J. Microbiol. Biotechnol. (2007), **17**(4), 571–578

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Production and Characterization of Monoclonal and Recombinant Antibodies Against Antimicrobial Sulfamethazine

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Received: July 16, 2006
Accepted: October 13, 2006

Abstract A monoclonal antibody (mab) against the antimicrobial sulfamethazine was prepared and characterized by an indirect competitive enzyme-linked immunosorbent assay (IC-ELISA). Sulfamethazine in the range of 0.2 and 45 ng/ml could be determined with the mab by IC-ELISA. cDNAs encoding a variable heavy chain and variable light chain of the mab were cloned to produce recombinant antibodies using phage display technology. Following phage rescue and three rounds of panning, a single-chain variable fragment (scFv) antibody with high sulfamethazine-binding affinity was obtained. ELISA analysis revealed that scFv antibody and parent mab showed similar, but not identical, characteristics. The IC_{50} value by IC-ELISA with scFv antibody was 4.8 ng/ml, compared with 1.6 ng/ml with the parent mab. Performances of the assays in the presence of milk matrix were compared; the mab-based assay was less affected than the scFv-based assay. Sixty milk samples were analyzed by mab-based IC-ELISA, and four samples were sulfamethazine positive; these results were favorably correlated with those obtained by HPLC.

Keywords: ELISA, sulfamethazine, antimicrobial, monoclonal antibody, scFv antibody

Sulfonamides are antimicrobial agents widely used in veterinary medicine for prophylactic, therapeutic, and growth-promoting purposes [15]. Improper use or improper time of withdrawal can lead to the presence of illegal residues in milk, meat, and other animal byproducts [22]. Sulfamethazine has been identified as the major agent in 95% of all sulfonamide tissue violations [18]. The safety of sulfamethazine for consumers has been questioned because of its potential toxic effects. Adverse effects known to be associated with high levels of antibiotic residues include allergenic effects, carcinogenicity, and antibiotic resistance of microorganisms. To prevent any problem with consumers, the maximum residue limit (MRL) for the sum of sulfonamides has been set to 100 μg/kg in the United States and the European Union member states, and the Codex Committee of FAO/WHO has specified a maximum residue limit to 25 μg sulfamethazine per kg of milk [23]. To comply with these regulations, it is necessary to use simple, fast, and sensitive methods to screen for sulfamethazine in food samples.

Generally, high-performance liquid chromatography (HPLC) and/or HPLC/mass spectrometry (HPLC/MS) have been used to quantify the amount of sulfamethazine residues in samples [1, 5, 6, 23]. Although these instrumental analytical methods are highly accurate and sensitive, they are also time-consuming and labor-intensive. On the other hand, immunoassays are analytical methods based on the interaction of an analyte with an antibody that recognizes it with high affinity and specificity. They are simple, cost-effective, do not require sophisticated instrumentation, and are able to analyze many samples simultaneously [15]. These features make ELISAs very powerful tools for sulfonamide residues analysis [4, 14, 16, 18].

Currently, recombinant antibody technology enables one to manipulate antibody genes for the engineering of single-chain variable fragment antibodies consisting of the variable heavy chain (V_H) and variable light chain (V_L) of an antibody

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[9, 17, 21, 25], both of which are connected by a flexible peptide linker, and for the large-scale production of recombinant antibodies in *Escherichia coli.*

In the present study, we produced and characterized monoclonal and recombinant antibodies against the antimicrobial sulfamethazine, and used these antibodies in the construction of an immunoassay to detect sulfamethazine in samples.

Materials and Methods

Chemicals

Sulfamethazine and other sulfonamides were purchased from Sigma Chemical Co. (MO, U.S.A.). Sulfamethazine haptens S1 and S2 (Fig. 1) were synthesized by Dr. Sergey A. Eremin (Lomonosov Moscow State University, Russia). Keyhole limpet hemocyanin (KLH), soybean trypsin inhibitor type II (STI), and ovalbumin (OVA) were purchased from Sigma Chemical Co. (MO, U.S.A.). DNA polymerase and DNA restriction endonuclease were purchased from TaKaRa Bio, Inc. (Shiga, Japan). HRP-conjugated anti-E tag antibody, HRP-conjugated anti-M13 antibody, phagemid pCANTAB5E, *E. coli* TG1, and *E. coli* HB2151 were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). The thio-affinity T-gel was purchased from Pierce (Rockford, IL, U.S.A.). All other chemicals and organic solvents were commercially available and were of reagent grade or better.

