-0217 and Mab-4444, respectively. Mab-4444 cross-reacted with Co-PCB congeners, except for PCB77 and PCB81. Mab-0217 reacted with PCB80 and cross-reacted with PCB111. A single-chain variable fragment (scFv) antibody derived from Mab-4444 was produced in recombinant *Escherichia coli* cells. The scFv antibody showed nearly the same sensitivity toward PCBH as the parent monoclonal antibody in ELISA. These results clearly suggested that Mab-4444 and its scFv antibodies were suitable for monitoring the representative congeners of Co-PCBs.

KEYWORDS: *enzyme-linked immunosorbent assay, monoclonal antibody, coplanar polychlorinated biphenyl, single-chain variable fragment antibody*

INTRODUCTION

Polychlorinated biphenyls (PCBs) consist of 209 congeners with 1−10 chlorine substituents on biphenyl rings. PCBs show nonflammability, low electrical conductivity, and stability to chemical breakdown. Therefore, they had been particularly suited for usage in electrical equipments, hydraulic equipment, and heat transfer systems. PCBs were released into the environment from PCB-containing residential and industrial wastes. Because these residues were highly persistent in the environment and adsorbed onto soils and organic materials, PCBs tended to be accumulated especially in the sediment of rivers, lakes, and seas and then bioaccumulated at the tops of aquatic food chains. Coplanar PCBs (Co-PCBs) consisting of non-*ortho*- and mono-*ortho*-chlorine-substituted congeners are dioxin-like compounds. Certain Co-PCB congeners selectively bound to an aryl hydrocarbon receptor and exhibited dioxin-like toxicity.[1]

PCB residue analyses have been performed by the use of instruments such as gas chromatography (GC) and GC−mass spectrometry (GC-MS).[2] In addition, immunoassays with polyclonal antibodies for PCBs such as an enzyme-linked immunosorbent assay (ELISA), which is a rapid, sensitive, and cost-effective assay, were reported.[3,4] Chiu et al. developed a monoclonal antibody (Mab)-based enzyme immunoassay (EIA).[5] The EIA was highly selective to PCB77 and PCB126 (0.9 and 1.2 ng mL^{-1} 50% inhibition concentration (IC_{50}) values, respectively), but did not detect more prevalent and much less toxic noncoplanar PCB congeners. In addition, the EIA had a relatively low tolerance toward methanol. A polyclonal antibody-based ELISA was also reported by Fránek et al.[6] The ELISA was selective to PCB77 and PCB126 (2.0 and 5.2 ng mL^{-1} IC_{50} values, respectively). However, tolerance toward some organic solvents such as dimethyl sulfoxide (DMSO) was also low. ELISA kits selective to PCB118,[7] PCB77, and PCB169[8] are commercially available. These immunoassays seemed to be suitable for a rapid screening of environmental samples contaminated with representative Co-PCBs.

The objective of this study was to establish a more sensitive and selective ELISA system toward Co-PCBs for monitoring of contamination in the environment. A Co-PCB hapten derived from PCB80 was used for the preparation of monoclonal antibodies selective to Co-PCBs. In addition, single-chain variable fragment (scFv) antibodies were designed and prepared for possible practical usage relative to monoclonal antibodies.

MATERIALS AND METHODS

Chemicals. 3,3′,5,5′-Tetrachlorobiphenyl (PCB80) and 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA) and Sigma-Aldrich

Received: September 6, 2011
Revised: January 17, 2012
Accepted: January 24, 2012
Published: January 24, 2012

ACS Publications © 2012 American Chemical Society

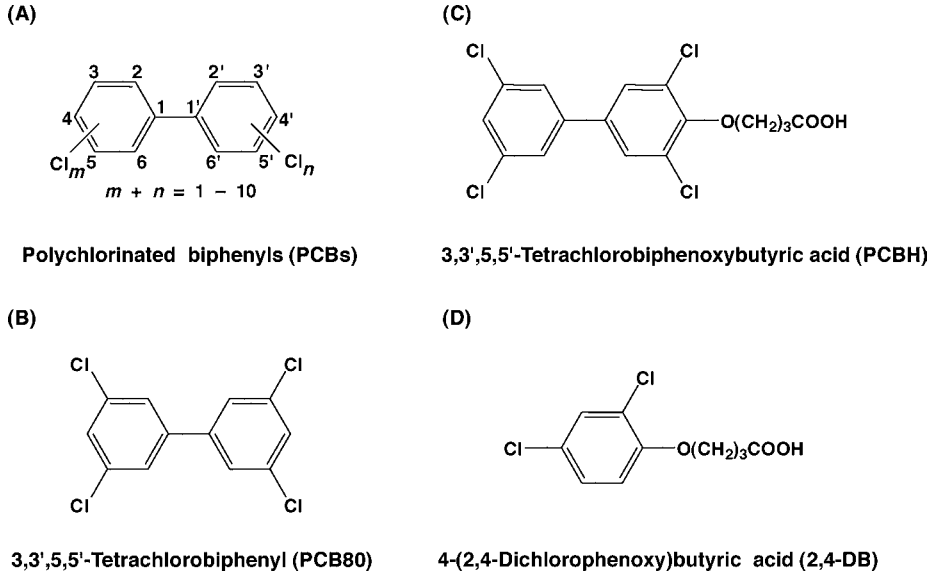

Figure 1. Structures of polychlorinated biphenyls (PCBs, A), 3,3′,5,5′-tetrachlorobiphenyl (PCB80, B), Co-PCB hapten 3,3′,5,5′-tetrachlorobiphenoxybutyric acid (PCBH, C), and PCB hapten, 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB, D).

