Antibodies bind antigens, including microorganisms, with high specificity and have been used in immunoassays for the rapid detection of pathogens. The use of antibodies may shorten the time required for microbial enrichment and isolation from a few days to a few hours. Several immunoabsorption approaches have been used for detection of microorganisms in food systems. Pathogens can be bound by dye-conjugated free antibodies and can subsequently be counted by fluorescence microscopy (14) or flow cytometric technology (25). Target microorganisms can also be trapped by an immobilized antibody and detected by enzyme-linked immunosorbent assay (26). Recently, immunomagnetic separation technology (11) has broadened the use of antibodies in detection or isolation of food-borne pathogens (22, 36). These immunomagnetic beads are able to bind the target microorganisms, thus allowing collection of the bead-bound microbes simply by applying a magnetic field. These magnetically recovered microorganisms have been detected by luminescence assay (39) or PCR (8) or have been simply identified or counted from selective medium (36).

Traditionally, antibodies can be obtained only from immunized animals; however, recent progress in molecular biology has made it possible to produce monoclonal antibody fragments from bacteria (35). To date, most of the antibody fragments produced from recombinant technology have been single-chain antibodies, consisting only of the variable-region domains of the heavy and light chains of the parent antibody and a short peptide linker used to connect these two domains. An effector protein can be genetically fused with the single-chain antibody to allow expression as a bifunctional antibody. For example, single-chain antibodies have been fused with alkaline phosphatase and used for diagnosis and immunoassays (5). Some affinity tags such as the FLAG tag (23), strep tag (33), His tag (34), calmodulin (28), or streptavidin (7) can be attached to the single-chain antibodies for direct detection by commercially available detection systems and for recovery of recombinant antibodies from the cell lysate by affinity chromatography.

Spore-forming bacteria such as Bacillus cereus may cause food-borne illness or spoilage and are problematic because they can survive mild heat treatment. Detection and control in food processing are exacerbated for bacterial spores because they typically are present in low numbers and are metabolically inactive. A procedure to concentrate and detect low numbers of these metabolically inactive yet significant organisms would be useful. In the present study, a truncated streptavidin gene (3) was amplified by PCR to introduce unique restriction enzyme sites. It was connected with the gene of single-chain anti-B. cereus spore antibody (19) to form a fusion protein gene. This bifunctional single-chain antibody gene was expressed by Escherichia coli. Both native and recombinant monoclonal antibodies revealed similar antigen specificities. This streptavidin-conjugated antibody can be immobilized on the surface of biotinylated magnetic beads, and its spore binding ability was demonstrated.

**MATERIALS AND METHODS**

**Bacterial spores and cells.** E. coli JM109 (endA1 recA1 gyrA96 thi hsdR17 (rK- mB-) relA1 supE44 Δlac-proAB Δ(lac-proAB) [F' traD36 proAB lacIqZΔM15]) was provided by Promega (Madison, Wis.). E. coli BL21 (DE3), which carries the T7 RNA polymerase gene under lacUV5 promoter control, was purchased from Novagen (Madison, Wis.). The competent E. coli cells used for gene transformation were prepared by a simple polyethylene glycol dimethylsulfoxide protocol (6). Spores of B. cereus T were prepared on fortified nutrient agar sporulation medium (15). After collection and washing, the spore suspension was stored at ~20°C. The numbers of spores were enumerated on Trypticase soy agar (Difco, Detroit, Mich.) plates and by direct microscopic counting.

**DNA manipulation and sequencing.** Most of the gene cloning procedures were based on the protocols described by Maloy (24). The DNA fragments generated from PCR or restriction enzyme digestion were purified by a diatomaceous earth-based protocol. The DNA sequences of PCR products and the fusion protein gene were obtained by the cycle sequencing method (20) and were
detected by a nonradioactive silver-staining protocol (2). The DNA-sequencing-grade Tag DNA polymerase and nucleotides were purchased from Promega. For accuracy, both strands of the DNA were sequenced.