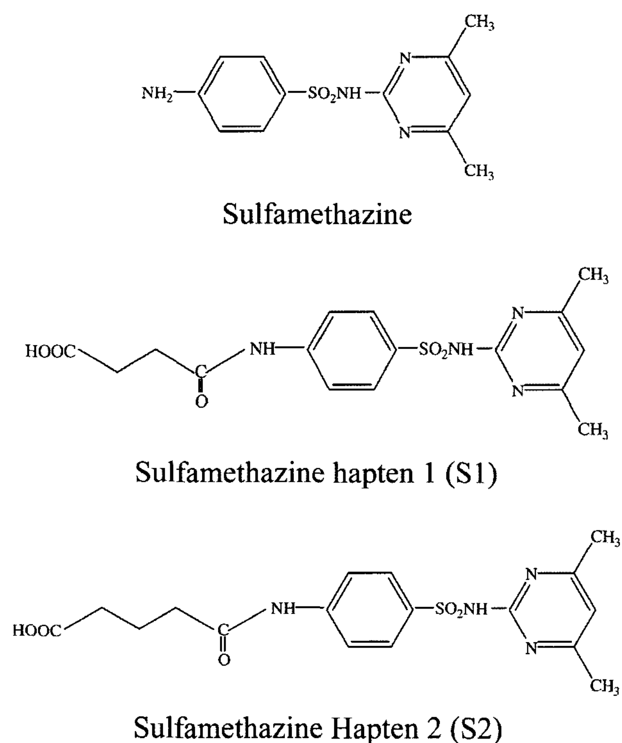

Fig. 1. Chemical structures of sulfamethazine and two haptens synthesized in this study.

Preparation of Hapten-Protein Conjugates

Sulfamethazine haptens S1 and S2 were covalently attached to carrier proteins KLH, STI, and OVA, according to the active esters method described by Schneider and Hammock [19]. The conjugates formed were purified by dialysis in 0.05 M phosphate-buffered saline (pH 7.4) for three days. The resulting conjugates were used as immunogens for mice, except for S1-OVA and S2-OVA used as coating antigens in ELISA.

Production of Monoclonal Antibodies

Seven-week-old BALB/c female mice were immunized with S1-KLH, S1-STI, S2-KLH, or S2-STI as described previously [2]. Immunized mouse spleen cells (6×10^7) were fused with myeloma cells (P3-X63-Ag8.653) according to standard procedures [7]. ELISA-positive hybridoma cells were cloned twice by the limiting dilution method. Then, the hybridoma cells were injected into BALB/c mice, and ascites were produced and collected. Mabs were purified from mice ascites by ammonium sulfate precipitation, followed by protein G affinity chromatography. The purified mabs were analyzed by SDS-PAGE and stored at -20°C. The protein concentration of purified mabs was determined using a protein assay kit (Bio-Rad). The isotype of a cloned mab was determined with a mouse monoclonal antibody isotyping kit (Roche Applied Science, U.S.A.) according to the instructions of the manufacturer.

Production of ScFv Antibodies

For cloning of monoclonal antibody variable chain fragment genes, mRNA was extracted from 2×10^7 hybridoma cells using mRNA purification kit (Amersham Biosciences, U.S.A.). About 1 µg of mRNA was reverse transcribed using an random hexamer primers and the first-strand cDNA synthesis kit (MBI Fermentas). The scFv DNA fragments were constructed by assembling the amplified V_H and V_L genes with a linker coding for a $(Gly_4Ser)_3$ peptide and ligated into pCANTAB5E vector (Amersham Biosciences, U.S.A.) as described by Marks *et al.* [12]. *E. coli* TG1 cells were transformed with the recombinant pCANTAB5E vector and used to prepare phage particles for panning by rescue using the M13K07 helper phage, as described by Yuan *et al.* [26].

Sulfamethazine-specific recombinant phages were isolated by three rounds of panning with sulfamethazine-OVA coated cell culture flasks as described by Choi *et al.* [3] and used to infect *E. coli* HB2151 to produce soluble scFv antibodies. Colonies raised from infected *E. coli* HB2151 cells on the plates were picked into 96-deep, well cell culture plates (Bioneer, Korea) containing 0.75 ml of 2×YT medium with ampicillin (100 µg/ml) and glucose (0.1%, w/v).