Table 1. Cross-Reactivity of Mab-0217 and Mab-4444 toward Co-PCB Congeners and Structurally Related Compounds in ic-ELISA

			Mab-0217		Mab-4444	
IUPAC no.	compound	WHO-TEF[a]	IC_{50} (ng mL^{-1})	cross-reactivity (%)	IC_{50} (ng mL^{-1})	cross-reactivity (%)
non-*ortho* PCB						
80	3,3′,5,5′-tetrachlorobiphenyl		2.6	100	0.46	100
77	3,3′,4,4′-tetrachlorobiphenyl	0.0001	>1000	<0.3	>1000	<0.1
81	3,4,4′,5-tetrachlorobiphenyl	0.0003	>1000	<0.3	>1000	<0.1
126	3,3′,4,4′,5-pentachlorobiphenyl	0.1	>1000	<0.3	2.3	20
169	3,3′,4,4′,5,5′-hexachlorobiphenyl	0.03	>1000	<0.3	0.63	73
mono-*ortho* PCB						
105	2,3,3′,4,4′-pentachlorobiphenyl	0.00003	>1000	<0.3	51	0.91
111	2,3,3′,5,5′-pentachlorobiphenyl		22	12	2.2	21
114	2,3,4,4′,5-pentachlorobiphenyl	0.00003	>1000	<0.3	320	0.15
118	2,3′,4,4′,5-pentachlorobiphenyl	0.00003	>1000	<0.3	95	0.48
123	2′,3,4,4′,5-pentachlorobiphenyl	0.00003	>1000	<0.3	63	0.74
156	2,3,3′,4,4′,5-hexachlorobiphenyl	0.00003	>1000	<0.3	11	4.1
157	2,3,3′,4,4′,5′-hexachlorobiphenyl	0.00003	>1000	<0.3	11	4.4
167	2,3′,4,4′,5,5′-hexachlorobiphenyl	0.00003	>1000	<0.3	43	1.1
189	2,3,3′,4,4′,5,5′-heptachlorobiphenyl	0.00003	>1000	<0.3	1.9	24
di-*ortho* PCB						
133	2,2′,3,3′,5,5′-hexachlorobiphenyl		460	0.56	810	<0.1
180	2,2′,3,4,4′,5,5′-heptachlorobiphenyl		>1000	<0.3	>1000	<0.1
	hapten					
	PCBH		0.44	600	0.07	660
	4-(2,4-dichlorophenoxy)butyric acid		750	0.35	4100	<0.1

[a]TEF, toxic equivalency factor.

Co. (St. Louis, MO), respectively. 3,3′,5,5′-Tetrachlorobiphenoxybutyric acid (PCBH) was provided by Otsuka Chemical Co. (Tokushima, Japan). PCBH was used as a Co-PCB hapten based on PCB80 and conjugated to each of the carrier proteins, keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA), to give PCBH−KLH and PCBH−BSA, respectively, for immunization and coating antigen, respectively. 2,4-DB was also used as a PCB hapten based on a part of PCB molecules and conjugated to BSA to give 2,4-DB−BSA for a coating antigen. Conjugation reactions were carried out by the active ester method as described previously.[10] Excess amounts of the haptens PCBH and 2,4-DB were used for preparation of the corresponding conjugates, which seemed to confer enough binding of the haptens to obtain antibodies.[11] Chemical structures of these compounds are shown in Figure 1. PCB congeners listed in Table 1 were obtained from AccuStandard Inc. (New Haven, CT).

Immunization. Four-week-old Balb/c mice purchased from Nippon SLC Co. (Shizuoka, Japan) were interperitoneally immunized with 100 μL of the conjugate PCBH−KLH emulsion. Immunizing doses consisted of 100 μg of the conjugate in 50 μL of phosphate-buffered saline (PBS) emulsified with an equal volume of TiterMax Gold adjuvant (CytRx Co., Los Angeles, CA). One month after the first immunization, secondary immunization was performed with 25 μg

dx.doi.org/10.1021/jf203585b | J. Agric. Food Chem. 2012, 60, 1605−1612

of the conjugate. After 1 week, blood samples of immunized mice were taken by tail bleeding and left for 30 min at room temperature. Then, blood samples were centrifuged for 15 min at approximately 10000*g*. Sera were collected and diluted 50 times with PBS. Titers of antisera were examined in indirect noncompetitive (in)-ELISA, and inhibition test for PCBH was performed in indirect competitive (ic)-ELISA. Mice producing anti-Co-PCB antibodies were selected as spleen donors for cell fusion and injected interperitoneally with 25 μg of the conjugate in 50 μL of PBS 4 days prior to cell fusion.

Cell Fusion. Procedures for cell fusion and hybridoma production were based on the method by Köler and Milstein with slight modifications.[12] Mouse spleen cells were fused with P3-X63-Ag8.653 myeloma cells provided from Iatron Laboratories Inc. (Tokyo, Japan) at 5:1 ratio by the use of PEG1500 (Nacalai Tesque, Inc., Kyoto, Japan) as a fusing agent. Fused cells were distributed in 96-well culture plates at a density of 2×10^{55} cells well^{-1} in hypoxanthine−aminopterin−thymidine (HAT) selection medium [Dulbecco's Modification of Eagle's Medium (DMEM)] supplemented with 8 μg mL^{-1} insulin, 50 U mL^{-1} penicillin−streptomycin, 100 μM hypoxanthine, 0.4 μM aminopterine, 16 μM thymidine, and 10% (v/v) fetal bovine serum and cultured at 37 °C under air containing 5% carbon dioxide gas. Seven days after cell fusion, half of the culture medium of wells was replaced by fresh HAT medium.

Screening and Cloning of Hybridoma Cells. Ten to 14 days after cell fusion, culture supernatants were screened in both in-ELISA and ic-ELISA for the presence of antibodies that recognize the hapten PCBH. Hybridoma cells producing antibodies with a high affinity to PCBH selected by ic-ELISA were cloned by a twice limiting dilution method and cultured in HAT medium without aminopterine (HT medium). Stable antibody-producing clones were expanded and cryopreserved under liquid nitrogen. Cell culture supernatants were used as monoclonal antibodies without further purification.

in-ELISA. Ninety-six-well microtiter plates were coated with 100 μL well^{-1} of the coating antigen 2,4-DB−BSA (3 μg mL^{-1}) in PBS overnight at 4 °C. Wells were washed three times with PBS. Sites uncoated with the coating antigen were blocked with 250 μL well^{-1} of 25% (v/v) Blocking One (Nacalai Tesque, Inc.) in distilled water. After plates were incubated overnight at 4 °C, wells were washed as described above. A mouse antiserum or a culture supernatant containing monoclonal antibodies diluted in PBS containing 10% (v/v) methanol was added to a well (100 μL), and plates were incubated for 1 h at 25 °C. After washing, 100 μL of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Thermo Fisher Scientific Inc., Rockford, IL) diluted at 1:2000 in PBS containing 10% (v/v) Blocking One was added to wells, and plates were incubated for 1 h at 25 °C. After washing, 100 μL of an enzyme substrate solution [2 mg mL^{-1} (w/v) *o*-phenylenediamine (Nacalai Tesque, Inc.), 0.03% (v/v) hydrogen peroxide in 100 mM citrate−phosphate buffer, pH 5.0] was added to wells, and color was developed for 10 min at room temperature. Enzymatic reaction was stopped by adding 50 μL of 1 N sulfuric acid. Absorbance was measured at 492−630 nm with the microplate reader MTP-500 (Corona Electric Co. Ltd., Ibaraki, Japan).

ic-ELISA. Ninety-six-well microtiter plates were coated and blocked as described for in-ELISA. After the plates had been washed, 50 μL of standard solution of the Co-PCB hapten PCBH or PCB80 diluted in distilled water containing 20% (v/v) methanol was added to wells, and 50 μL of an antibody solution diluted in 2 times concentrated PBS was added. The latter procedure was described above. Values of absorbance were converted to B/B_0 (%) values according to the following formula: B/B_0 (%) = $(A/A_0) \times 100$, where A is a value of absorbance for the standard and A_0 is a value of absorbance for the control. Reactivity of antisera and culture supernatants was evaluated by IC_{50} value (IC_{50} value was determined as a concentration of a compound required for reducing the maximum absorbance to 50%.).