Construction of expression vectors. (i) Plasmid DNA and oligonucleotides. The plasmid pGEM-SZ, which was used for gel-cleaning and sequencing purposes, was obtained from Promega. The pET22(-) derivative plasmid pET22IgTag (19) was used as the single-chain antibody gene source. This plasmid contains a T7/8 promoter (37), a pelB signal sequence (16) for protein relocalization, the complete anti-B. cereus single-chain antibody gene, and the T7 transcription terminator. The oligonucleotides used for PCR primers or DNA sequencing were synthesized at the Molecular Biology Center, North Carolina State University, Genosys Biotechnologies, Inc. (Woodlands, Tex.), or Gibco BRL (Gaithersburg, Md.).

(ii) Modification of streptavidin gene. The plasmid pUC8-SZ (a gift from C. E. Argargar), which contained the complete streptavidin gene (1), was modified by colony PCR (9) with primers STREP3 (5'-CATCGGATCCGCCATATCGGGCACCTGGTAGTACAAC) and STREP5 (5'-GGAGAAGTCTGGCTTACCTGGTAGGAGGATGATC). Colonies of pUC-SZ transformants were picked from a Luria-Bertani (LB)-ampicillin plate and were used as the gene source. Each colony was suspended with 50 μl of distilled water in a microcentrifuge tube and then heated in boiling water for 5 min to lyse the cells. The cell lysate was centrifuged at 12,000 × g for 5 min, and 10 μl of the supernatant containing the target DNA was mixed with a deoxyoxynucleoside triphosphate mixture (Boehringer Mannheim, Indianapolis, Ind.), 10 μl of PCR buffer (Sigma, St. Louis, Mo.), and 30 pmol of each primer STREP3 and STREP5. The mixture was heated in a Perkin-Elmer Thermal Cycler 480 (Norwalk, Conn.) at 95°C for 6 min, and then the Tag DNA polymerase was added. The target gene was amplified by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for the STRP3 primer. The 72°C step was replaced by 40°C for 1 min at the end of the cycle for the STREP3 primer. The amplified fragments introduced into the T7/8 promoter sites at the boundaries of the biotin binding-domain gene. The STREP3 primer also encoded a stop codon (TAA) for the C-terminal end of the streptavidin-conjugated single-chain antibody. The protein concentration of the fusion protein present in the final solubilized cellular fraction was quantitated by the SDS-PAGE gel by using the Protein Densitometer SI and Fragment NT software (Molecular Dynamics, Sunnyvale, Calif.).

Determination of protein concentrations. The protein concentrations of purified native monoclonal antibody and the final solubilized cellular fraction from the E. coli culture were determined by the Bradford dye-binding method (4) with ponceau B. The percentage of fusion protein present in the final solubilized cellular fraction was quantitated by a microsequencing method at the Medical Center, University of North Carolina at Chapel Hill.

Purification of native monoclonal antibody. The parent, native monoclonal antibody against B. cereus spores (31) was purified from tissue culture supernatant by thiolphilic gel affinity chromatography as described by Hutchens and Porath (13). The purified monoclonal antibody was diazylated against phosphate buffer and then concentrated with an Amicon Centricron-10 ultrafiltration concentrator (Amicon, Beverly, Mass.).

DOT blot immunoassay. The immunoassay protocol was modified from the method described by Phillips (29). Fifty microliters of a B. cereus T spore suspension (approximately 10⁶ spores/ml) was applied into each well of a dot blot apparatus (Bio-Rad) and allowed to dry for 2 h. The immunoblotting procedure was performed using a 0.125-nM concentration of monoclonal antibody as the primary antibody, followed by biotinylated horseradish peroxidase (Sigma catalog no. P2907). To reduce the background signal from the membrane, 4-chloro-1-naphthol (Bio-Rad) was chosen as the peroxidase substrate.

Functional assays. The antigen specificities of native monoclonal and streptavidin-conjugated single-chain antibodies were determined by the dot blot immunoassay method described above. The final concentrations of both types of antibodies were adjusted by PBS.