Following 4 h of incubation at 37°C, 0.25 ml of 2×YT medium containing IPTG (3 mM) was added to each well, and incubation was continued for 20 h at 28°C. Finally, 1 ml of PBST was added to each well, and the plates were centrifuged at 1,000 ×*g* for 20 min. The supernatant in each well was tested for the antibody activity to sulfamethazine by screening ELISA using an HRP-conjugated anti-E tag antibody (Amersham Biosciences).

One selected clone of *E. coli* HB2151 that can produce sulfamethazine-specific scFv antibody was inoculated into a 2-l flask containing 1.5 l of SB medium supplemented with 100 μg/ml of ampicilin for large-scale preparation of scFv antibody. Culture supernatant containing the extracellular soluble scFv antibody was separated from the cell pellets by centrifugation at 2,500 ×*g* for 15 min and filtered through a 0.45-μm-pore-size filter. The cell pellets were used to prepare cytoplasmic and periplasmic extracts using the protocol of McCarthy and Hill [13]. The scFv antibody in periplasmic extracts was purified by thiophilic adsorption chromatography, as described by Schulze *et al.* [20]. The purified scFv antibody was analyzed by SDS-PAGE. Protein concentration of the purified scFv was determined using a protein assay kit (Bio-Rad).

SDS-PAGE and Western Blot Analysis of ScFv Antibody

Proteins in 15 μl of concentrated supernatant (0.3 ml of supernatant concentrated by 10% trichloroacetic acid), 15 μl of periplasmic extract, or 15 μl of cytoplasmic extract were separated on 12.5% SDS-polyacrylamide and transferred to nitrocellulose membranes. The membranes were blocked with 3% skim milk in PBS for 1 h at room temperature. HRP-conjugated anti-E tag antibody (diluted 1:2,000 with PBS) was incubated with the membrane for another 1 h. The membrane was washed twice with PBST for 10 min at room temperature. The Western blot was visualized using the ECL Western blotting analysis system (Pharmacia Biotech) according to the manufacturer's instruction.

DNA Sequence Analysis

The clone producing scFv antibody with the highest affinity for sulfamethazine in *E. coli* HB2151 was chosen for DNA sequencing by an automated DNA sequencer (PerkinElmer Life Sciences).

IC-ELISA Based on Mab

Each well of microtiter plates (Nunc, Denmark) was coated overnight at 4°C with 100 μl of sulfamethazine-OVA (1 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and blocked with 1% skim milk in PBS at 37°C for 1 h. After the plates were washed with PBST, 50 μl of standard solutions or samples and 50 μl (1.5 μg/ml) of mab were added to each well, followed by incubation for 1 h at 37°C. After washing, 100 μl of goat anti-mouse IgG conjugated to HRP antibody (diluted 1:5,000 in PBS) was added and incubated for 1 h at 37°C. Then, 100 μl/well of ABTS solution containing 0.03% H_2O_2 was added and incubated for 30 min in the dark at room temperature. Absorbance was measured at 405 nm using a microtiter plate reader (Bio-Rad, Benchmark 550).

IC-ELISA Based on ScFv Antibody

The ELISA assay was performed with an incubation for 1 h at 37°C as described above, except for addition of 50 μl (4 μg/ml) of a scFv antibody in place of a mab, followed by washing and adding 100 μl/well of HRP-conjugated anti-E tag mouse monoclonal antibody (diluted 1:2,000 with PBS) with incubation for another 1 h at 37°C.

Cross-Reactivity

To measure the cross-reactivity of the compounds structurally related to sulfamethazine with mab and scFv antibody, standard curves of sulfadiazine, sulfapyridine, sulfachloropyridazine, sulfamethizole, sulfamerazine, sulfamethoxazole, sulfaquinoxaline, sulfisozole, sulfathiazole, and sulfadimethoxine were constructed in the same manner as for IC-ELISA. The cross-reaction was calculated as the ratio of the mass of sulfonamides giving 50% inhibition of the maximum responses to the concentration of sulfamethazine as standard.

Sample Preparation

Milk samples were obtained from local markets. Before the spike and recovery studies, each test sample was verified by HPLC to contain <1 ng/ml of sulfamethazine. For a spiking study, 10 ml of milk samples were spiked with different levels of sulfamethazine dissolved in methanol, thoroughly mixed, and then 10 ml of acetone was added and shaken by hand. After centrifugation for 5 min at 2,500 ×*g*, the middle clear liquid was diluted 1:20 with PBST and analyzed by IC-ELISA, based on mab 1H11 or scFv antibody.