Cross-Reactivity to PCB Congeners. Fifteen PCB congeners containing Co-PCB congeners were used for the assay of cross-reactivity of Mab-0217 and Mab-4444 in ic-ELISA. PCB standard solutions were prepared in distilled water containing 20% (v/v) methanol and mixed with a monoclonal antibody solution diluted in 2 times concentrated PBS (pH 8). Cross-reactivity (%) was calculated according to the following formula:

$$\text{cross-reactivity (\%)} = (IC_{50} \text{ for PCB80}) / (IC_{50} \text{ for the other congeners}) \times 100$$

Cloning of cDNAs for Mab-4444. Cloning of cDNAs encoding variable fragments of light (VL) and heavy (VH) chains of Mab-4444 was carried out according to previous studies with slight modifications.[13,14] cDNAs were each amplified by the 5′-RACE method using a SMART-RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA) with a universal primer mixture and gene-specific primers for constant regions of murine IgG 1 or IgG 2a heavy chain or κ light chain. The primer sequences were as follows: 5′-ACCGATGGGGCTGTTGTTTTGG-3′ for IgG 1 chain; 5′-AGATGGATACAGTTGGTGCAGCATCAGC-3′ for κ chain; 5′-GACAGATGGGGGTGTCGTTTTGGC-3′ for IgG 2a chain. 5′-RACE-PCR was performed under the following conditions: 30 s at 94 °C and 3 min at 72 °C for 5 cycles, 30 s at 94 °C and 30 s at 72 °C for 5 cycles, and 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C for 20 cycles. Amplified cDNA fragments encoding VL and VH were cloned into pSTBlue-1 (EMD Chemicals, Inc., San Diego, CA) and then sequenced.

Construction of Genes for a scFv VL−VH (LH)-FLAG Antibody. A gene encoding a scFv LH-FLAG antibody containing VL and VH in order was constructed by two-step PCR as reported previously with slight modifications.[13] cDNAs for VL and VH were each amplified in the first PCR, using two primers: 5′-CCATGGATGACATCCTGATGACCC-3′ and 5′-AGAGCCACCTCCGCCTGAACCGCCTCCACCCCGTTTTATCTCCAGCTTG-3′ for VL cDNA and 5′-GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAGATCCAGCTGCA-3′ and 5′-CTCCGAG*TTTATCATCATCATCTTTATAAT*CTGCAGAGACAGTGACCAGAG-3′ for VH cDNA. Sequences corresponding to the $(Gly_4Ser)_3$ linker are underlined, and sequences corresponding to the FLAG tag are italicized. The first PCR was carried out under conditions of 30 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C for 30 cycles. The second PCR without primers was also carried out under conditions of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for 7 cycles, and then assembling of VL and VH cDNAs was performed under conditions of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for 20 cycles after the addition of primers. Resulting gene products were cloned into pSTBlue-1 as a scFv LH-FLAG antibody fragment.

Expression of a scFv LH-FLAG Gene Derived from Mab-4444 in Recombinant *Escherichia coli* Cells. A gene encoding a scFv LH-FLAG antibody of Mab-4444 was inserted into pET-26b(+) (EMD Chemicals, Inc.) containing a *pel*B leader sequence for periplasmic secretion and a hexahistidine tag sequence (Figure 5A). The recombinant plasmid was introduced into the host strain *E. coli* Rosetta (DE3) pLysS cells (EMD Chemicals, Inc.) for isopropyl-β-D-thiogalactopyranoside (IPTG)-induced expression.

Recombinant *E. coli* cells were grown overnight at 25 °C in 2 mL of 2× yeast extract/tryptone (YT) medium [1.6% (w/w) tryptone, 1% (w/w) yeast extracts, and 5% (w/w) sodium chloride] containing 25 μg mL^{-1} kanamycin and 33 μg mL^{-1} chloramphenicol, and induced by adding IPTG to the culture medium at a final concentration of 1 mM. After induction for 4 h at 25 °C, *E. coli* cells were harvested for the periplasmic fraction by an osmotic shock, and a soluble fraction was extracted by freeze−thaw treatment.[15] Titration of scFv antibodies from each fraction was tested in in-ELISA.

Production of a scFv LH-FLAG Antibody of Mab-4444. Recombinant *E. coli* cells producing an active scFv-FLAG antibody in in-ELISA were inoculated into 200 mL of SB medium supplemented with 25 μg mL^{-1} kanamycin and 33 μg mL^{-1} chloramphenicol and grown overnight at 25 °C. After the production of scFv antibodies was induced by adding IPTG, periplasm or soluble/insoluble fractions were extracted. Then, periplasmic extracts were prepared by osmotic shock, and soluble/insoluble fractions were extracted by the use of alumina. Briefly, *E. coli* cells were harvested by centrifugation, and then alumina was added at a rate of 2.5 g per gram weight of pellets. *E. coli*

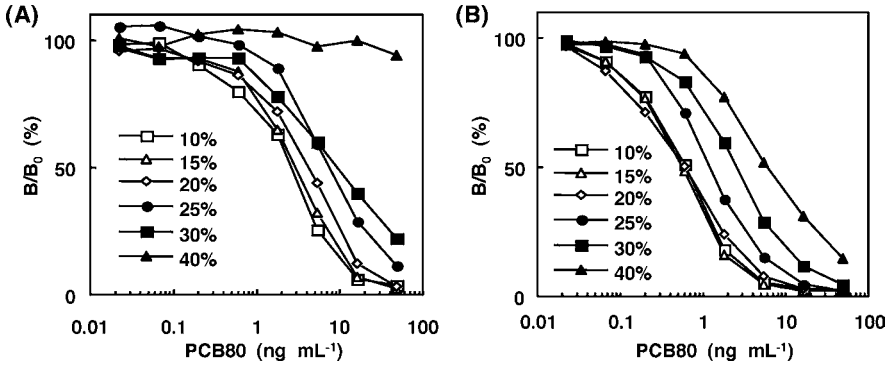

Figure 2. Methanol tolerance of the monoclonal anti-Co-PCB antibodies Mab-0217 (A) and Mab-4444 (B) in ic-ELISA for PCB80. Final concentrations of methanol in the competitive reaction are indicated as percentage.