(i) Spore binding experiment. The purity-binding specificities of both native monoclonal and streptavidin-conjugated single-chain antibodies were tested by using different species of spores. Spores of Bacillus subtilis A, B. subtilis subsp. subtilis, Bacillus cereus, Bacillus stearothermophilus, and Clostridium perfringens spores were obtained and prepared as described by Quinlan and Foegeding (31).

(ii) Competition experiment. The ability of streptavidin-conjugated single-chain antibody to bind B. cereus spores was tested by a dot blot immunoassay with streptavidin-conjugated single-chain antibody (22) preincubated with native or anti-B. cereus monoclonal antibody as a competitor protein. The binding of streptavidin-conjugated single-chain antibody was detected by biotinylated horseradish peroxidase. The concentrations of competitor ranged from 10 to 100 μg/ml.

Preparation of biotinylated matrices. Magnetic beads that were coated covalently with sheep anti-mouse IgG antibodies (M-280; Dynal, Oslo, Norway) were used as the solid support for immobilization of single-chain antibodies. N-Hydroxysuccinimimidyl (NHS)-biotin (Pierce Chemical Co.) was used as the biotinylation reagent. Approximately 450 μl of beads was washed three times with 1 ml of PBS, and then the beads were resuspended in 250 μl of 0.12 M borate buffer (pH 8.8). Fifty microliters of N-hydroxysuccinimimidyl-dimethyl sulfoxide solution (10 mg/ml) was added, and the suspension was mixed on a horizontal sample mixer (Dynal) at room temperature for 4 h.

The residual N-hydroxysuccinimimidyl biotin in the bead suspension was removed by washing the beads three times with 3 volumes of PBS. The cleaned biotinylated beads were stored in a storage solution (0.1% BSA in PBS, filtered by a 0.22-μm-pore-size membrane, with 0.02% [vol/vol] sodium azide added) at 4°C. The beads were checked by a simple enzyme-linked assay to validate the presence of functional biotin groups. Approximately 20 μl of biotinylated beads was transferred into a microcentrifuge tube and washed with 200 μl of PBS, and then the beads were resuspended in 200 μl of 1% BSA in PBS for 10 min to block the nonspecific binding sites. After the BSA-blocked beads were washed with 200 μl of TBST three times, 200 μl of streptavidin-conjugated horseradish peroxidase (Sigma) in TBPS was added and mixed with the beads at room temperature for 20 min. Unbound enzyme was removed by washing the beads with 200 μl of TBPS three times. One milliliter of o-phenylene-diamine–H₂O₂ (21) was added as the peroxidase substrate. After the reaction was suspended for 5 min, the suspension was mixed with 1 ml of substrate solution in the biotinylated antibody P membrane turned yellow while the nonbiotinylated beads that were used as a control were colorless.

Immobilization of recombinant antibody. The biotinylated beads in storage solution were mixed with streptavidin-conjugated single-chain antibody (0.1 mg/ml) in phosphate buffer at 4°C for 1 h at room temperature before using a horizontal sample mixer. The beads were cleaned by being washed three times.
times with storage solution. The single-chain antibody-coated magnetic beads were resuspended in storage solution at a level of 6 × 10^10 beads per μl of suspension (10 μg/μl) and stored at 4°C. Nonbiotinated beads treated by the same procedure were used as a control. For qualitative assay of the immobilized streptavidin-conjugated single-chain antibody on the beads, an ELISA method was used. The details of procedure were similar to the detection of the biotinyl group given previously. A rabbit antistreptavidin antiserum was used to document that the beads were coated with streptavidin fusion proteins. Bound primary antibody was detected by goat antirabbit antibody conjugated to horseradish peroxidase with o-phenylenediamine–H₂O₂ as the substrate.