HPLC Assay for Sulfamethazine

The test sample was verified using DIONEX (Germany) HPLC equipped with a P580 pump with UVD and ASI-100 automated sample injector. A C18 column (250 mm× 4.6 mm i.d., 5 μl) was used. The analyses were performed at 265 nm, and the mobile phase was 0.02 M PBS buffer (pH 3.1)/ACN (155: 47) at a flow rate of 1.0 ml/ml.

RESULTS AND DISCUSSION

Production and Characterization of Anti-Sulfamethazine Mab

Six female BALB/c mice were immunized with S1-KLH or S1-STI and six female BALB/c mice were immunized with S2-KLH or S2-STI. Following a series of boosts, the titers

of antisera from mice were examined by a noncompetitive ELISA. All mice immunized with S2-KLH showed a good antibody titer and reactivity to sulfamethazine. However, mice immunized with S1 conjugates or S2-STI exhibited an insufficient antibody titer, and three of six antisera raised against S1 conjugates did not react to sulfamethazine. In theory, immunogens with longer spacers between hapten and carrier protein are preferable to those with shorter spacers [8]. In this study, hapten S1 had a two-methylene chain as the linker arm for the conjugation with carrier protein, whereas hapten S2 had a three-methylene chain for the conjugation with carrier protein, and this might be the reason that mice immunized with S1 conjugates exhibited an insufficient antibody titer. Considering that mice immunized with S1-STI and S2-STI conjugates did not produce anti-sulfamethazine antibodies, STI protein might not be a good sulfamethazine carrier protein for mice immunization.

Cell fusion between myeloma cells and spleen cells from the immunized mice was carried out, and fifteen hybridoma cell lines secreting anti-sulfamethazine monoclonal antibodies were obtained. The most reactive mab with sulfamethazine was named 1H11 that was derived from the mouse immunized with S2-KLH. The hybridoma cell line 1H11 was adapted to an ascites preparation. Monoclonal antibody 1H11 was purified by ammonium sulfate precipitation, followed by protein G affinity chromatography. The purified mab 1H11 was confirmed by SDS-PAGE analysis (Fig. 2). The protein concentration of purified mab 1H11 was 1.5 mg/ml, as determined by the protein assay kit (Bio-Rad). The purified mab was used in indirect competitive

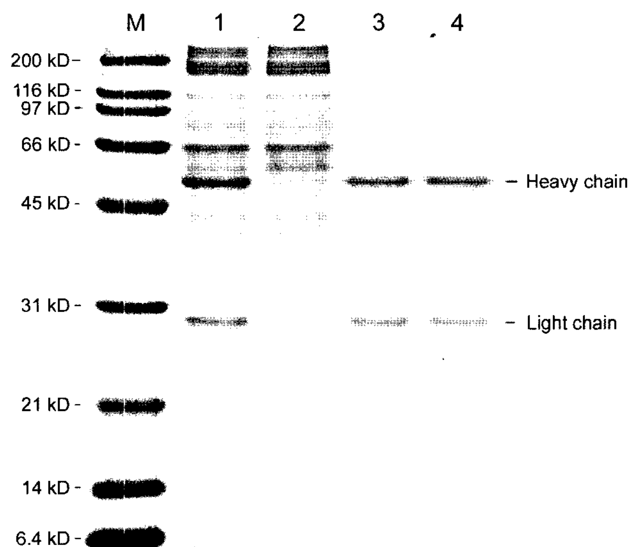

Fig. 2. SDS-PAGE analysis of purified mab 1H11.
Lane M, standard protein marker; lane 1, mab purified by ammonium sulfate; lane 2, unbound proteins by Protein G column; lane 3 and lane 4, eluted fractions of purified mab 1H11.

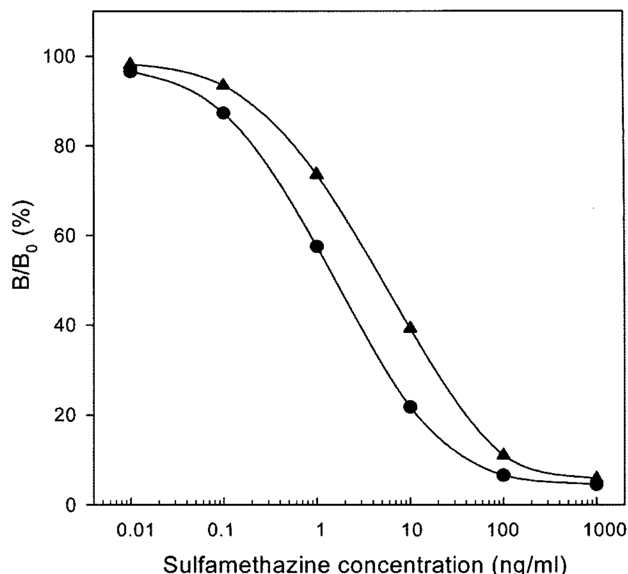

Fig. 3. Standard curves for mAb 1H11 (●) and SP4scFv (▲).
The reactivity of SP4scFv and mab against sulfamethazine was determined by IC-ELISA, as described in Materials and Methods.