cells were disrupted by grinding with alumina and resuspended in a phosphate buffer [50 mM sodium hydrogenphosphate, 300 mM sodium chloride, 10% (w/v) glycerol, pH 7.0]. A cell lysate was centrifuged (10000*g*, 20 min), and supernatant was collected as soluble protein extracts. Pellets were resuspended in a phosphate buffer containing 8 M urea and centrifuged. Resulting supernatants were again partitioned as insoluble protein extracts.

Purification of a scFv LH-FLAG Antibody of Mab-4444. A scFv LH-FLAG antibody containing a hexahistidine tag was extracted from three fractions and was further purified by an immobilized metal affinity chromatography using TALON resin (Clontech Laboratories Inc.). Preparation of affinity media was performed following the manufacturer's instruction. Proteins containing a scFv LH-FLAG antibody in a phosphate buffer were added to 1 mL of the resin. The resin was gently agitated (end-over-end) at room temperature for 20 min for binding a scFv LH-FLAG antibody to the resin and centrifuged. Supernatant was carefully removed, and the resin was washed with a phosphate buffer. The suspension was gently agitated at room temperature for 10 min to promote thorough washing and centrifuged. A phosphate buffer was added to the resin, and resuspension was transferred to a 1 mL polypropylene column (QIAGEN, Hilden, Germany). Then, a scFv antibody was eluted by adding an elution buffer (a phosphate buffer containing 150 mM imidazole, pH 7.0) to the column, and eluates were collected in 400 μL fractions. All fractions were evaluated by using a Bradford protein assay[16] and in-ELISA to test for the presence of a scFv LH-FLAG antibody.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples of scFv LH-FLAG antibodies in periplasmic, soluble, and insoluble fractions were loaded to 12% SDS-PAGE and then stained by CBB Stain One (Nacalai Tesque, Inc.).

ic-ELISA Based on a scFv LH-FLAG Antibody of Mab-4444. Microtiter plates were coated with 100 μL well^{-1} of PCBH–BSA (10 μg mL^{-1}) in PBS overnight at 4 °C, washed three times with PBS, and blocked with 2% BSA (250 μL well^{-1}) for 2 h at room temperature. After the plates had been washed, 50 μL of standard solution of the Co-PCB hapten PCBH in distilled water and 50 μL of a scFv LH-FLAG antibody diluted in 6× PBS containing 10% Blocking One were added to wells and incubated for 1 h at 25 °C. After washing with PBS, antibody cocktail (100 μL well^{-1}) containing 4000 fold-diluted mouse anti-FLAG M2 monoclonal antibody (Sigma-Aldrich Co.) and 2000-fold diluted HRP-labeled goat anti-mouse IgG (Thermo Fisher Scientific Inc.) in PBS with 10% Blocking One was incubated for 1 h at 25 °C. The subsequent procedure was described above.

RESULTS

Production of Anti-Co-PCB Monoclonal Antibodies. For the preparation of monoclonal antibodies selective to representative congeners of Co-PCBs, the Co-PCB hapten PCBH based on PCB80 was used. After the third or fourth immunization with the conjugate PCBH–KLH, antisera taken from 10 mice were each tested for titration in in-ELISA. Then, inhibition tests for the Co-PCBH hapten PCBH were performed with these antisera in ic-ELISA. All antisera showed high antibody titers regardless of existence of 10% methanol in antisera diluents and also showed competitive binding between the coating antigen PCBH–BSA or 2,4-DB–BSA and PCBH at low parts per billion levels (data not shown). On the basis of the results, it was found that certain positive wells contained hybridoma cells producing antibodies, which reacted with the coating antigens 2,4-DB–BSA for Mab-0217 and PCBH–BSA for Mab-4444 in ic-ELISA. After cloning, the hybridoma cell lines producing the anti-Co-PCB monoclonal antibodies Mab-0217 and Mab-4444 were established.

Optimization of Assay Conditions in ic-ELISA. With the monoclonal antibody Mab-4444, a heterologous ic-ELISA using 2,4-DB–BSA as a coating antigen was found to be about 200 times more sensitive than a homologous ic-ELISA using PCBH–BSA (data not shown). Therefore, assay conditions of the heterologous ic-ELISA were optimized for PCBH and PCB80.

When effects of concentration of the coating antigen 2,4-DB–BSA were examined in ic-ELISA for PCB80, the plates coated with <5 μg mL^{-1} of 2,4-DB–BSA were more sensitive than those coated with >5 μg mL^{-1} of 2,4-DB–BSA (data not shown). The results also showed a similar pattern between Mab-0217 and Mab-4444 on assays coated with 2–4 μg mL^{-1} of 2,4-DB–BSA (data not shown). On the basis of the results, 3 μg mL^{-1} of 2,4-DB–BSA was selected as the optimal concentration of the coating antigen in ic-ELISA.

Changes of assay temperature and time did not significantly affect the sensitivity with Mab-0217 (Supplementary Figure 1A in the Supporting Information). On the other hand, the assay based on Mab-4444 at 4 °C for 2 h was 3 times more sensitive to PCBH than that at 25 °C for 1 h in comparison with IC_{50} values (Supplementary Figure 1B in the Supporting Information). Considering sensitivity and assay convenience, the assay conditions for 1 h at 25 °C and for 2 h at 4 °C for Mab-0217 and Mab-4444, respectively, were selected.

The best IC_{50} value with Mab-4444 was obtained from the assay at pH 8 (IC_{50} = 0.4 ng mL^{-1}), although the IC_{50} value with Mab-0217 was 2.3–2.9 ng mL^{-1} at the range of pH from 5 to 8 (data not shown). However, the absorbance at 492 nm was decreased under low-pH conditions. Thus, the phosphate buffer at pH 8 was used as an assay buffer for both antibodies.

Because PCBs are highly hydrophobic, certain amounts of organic solvents were needed for preparing PCB diluents.