**Evaluation of spore-binding ability by using immobilized single-chain antibody.** The *B. cereus* spore stock suspension was diluted with filter-sterilized (0.22-μm pore-size membrane) 0.1% BSA in PBS or pasteurized whole milk (purchased from a local supermarket and then stored at 4°C for various periods of time) to a final spore concentration of approximately 5 × 10⁶ CFU/ml. One milliliter of diluted spores was transferred into a siliconized sterile microcentrifuge tube for the spore binding test. Approximately 50 μl of antibody-coated beads was mixed with the spore suspension at room temperature (23°C) or at 4°C for 1 h with a horizontal sample mixer at a speed of 25 rpm. The magnetic particle concentrator for the microcentrifuge tube (MPC-E-1; Dynal) was used to collect the magnetic beads. The supernatant was removed carefully, and the spores present were defined as the unbound fraction. The pelletted beads were washed three times by resuspending the bead pellet with 500 μl of sterilized PBS and vortexing for each wash. Spores in the washing solutions were designated the bound but removable fraction. The buffer-washed beads were resuspended in 500 μl of PBS, and the spore fraction remaining was designated the bound fraction.

The spore number was determined by the spread plating method with Trypticase soy agar plates. One hundred microliters of serial dilutions from each fraction was plated directly. Mannitol yolk polymyxin agar plates were resuspended in storage solution at a level of 6 × 10⁹ beads per μl of PBS, and the spore fraction remaining was designated the unbound fraction. The pelleted beads were washed with PBS, and the spore fraction was designated the bound fraction. The buffer-washed beads were resuspended in 500 μl of PBS, and the spore fraction remaining was designated the bound fraction.

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**RESULTS AND DISCUSSION**

**Construction of the streptavidin-conjugated single-chain antibody gene and expression vector.** The interaction of streptavidin and biotin is one of the strongest noncovalent affinities known in biology. It can be used not only as a reporter for use in immunoassays but also as a domain for bioselective immobilization (41). For this reason, it was chosen as the affinity domain in this study. Native streptavidin is sensitive to the action of proteolytic enzymes at both the N and the C termini (3). However, enzymatically truncated streptavidsins, which are also called core streptavidins and which include residues 12 through 140 of native streptavidin, still have strong biotin-binding ability. Like streptavidin, core streptavidin can associate into tetramers (3, 7, 32), but these are less prone to aggregation than is streptavidin (32). In this case, the PCR-modified core streptavidin gene segment. Fortunately, only a silent mutation (GGC to GGT; the 68th amino acid in the wild-type streptavidin gene [1]) was detected, and the primary structure of core streptavidin protein was identical to that originally reported (1). In this case, the streptavidin-conjugated single-chain antibody structure included V₅₁-linker-V₁₂-core streptavidin. Thus, the PCR-derived V₅₁-core streptavidin gene fragments were assembled into the pET22lgTag vector (which contained V₁₆, V₁₃, and strep tag peptide genes) to form a new pET22lgSAV streptavidin-conjugated single-chain antibody expression vector (Fig. 1).

**Expression and recovery of bifunctional single-chain antibody from E. coli.** The T7 RNA polymerase-T7 promoter expression system is a tightly controlled bacterial expression system. It has been used for production of core streptavidin, which is potentially lethal to host cells (32). In the present study, the streptavidin-conjugated single-chain antibody was expressed successfully within a few hours by this system. Although the fusion protein was produced mainly as an insoluble form, the active bifunctional fusion antibody was recovered by simple denaturation, renaturation, and dialysis operations. The SDS-PAGE and Western blotting analyses (Fig. 2) showed that most of the single-chain antibody existed in the final solubilized cellular fraction, while a small amount of the fusion protein was detected in the soluble cytoplasmic fraction. Densitometry data

![FIG. 1. Construction of the streptavidin-conjugated single-chain antibody gene and expression vector.](http://example.com/figure1.png)

**FIG. 1.** Construction of the streptavidin-conjugated single-chain antibody gene and expression vector.

![FIG. 2. Protein fractionation analysis of streptavidin-conjugated single-chain antibody from E. coli.](http://example.com/figure2.png)