ELISA. Isotyping experiment revealed that the isotype of mab 1H11 was IgG1 with a kappa light chain.

The anti-sulfamethazine mab 1H11 was characterized by IC-ELISA. The representative standard curve of 1H11 toward sulfamethazine is shown in Fig. 3. The IC_{50} value (concentration of analyte giving 50% inhibition) value for sulfamethazine was 1.6 ng/ml, and the detection range was from 0.2 to 45 ng/ml. When a hybridoma is used as an mRNA source for construction of scFv antibody, it is highly desirable to use a cell line that produces a high-affinity antibody for target antigen [26]. The hybridoma cell line 1H11 produced a monoclonal antibody with high affinity to sulfamethazine, which made the cloning of high-affinity scFv antibody against sulfamethazine possible.

Production and Characterization of Anti-Sulfamethazine ScFv Antibodies

To clone the genes of coding for V_H and V_L domains of mab 1H11, cDNAs were synthesized from mRNA isolated from hybridoma cells. Based on the constructed cDNAs, we generated the anti-sulfamethazine scFv antibodies by phage display techniques, as described in Materials and Methods. The prepared scFv genes were inserted into the expression vector pCANTAB5E and introduced into *E. coli* TG1 cells to produce the corresponding fusion protein, scFv-g3p, displayed on the outer coat protein of the recombinant phages.

The prepared recombinant phages displaying scFv antibodies, which bound to sulfamethazine-OVA, were isolated by the panning method. The isolation process was

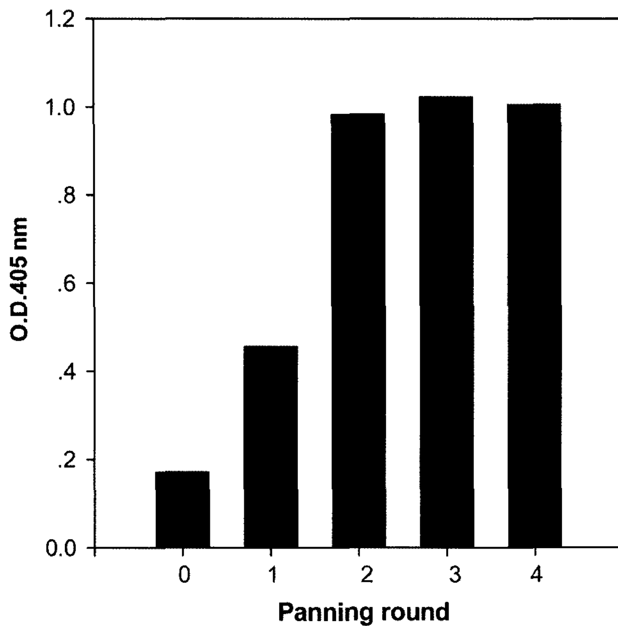

Fig. 4. Sulfamethazine-OVA binding activities of recombinant phages, following each round of panning, measured by ELISA.
Panning round "0" represents period of panning.

repeated through three panning rounds, and the phage titers were determined for each round. An increasing number of phage binders were obtained following each panning cycle. After the first round of panning, 3×10^3 binders were obtained, whereas the binders numbers were 6×10^5 after the second cycle. The affinity of recombinant phage antibodies to sulfamethazine-OVA after each round of panning was tested. Prior to panning, the pooled recombinant phage antibodies exhibited very low binding activity to sulfamethazine-OVA (Fig. 4). Increase of binding affinity was observed after the first and second pannings. No substantial increase was obtained after the third round of panning.