Therefore, tolerance of the antibodies toward methanol was examined in ic-ELISA for PCB80 (Figure 2). Although >15% of methanol decreased color developments in the assay with both monoclonal antibodies, the absorbance was increased by 40% methanol with Mab-0217 and Mab-4444 (data not shown). With both Mab-0217 and Mab-4444, assay sensitivity did not change in the presence of 10−20% methanol (IC_{50} values for PCB80 of 2.7−3.1 ng mL^{-1} and 0.55−0.58 ng mL^{-1}, respectively), although competitive binding was not observed with >40% methanol with Mab-0217. The reproducible reactions with Mab-0217 and Mab-4444 were obtained from the assays of PCB80 standard solutions containing 10−20% of methanol. On the basis of the results, 20% of methanol in PCB standard solutions, namely, 10% in the competitive reaction, was used for both antibodies.

Standard curves in ic-ELISA for PCB80 under the optimal conditions are shown in Figure 3. The IC_{50} values with Mab-0217 and Mab-4444 were 2.6 and 0.46 ng mL^{-1}, respectively, and the detection ranges were 0.7−7.0 and 0.15−1.3 ng mL^{-1}, respectively. Therefore, it was found that ic-ELISA with Mab-4444 was more sensitive to PCB80 than ic-ELISA with Mab-0217.

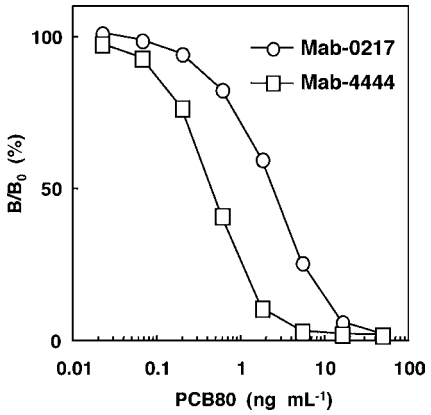

Figure 3. Standard curves for PCB80 in ic-ELISA with the anti-Co-PCB antibodies Mab-0217 and Mab-4444 under optimized conditions.

Cross-Reactivity of Anti-Co-PCB Monoclonal Antibodies in ic-ELISA. Cross-reactivity of Mab-0217 and Mab-4444 in ic-ELISA was examined for Co-PCB congeners and structurally related compounds as shown in Table 1. Mab-0217 reacted with PCB80 and cross-reacted with PCB111 having a structure similar to that of PCB80. In contrast, Mab-4444 reacted with PCB80 and showed high cross-reactivity toward PCB169 (73%), PCB189 (24%), PCB111 (21%), and PCB126 (20%). Also, the antibody slightly cross-reacted with PCB157, PCB156, and PCB167, but did not react with PCB77 and PCB81. These were non-*ortho*- and mono-*ortho*-chlorinated PCB congeners. However, the antibody did not react with di-*ortho*-chlorinated PCB congeners. When 2,4-DB was used for assay, it almost did not cross-react with both antibodies, although PCBH showed >600% of cross-reactivity. On the basis of the results, it was found that Mab-0217 was selective to 3,3′,5,5′-tetrachloro substituents including PCB80 and PCB111, but not including PCB169 and PCB189. Mab-4444 showed selectivity toward non-*ortho*- and mono-*ortho*-chlorinated Co-PCB congeners with toxic equivalency factor (TEF) values except for PCB77 and PCB81.

Cloning of VL and VH cDNAs from Mab-0217 and Mab-4444. mRNA fractions extracted from 1×10^7 cells of two hybridoma cell lines producing Mab-0217 and Mab-4444 were used as sources of anti-Co-PCB immunoglobulin (IgG) cDNAs. Each of the mRNA fractions was used for the first-strand cDNA synthesis. To isolate IgG cDNAs, the 5′-RACE method was employed, resulting in the expected sizes of cDNA fragments. Then, cDNA fragments were amplified with each of gene-specific primers (data not shown). DNA sequencing revealed that all of the cDNA fragments were murine IgG genes. The cloned VL cDNAs of Mab-0217 and Mab-4444 contained 324 bp encoding 108 amino acids (Figure 4A,C). In contrast, the VH cDNAs of Mab-0217 and Mab-4444 contained 363 bp encoding 121 amino acids and 351 bp encoding 117 amino acids, respectively (Figure 4B,D). The identity in the amino acid sequences of VL and VH between two antibodies was 60.2 and 38.0%, respectively (Supplementary Figure 2 in the Supporting Information).

Production of Mab-4444 scFv LH-FLAG Antibody. The recombinant *E. coli* cells expressing genes encoding Mab-4444 scFv LH-FLAG antibody (Figure 5A) were partitioned into periplasmic, soluble, and insoluble fractions. SDS-PAGE showed that a band around 29 kDa corresponding to the scFv LH-FLAG antibody was present in all fractions (Figure 5B). The periplasmic fractions were further purified. Among them, a band corresponding to the LH-FLAG antibody was also detected in Western blot analysis by the use of anti-FLAG antibody in these three fractions (data not shown). A similar band was found in a scFv VH and VL (HL)-*Herpes simplex* virus (HSV) antibody derived from Mab-0217 in which a band corresponding to the scFv HL-HSV antibody was detected in SDS-PAGE and Western blot analyses (data not shown). In contrast, weak bands were observed in Western blot analysis by the use of the scFv LH-FLAG antibody from Mab-0217 and the scFv HL-HSV antibody from Mab-4444, but not in SDS-PAGE (data not shown).

ic-ELISA. Inhibition curves for the Co-PCB hapten PCBH with Mab-4444 scFv LH-FLAG antibody and Mab-4444 in ic-ELISA are shown in Figure 5C. The reactivity of the scFv LH-FLAG antibody was similar to that of the corresponding parent monoclonal antibody Mab-4444. The IC_{50} value of PCBH for the scFv LH-FLAG antibody was 3.0 ng mL^{-1}, whereas that for Mab-4444 was 4.6 ng mL^{-1}. In contrast, the scFv LH-FLAG antibody of Mab-0217 and the scFv HL-HSV antibodies of Mab-0217 and Mab-4444 did not show any competitive reactions toward PCBH (data not shown).

On the basis of the results, it was found that both Mab-4444 and the corresponding scFv LH-FLAG antibodies showed a similar reactivity toward the Co-PCB hapten PCBH in ic-ELISA. Therefore, it seemed that both Mab-4444 and the scFv LH-FLAG antibodies were similarly sensitive to Co-PCB congeners in ic-ELISA.