After three rounds of panning, the pooled phages were used to infect logarithmic phase *E. coli* HB2151 for production of soluble scFv antibodies. Total 480 colonies were randomly selected and respectively cultured in 2×YT medium. After induction by IPTG, the culture supernatant from each clone was used directly for detection of antibody activity to sulfamethazine-OVA by noncompetitive ELISA. As a result, the scFv antibodies produced by 15% of the 480 clones showed binding significantly above background, and eight clones gave high binding. The eight selected scFv antibodies were further assessed by IC-ELISA for binding to free sulfamethazine. One of the scFv antibodies, named SP4scFv produced by clone SP4, showed high binding affinity to free sulfamethazine. The other seven scFv antibodies showed less or no recognition of free sulfamethazine.

Clone SP4, producing SP4scFv with high binding affinity to free sulfamethazine in *E. coli* HB2151, was

Heavy chain variable region

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caggtgaagctgcaggagtctggggggaggcttagtgcagcctggagggtcccggaaactc 60
Q  V  K  L  Q  E  S  G  G  G  L  V  Q  P  G  G  S  R  K  L
tcctgtgcagcctctggattcaccttcagtaactttggattgcactgggttcgtcaggct 120
S  C  A  A  S  G  F  T  F  S  N  F  G  L  H  W  V  R  Q  A
                         CDR H1
ccagacaaggggctggactgggtcgcatacatcagttctggcagtaaaaccacctactat 180
P  D  K  G  L  D  W  V  A  Y  I  S  S  G  S  K  T  T  Y  Y
                                          CDR H2
gcagacactgtaaagggccgattcaccgtctccagagacaatcccacgaacaccctgttc 240
A  D  T  V  K  G  R  F  T  V  S  R  D  N  P  T  N  T  L  F
ctgcaaatgaccagtctacggtctgaggacacggccatttattactgtgcaagatattat 300
L  Q  M  T  S  L  R  S  E  D  T  A  I  Y  Y  C  A  R  Y  Y
                                              CDR H3
gataacgacggtatatcttactggggccaaggcaccctggtcaccgtctcctca 348
D  N  D  G  I  S  Y  W  G  Q  G  T  L  V  T  V  S  S
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Light chain variable region

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gacattgtgatgacccagtctccagcaataatggctgcctctctggggcagaaggtcacc 60
D  I  V  M  T  Q  S  P  A  I  M  A  A  S  L  G  Q  K  V  T
atgacctgcagtgccagctcaagtgtaagttccagttacttgcactggtaccagcagaag 120
M  T  C  S  A  S  S  S  V  S  S  S  Y  L  H  W  Y  Q  Q  K
                 CDR L1
tcaggcgcttcccccaaacccttgattcataggacatccaacctggcttctggagtccca 180
S  G  A  S  P  K  P  L  I  H  R  T  S  N  L  A  S  G  V  P
                                 CDR L2
gctcgcttcagtggcagtgggtctgggacctcttactctctcacaatcagcagcgtggag 240
A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  S  V  E
gctgaagatgatgcaacttattactgccagcagtggagtggttacccatccatcacgttc 300
A  E  D  D  A  T  Y  Y  C  Q  Q  W  S  G  Y  P  S  I  T  F
                                    CDR L3
ggcggagggaccaagctggagatcaaacgt 330
G  G  G  T  K  L  E  I  K  R
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Fig. 5. Nucleotide and deduced amino acid sequences of the heavy and light chain variable regions of SP4scFv.
The complementarity determining regions are underlined which were determined according to Kabat *et al.* [10]. The nucleotide sequence in this study was submitted to the GenBank Data Bank with accession number DQ838798.

chosen for DNA sequencing. The nucleotide and deduced amino acid sequences of the heavy and light chain variable regions in SP4 are shown in Fig. 5. The DNA coding for V_H domain of SP4scFv comprised 348 bp encoding 116 amino acid residues, and appeared to be a member of the mouse heavy chain subgroup III according to the classification of Kabat *et al.* [10]. The V_L gene comprised 330 bp encoding 110 amino acid residues, belonging to the mouse light chain subgroup IA. The complementarity determining regions (CDRs) 1, 2, and 3 of V_H and V_L were positioned as depicted in Fig. 5.