DISCUSSION

It was attempted to establish ic-ELISA with monoclonal and corresponding scFv LH-FLAG antibodies selective to the representative congeners of Co-PCBs, because these residues were widely contaminated in the environment and highly accumulated at the tops of aquatic food chains. Several ELISA kits so far developed were reported to contain antibodies selective to PCB77, PCB81, PCB118, PCB126, PCB156, and PCB169.[7,8] Detection levels of these kits were at parts per billion levels. In this study, the Co-PCB hapten PCBH based

(A)

```
1- GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACC -60
    D  I  Q  M  T  Q  T  T  S  S  L  S  A  S  L  G  D  R  V  T
61- ATCAGTTGCAGGACAAGTCAGGACATTAGGACTTATTTAAACTGGTATCAGCAGAAACCA -120
    I  S  C  R  T  S  Q  D  I  R  T  Y  L  N  W  Y  Q  Q  K  P
121- GATGGAACTGTTACCCTCCTCATCTACTACACATCAAGATTACACTCAGGAGTCCCATCA -180
    D  G  T  V  T  L  L  I  Y  Y  T  S  R  L  H  S  G  V  P  S
181- AGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCTGGAGCAA -240
    R  F  S  G  S  G  S  G  T  D  Y  S  L  T  I  S  N  L  E  Q
241- GAAGATGTTGCCACTTACTTTTGCCAACAGGTTAATACGCTTCCGTGGACGTCCGGTGGA -300
    E  D  V  A  T  Y  F  C  Q  Q  V  N  T  L  P  W  T  F  G  G
301- GGCACCAAACTGGAAATCAAACGG
    G  T  K  L  E  I  K  R
```

(B)

```
1- GACGTGAACCTCGTGGAGTCTGGGGGAGGCTCAGTGAAGCTTGGAGGGTCCCTGAAACTC -60
    D  V  N  L  V  E  S  G  G  G  S  V  K  L  G  G  S  L  K  L
61- TCCTGTGCAGCCTCTGGATTCACTTTCAGTACCTATTACATGTCTTGGGTTCGCCAGACT -120
    S  C  A  A  S  G  F  T  F  S  T  Y  Y  M  S  W  V  R  Q  T
121- CCTGAGAAGAGGCTGGAGGTGGTCGCAGCCATTAATAGTAATGGTGGTAGGTCATACTAT -180
    P  E  K  R  L  E  V  V  A  A  I  N  S  N  G  G  R  S  Y  Y
181- CCAGACACTATGAGGGGCCGATTCACGATCTCCAGAGACAATGCCAAGAACACCCTGTAC -240
    P  D  T  M  R  G  R  F  T  I  S  R  D  N  A  K  N  T  L  Y
241- CTGCAAATGAGCAGTCTGAAGTCTGAGGACACAGCCTTGTATTACTGTGCAAGGGCCTAC -300
    L  Q  M  S  S  L  K  S  E  D  T  A  L  Y  Y  C  A  R  A  Y
301- TATGGTAATAACGTGGGGTACTTTGACTACTGGGGCCAAGGCACCGCTCTCACAGTCTCC -360
    Y  G  N  N  V  G  Y  F  D  Y  W  G  Q  G  T  A  L  T  V  S
361- TCA
    S
```

(C)

```
1- GACATCCTGATGACCCAATCTCCATCCTCCATGTCTGTCTCTCTGGGAGACACAGTCAGC -60
    D  I  L  M  T  Q  S  P  S  S  M  S  V  S  L  G  D  T  V  S
61- ATCACTTGCCATGCAAGTCAGGGCATTAGCAGAAATATAGGGTGGTTGCAGCAGAAACCA -120
    I  T  C  H  A  S  Q  G  I  S  R  N  I  G  W  L  Q  Q  K  P
121- GGGAAATCATTAAAGGGCCTGATCTATCATGGAACCACCTTGGAAGATGGAGTTGCATCA -180
    G  K  S  L  K  G  L  I  Y  H  G  T  T  L  E  D  G  V  A  S
181- AGGTTCAGTGGCAGTGGATCTGGAGCAGATTATTCTCTCACCATCAGCAGCCTGGAATCT -240
    R  F  S  G  S  G  S  G  A  D  Y  S  L  T  I  S  S  L  E  S
241- GAAGACTTTGCAGACTATTACTGTGTACAGTATGCTCAGTTTCCGTACACGTTCGGAGGG -300
    E  D  F  A  D  Y  Y  C  V  Q  Y  A  Q  F  P  Y  T  F  G  G
301- GGGACCAAGCTGGAGATAAAACGG
    G  T  K  L  E  I  K  R
```

(D)

```
1- GAGATCCAGCTGCAGCAGTCTGGACCTGAGTTGGTGAAGCCTGGGGCTTCAGTGAAGGTA -60
    E  I  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K  V
61- TCCTGCAAGCCTTCTGGTTACTCATTTACTGACTTCAACATATTCTGGGTGAAGCAGAGC -120
    S  C  K  P  S  G  Y  S  F  T  D  F  N  I  F  W  V  K  Q  S
121- CATGGAAAGAGTCCTGAGTGGATTGGATATATTGCTCCTTATAATGGTGGTTCTACTTAC -180
    H  G  K  S  P  E  W  I  G  Y  I  A  P  Y  N  G  G  S  T  Y
181- AATCAGAAGTTCAAGGGCAAGGCCACATTGACTGTTGACAAGTCCTCCAGCACAGCCTTC -240
    N  Q  K  F  K  G  K  A  T  L  T  V  D  K  S  S  S  T  A  F
241- ATGCATCTCAACAGCCTGACATCTGAAGACTCTGCAGTCTATTACTGTGCAAGATGGAAT -300
    M  H  L  N  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  W  N
301- TACTATAGAGGAACTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA
    Y  Y  R  G  T  Y  W  G  Q  G  T  L  V  T  V  S  A
```

Figure 4. Nucleotide and deduced amino acid sequences of light (A, C) and heavy (B, D) chains in variable regions of the anti-Co-PCB antibodies Mab-0217 (A, B) and Mab-4444 (C, D).