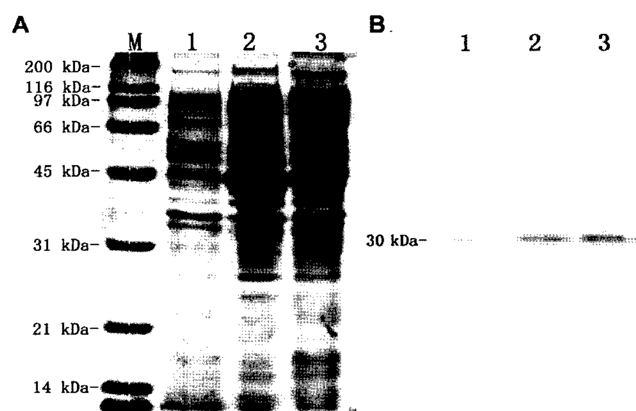

Fig. 6. SDS-PAGE (**A**) and Western blot (**B**) analysis of anti-sulfamethazine SP4scFv antibody expressions.
Lane 1, SP4scFv in concentrated supernatant (0.3 ml supernatant concentrated by 10% trichloroacetic acid); lane 2, SP4scFv expression in the periplasm; lane 3, SP4scFv expression in the cytoplasm; lane M, protein markers.

SP4scFv in a large scale was prepared in SB medium, and its location in the cell fraction was analyzed by Western blot. As shown in Fig. 6, the location of the expressed SP4scFv was found to be in the periplasm as well as in the cytoplasm. The expression of SP4scFv in culture supernatant was very low, and the event that used 20 times concentrated supernatant sample, gave only a weak band in Western blot analysis. The SP4scFv antibody in periplasmic extracts was purified by thiophilic adsorption chromatography. The

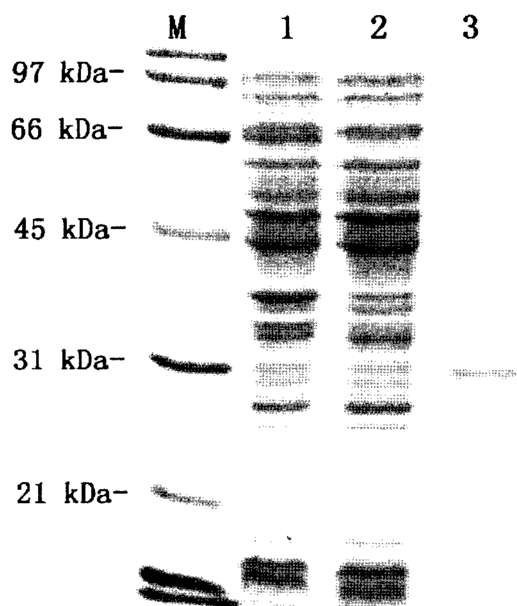

Fig. 7. SDS-PAGE analysis of purified SP4scFv antibody.
Lane M, standard protein marker; lane 1, cytoplasmic extracts; lane 2, periplasmic extracts; lane 3, purified SP4scFv antibody by thiophilic adsorption chromatography.

purified SP4scFv antibody was confirmed by SDS-PAGE analysis (Fig. 7). The protein concentration of purified SP4scFv antibody was 1.2 mg/ml, as determined by protein assay kit (Bio-Rad). The purified SP4scFv antibody was used in the subsequent experiments.

The standard curve of SP4scFv toward sulfamethazine is shown in Fig. 3. The binding of SP4scFv to the immobilized antigen sulfamethazine-OVA was inhibited by sulfamethazine in a concentration-dependent manner. The IC_{50} value of SP4scFv was 4.8 ng/ml, and this was comparable to that obtained with the parent mAb 1H11 in the IC-ELISA (IC_{50}=1.6 ng/ml).

Cross-Reactivity

Cross-reactivity of mab 1H11 and SP4scFv was determined by comparing IC_{50} values for various sulfonamide compounds in IC-ELISA. The values were expressed as percent cross-reactivity (IC_{50} for sulfamethazine/IC_{50} for test compound ×100). The result of cross-reactivity tests with the sulfonamide compounds is shown in Table 1. The high cross-reactivity of both antibodies observed with sulfamerazine (54% and 59% for mab and scFv antibody, respectively) is quite reasonable, because of its similar aromatic structure to sulfamethazine. Relatively little reaction of sulfadiazine (8%) and sulfachlorpyridazine (4%) with SP4scFv was exhibited also; however, these two compounds did not react with mab 1H11 (Table 1). Both antibodies did not exhibit significant cross-reactivity with other structurally related sulfonamide compounds tested in this study.

Analysis of Milk Sample

Milk samples were examined in IC-ELISA to measure the content of sulfamethazine. To gain basic information on the matrix effect, conformity of the standard curve generated in PBS was compared with that of curves obtained using milk matrices. The inhibition curves generated in PBS were consistent with those prepared in 10, 20, 50, and 100 times

Table 1. Comparison of cross-reactivities of SP4scFv and monoclonal antibody 1H11 to sulfamethazine analogs.

Compound	Cross-reactivity (%)	
	mab	SP4scFv
Sulfamethazine	100	100
Sulfadiazine	<0.1	8
Sulfapyridine	<0.1	<0.1
Sulfachlorpyridazine	<0.1	4
Sulfamethizole	<0.1	<0.1
Sulfamerazine	5.4	5.9
Sulfamethoxazole	<0.1	<0.1
Sulfaquinoxaline	<0.1	<0.1
Sulfisozole	<0.1	<0.1
Sulfathiazole	<0.1	<0.1
Sulfadimethoxine	<0.1	<0.1

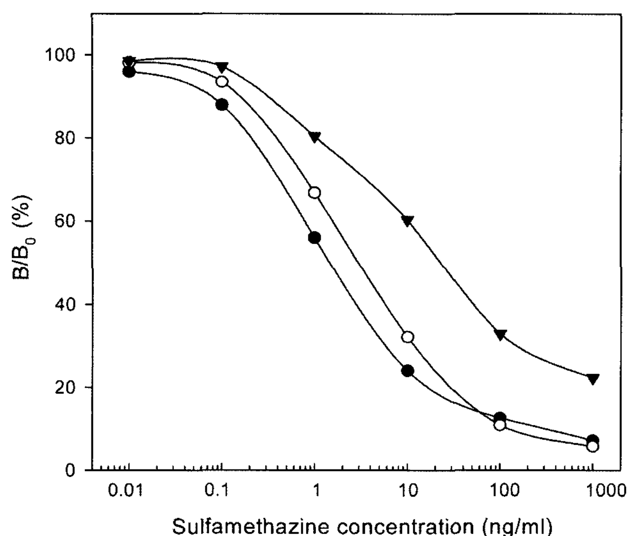

Fig. 8. Effect of milk matrix (treated with acetone and diluted 20 times in PBST) on mab 1H11 (○) and SP4scFv (▼) standard curves compared with the mab 1H11 (●) standard curve in PBS.

diluted milk matrices, but the OD value curves differed significantly. After removal of protein with acetone, the milk samples were diluted 10, 20, 50, and 100 times with PBST and used to generate standard curves. Twenty times dilution with PBST was found to be good enough to reduce the matrix effects on detection of sufamethazine by mab 1H11-based IC-ELISA (Fig. 8), and the IC_{50} of mab 1H11 under this condition was 2.2 ng/ml. However, the effect of milk matrix on SP4scFv-based IC-ELISA was significant (Fig. 8), and the IC_{50} of SP4scFv under this condition was 24 ng/ml. The stronger effect of milk matrix to recombinant antibody SP4scFv than mab 1H11 might be due to structural difference between scFv antibody and parent mab. Therefore, the IC-ELISA-based mab 1H11 was used to detect sulfamethazine in milk samples. The recoveries of sulfamethazine added to milk at 10, 50, and 100 ng/ml are shown in Table 2. The recoveries from the matrices were in the range of 94–104%. Concerning the reproducibility, the average interassay coefficient of variation (CV) values was 9.8%. Therefore, the mab 1H11-based indirect competitive ELISA presented herein could be used to determine sulfamethazine in milk samples.

Real milk samples were obtained from local markets for sulfamethazine screening and determination. Analysis of

Table 2. Results on the determination of sulfamethazine in spiked milk samples by mab-based IC-ELISA.

Spiked concentration (ng/ml)	Mean±SD (ng/ml)	Recovery (%)	CV (%)
10	10.4±1.1	104	11.3
50	47±2.3	94	8.9
100	96±5.4	96	9.1

60 milk samples by IC-ELISA showed that 4 samples were positive (sulfamethazine concentration ranged 5.1 to 8.1 ng/ml). HPLC analysis also confirmed the same positive samples, thus demonstrating that the developed assay can be used for analysis of real samples.

Acknowledgments

We are very grateful to Dr. Sergey A. Eremin from the Department of Chemical Enzymology, Chemistry Faculty, M.V. Lomonosov Moscow State University (Moscow, Russia) for kindly providing the haptens used in the study. This research was partially supported by the National Toxicology program of the National Institute of Toxicological Research, Brain Korea 21 Program from the Ministry of Education and the Korea Health 21 R&D Project (03-PJ1-PG1-CH11-003), Ministry of Health & Welfare, Republic of Korea.

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