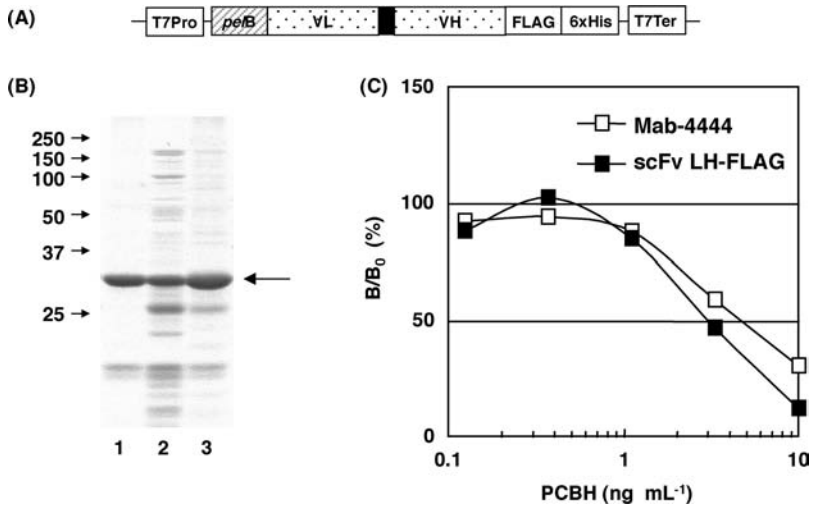

Figure 5. ic-ELISA for PCBH with Mab-4444 scFv LH-FLAG antibody produced in recombinant *E. coli* cells: (A) expression plasmid for Mab-4444 scFv LH-FLAG antibody gene (T7Pro, T7 promoter; *pel*B, *pel*B leader sequence; VL, variable region of light chain; VH, variable region of heavy chain; 6×His, hexahistidine tag; FLAG, FLAG tag, T7Ter, T7 terminator); (B) SDS-PAGE for periplasmic (lane 1), soluble (lane 2), and insoluble (lane 3) fractions purified by IMAC; (C) standard curves for PCBH in ic-ELISA with Mab-4444 and the scFv LH-FLAG antibody.

on PCB80 was used for preparation of monoclonal antibodies selective to Co-PCB congeners including PCB126 (TEF 0.1) and PCB169 (TEF 0.03). The Co-PCB hapten PCBH had a spacer arm with a carboxyl group at the para-position of the biphenyl structure. Chiu et al. and Fránek et al. also used the haptens with the same linker position for development of an immunoassay for Co-PCBs.[5,6] In the other immunoassays for PCBs, the haptens had a linker at the ortho-position of the biphenyl structure.[4,8] It seemed that a linker at the para-position is important for keeping the coplanarity of the hapten structure for obtaining Co-PCB-selective monoclonal antibodies.

By the use of PCBH−KLH for immunization, two anti-Co-PCB monoclonal antibodies, Mab-0217 and Mab-4444, were obtained. The heterologous ic-ELISA with 2,4-DB−BSA as the coating antigen was established, because hapten heterology was commonly used to eliminate problems from no or poor inhibition by the target analyte.[12] It usually resulted in

somewhat weaker recognition of a coating antigen on wells than that of a target analyte by an antibody. Thus, Co-PCBs at lower concentrations may compete with 2,4-DB−BSA under equilibrium conditions, resulting in a better assay sensitivity than that of homologous system. These were supported by the results that the IC_{50} values in ic-ELISA with 2,4-DB−BSA as the coating antigen for 2,4-DB were much higher than those for PCBH (Table 1). Interestingly, 2,4-DB did not also show higher affinity than PCBH did in the case of ic-ELISA with Mab-4444 scFv LH-FLAG antibody (data not shown). These results suggested that conformation of the scFv LH-FLAG antibody was kept without decrease of binding to PCBH.

Increased sensitivity toward PCBH in ic-ELISA with Mab-4444 incubated for 2 h at 4 °C suggested that under nonequilibrium conditions, Mab-4444 interacted with PCBH easily. Furthermore, it seemed that interaction between antigen and antibody was increased under low-temperature conditions. However, sensitivity toward PCB80 was low under low-temperature conditions as compared with PCBH because of its physicochemical property.

Although ELISA systems for Co-PCBs reported so far were tolerant toward organic solvents such as 5% of methanol and 1.3% of DMSO,[5,6] these tolerances in ELISA decreased sensitivity in ELISAs for PCBs. Both Mab-0217 and Mab-4444 showed tolerance to methanol up to 30 and 40%, respectively. Therefore, Co-PCB dilutions with PBS or distilled water containing 20% (v/v) of methanol were used in ic-ELISA (final concentration of methanol was 10%).

It is important to investigate cross-reactivity of both Mab-0217 and Mab-4444 with Co-PCB congeners and structurally related compounds. The results suggested that Mab-0217 selectively recognized 3,3′,5,5′-tetrachloro substituents including PCB80 and PCB111, but not PCB169 and PCB189, suggesting that chlorines at the 4- and 4′-positions were responsible for recognition of the antibodies. Furthermore, Mab-0217 highly reacted with the Co-PCB hapten PCBH. Therefore, the antibody may recognize not only 3,3′,5,5′-tetrachloro substituents but also a part of phenoxybutyric acid. In contrast, Mab-4444 seemed to mainly recognize 3,3′,5,5′-tetrachloro substituents, but not 2,2′-dichloro substituents. Therefore, Mab-4444 seemed to be selective to Co-PCB congeners except for PCB77 and PCB81. Neither antibody showed cross-reactivity toward PCB77, due to the structure of PCBH used for immunization. Docking models for PCB congeners showing cross-reactivity seemed to be useful for creation of PCB-binding antibodies with high specificity.[9]

To achieve simplicity for development of the PCB ELISA system, anti-Co-PCB scFv antibody genes were constructed and successfully produced for the scFv LH-FLAG antibody derived from Mab-4444 in the periplasmic fraction of the recombinant *E. coli* cells. Simple purification steps brought functional scFv LH-FLAG antibody. LH-FLAG of Mab-0217 and HL-HSV of Mab-4444, showing low expression levels, seemed to be unstable in recombinant *E. coli* cells, indicating that these orders of variable fragments were not suitable for stable expression. In contrast, HL-HSV did not show competitive binding toward PCBH, even in high expression, suggesting that a functional scFv antibody was not produced due to inappropriate protein folding. On the basis of these results, functional expression of an scFv antibody gene in recombinant *E. coli* cells with appropriate order of VL and VH to maintain a binding site for an antigen seemed to be one of the most important points to develop the scFv antibodies for an ELISA system. In previous papers, the scFv antibodies toward bisphenol A or the herbicide simetryn showed higher sensitivity than the parent monoclonal antibodies in ELISA.[13,14]

In this study, we produced two monoclonal antibodies with different Co-PCB selectivities and sensitivities and the scFv antibody for development of Co-PCB ELISA. The ELISA system with Mab-4444 may be useful for screening of environmental samples contaminated with low concentrations of Co-PCB congeners by the detection of the representative congeners of Co-PCBs. Preliminary results showed that Mab-4444 detected PCB80 in soils from agricultural fields spiked with 10 ng $(g\ soil)^{-1}$ with approximately 60% of a recovery rate. The corresponding scFv LH-FLAG antibody seemed to be also useful for an immunoaffinity column for cleanup of Co-PCB congeners in environmental samples because it was easily propagated and had a small molecular size.[15]

SAFETY

This study was approved by the Institutional Animal Care and Use Committee (permission no. 14-10) and the Committee for Safe Handling of Living Modified Organisms (permission no. 19-68) in Kobe University and carried out according to the Kobe University Animal Experimentation Regulations and the Guidelines of the Committee, respectively.

ASSOCIATED CONTENT

Supporting Information

Supplementary Figure 1. Effects of temperature and incubation time in competitive reaction in ic-ELISA with Mab-0217 (A) and Mab-4444 (B). Supplementary Figure 2. Alignments of amino acid sequences of light (A) and heavy (B) chains in variable regions of Mab-0217 and Mab-4444. Lines represent complementarity-determining regions. Asterisks represent identical amino acid residues between Mab-0217 and Mab-4444. Gaps are inserted to maximize alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81-78-582-1559. Fax: +81-78-582-1559. E-mail: hideo.ohkawa@gmail.com.

Present Addresses

△Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan.

◇Research and Development Division, HORIBA Ltd., 2 Kishoin Miyanohigashimachi, Minami-ku, Kyoto, Kyoto 601-8510, Japan.

Author Contributions

‖These authors contributed equally to the work.

Funding

This study was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Otsuka Chemical Co. (Osaka, Japan) for PCBH.

ABBREVIATIONS USED

BSA, bovine serum albumin; Co-PCB, coplanar PCB; DB, dichlorophenoxy butyric acid; DMSO, dimethyl sulfoxide; EIA,

enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; GC, Gas chromatography; HL, heavy chain−light chain; HSV, *Herpes simplex* virus; HAT, hypoxanthine−aminopterin−thymidine; HRP, horseradish peroxidase; ic, indirect competitive; in, Indirect noncompetitive; IPTG, isopropyl-β-D-thiogalactopyranoside; KLH, keyhole limpet hemocyanin; MS, mass spectrometry; LH, light chain−heavy chain; PBS, phosphate-buffered saline; PCB, polychlorinated biphenyl; PCBH, 3,3′,5,5′-tetrachlorobiphenoxybutyric acid; scFv, single-chain variable fragment; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEF, toxic equivalency factor; VH, variable fragment of heavy chain; VL, variable fragment of light chain.

REFERENCES

(1) Takeuchi, S.; Iida, M.; Yabushita, H.; Matsuda, T.; Kojima, H. In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron. *Chemosphere* **2008**, *74*, 155−165.

(2) Inui, H.; Wakai, T.; Gion, K.; Kim, Y. S.; Eun, H. Differential uptake for dioxin-like compounds by zucchini subspecies. *Chemosphere* **2008**, *73*, 1602−1607.

(3) Fránek, M.; Hruska, K.; Šisák, M.; Diblíková, I. Development of a microcolumn radioimmunoassay for screening of polychlorinated biphenyls in milk and in animal fats. *J. Agric. Food Chem.* **1992**, *40*, 1559−1565.

(4) Fránek, M.; Pouzar, V.; Kolář, V. Enzyme-immunoassays for polychlorinated biphenyls: structural aspects of hapten-antibody binding. *Anal. Chim. Acta* **1997**, *347*, 163−176.

(5) Chiu, Y. W.; Carlson, R. E.; Marcus, K. L.; Karu, A. E. A monoclonal immunoassay for the coplanar polychlorinated biphenyls. *Anal. Chem.* **1995**, *67*, 3829−3839.

(6) Fránek, M.; Deng, A.; Kolář, V.; Socha, J. Direct competitive immunoassays for the coplanar polychlorinated biphenyls. *Anal. Chim. Acta* **2001**, *444*, 131−142.

(7) Okuyama, A.; Takenaka, H.; Nishi, K.; Mizukami, H.; Kozaki, S.; Kirihata, M.; Miyatake, K.; Takigami, H.; Sakai, S.; Morita, M. Development of enzyme-linked immunosorbent assay for the pre-screening of coplanar polychlorinated biphenyls. *Organohal. Compd.* **2002**, *58*, 333−335.

(8) Glass, T. R.; Ohmura, N.; Saiki, H.; Sawadaishi, K.; Kataoka, C.; Takagi, Y.; Ohiwa, T. Development and characterization of new monoclonal antibodies specific for coplanar polychlorinated biphenyls. *Anal. Chim. Acta* **2004**, *517*, 161−168.

(9) Pellequer, J. L.; Chen, S. W.; Keum, Y. S.; Karu, A. E.; Li, Q. X.; Roberts, V. A. Structural basis for preferential binding of non-*ortho*-substituted polychlorinated biphenyls by the monoclonal antibody S2B1. *J. Mol. Recog.* **2005**, *18*, 282−294.

(10) Hsiao, H. Y.; Royer, G. P. Immobilization of glycoenzymes through carbohydrate side chains. *Arch. Biochem. Biophys.* **1979**, *198*, 379−385.

(11) Singh, K. V.; Kaur, J.; Varshney, G. C.; Raje, M.; Suri, C. R. Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjugate Chem.* **2004**, *15*, 168−173.

(12) Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **1975**, *256*, 495−497.

(13) Nishi, K.; Takai, M.; Morimune, K.; Ohkawa, H. Molecular and immunochemical characteristics of monoclonal and recombinant antibodies specific to bisphenol A. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 1358−1367.

(14) Nishi, K.; Ishiuchi, M.; Morimune, K.; Ohkawa, H. Molecular and immunochemical characteristics of monoclonal and recombinant antibodies selective for the triazine herbicide simetryn and application to environmental analysis. *J. Agric. Food Chem.* **2005**, *53*, 5096−5104.

(15) Inui, H.; Takehara, A.; Doi, F.; Nishi, K.; Takai, M.; Miyake, S.; Ohkawa, H. A scFv antibody-based immunoaffinity chromatography column for clean-up of bisphenol A-contaminated water samples. *J. Agric. Food Chem.* **2009**, *57*, 353−358.

(16) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.