**FIG. 2.** Protein fractionation analysis of streptavidin-conjugated single-chain antibody from E. coli. (A) SDS-12% PAGE; (B) Western blot. Fusion protein was probed with rabbit anti-streptavidin antiserum (Sigma S6390) and anti-rabbit IgG peroxidase conjugate (Sigma A8275). Lanes: MW, molecular weight standards; 1, total cell sample (without IPTG induction); 2, total cell sample (with 0.2 mM IPTG induction); 3, osmotic shock fraction; 4, soluble fraction from cell lysis; 5, final solubilized cellular fraction.
of the final solubilized cellular fraction revealed that approximately 70% of the soluble protein was streptavidin-conjugated single-chain antibody. The concentration of soluble streptavidin-conjugated single-chain antibody present was approximately 3 to 7 mg of fusion protein/liter of culture. N-terminal sequence analysis indicated that this protein was mature fusion protein. The high expression rate of the T7 RNA polymerase-T7 promoter system may be the cause for expression of the fusion protein mainly in an insoluble form (18).

Characteristics of streptavidin. The expressed streptavidin-conjugated recombinant antibody retained many of the characteristics of native streptavidin. Not only did this fusion protein bind biotin and the B. cereus spore antigen, but the core streptavidin also associated into a tetramer or higher order oligomers. The results from SDS-6% PAGE suggested that the core streptavidin-conjugated fusion protein remained as an oligomer when the final solubilized cellular fraction in 2× SDS sample buffer mixture (1:1) was heated at 55°C (Fig. 3, lane 2). However, SDS-PAGE results showed that by heating at 100°C, the apparent molecular mass of the protein in the final solubilized cellular fraction was shifted from 200,000 to 45,000 Da (39,800 Da; calculated from primary structure) (Fig. 3, lane 1). These data suggest that the protein associates into an oligomer probably composed of 4 or 5 U.

Binding properties of the monoclonal antibody and the single-chain antibody. Because of the bifunctional nature of the fusion protein, the spore binding ability can be detected simply by applying biotinylated horseradish peroxidase. Six different species of Bacillus and Clostridium spores were used to compare the antigen specificity of this streptavidin-conjugated single-chain antibody with that of its parent monoclonal antibody. The two types of antibodies exhibited similar spore-binding behaviors, with the exception that the single-chain antibody showed slight cross-reactivity with C. perfringens spores (Fig. 4). The unexpected cross-reaction with B. subtilis A spores observed with the strep tag (a 10-amino-acid peptide)-conjugated single-chain antibody has the same primary structure for the antigen-binding domains (19) was not detected in this single-chain antibody. A simple competitive dot blot assay with BSA confirmed that specific competition, rather than nonspecific blocking, occurred between the native and recombinant antibodies. Thus, they must have similar tertiary structures in the variable region domains, and the fusion with streptavidin must not have significantly altered the conformation of the antigen-binding domain. Furthermore, the formation of multimeric complexes might increase the apparent affinity of single-chain antibody (17) or stabilize the conformation of the single-chain antibody favoring antigen binding and mimic the antigen-binding behavior of native bivalent or multivalent antibodies.

Spore-binding tests. Spore binding was tested in phosphate buffer and in whole-milk systems. The performance of the immunomagnetic beads in the phosphate buffer system is indicated in Table 1. Controls, including the original magnetic beads with and without added fusion protein and biotinylated beads that were not coated with fusion protein, were tested to determine the degree of nonspecific binding. The results of controls without added fusion protein indicated a low level (<1%) of nonspecific binding of the spores. Nonbiotinylated original beads mixed with the streptavidin-conjugated single-chain antibody control were used as a control to evaluate interaction of the fusion protein directly with the beads. The results indicated that fusion protein did interact directly with the beads, resulting in low levels of specific (3.2%) and nonspecific (32.4%) interaction with the spores. These data coincided with the result of an ELISA for qualitative assaying of immobilized fusion protein on the beads. In the ELISA, original (nonbiotinylated) magnetic beads and the biotinylated beads turned the substrate faint yellow (A490, ~0.3). Fusion protein immobilization on the biotinylated beads was documented by the substrate turning orange (A490, ~2.0). Nonbiotinylated beads with added fusion protein turned the substrate solution light yellow (A490, ~0.8), indicating some nonspecific binding.

The immunomagnetic beads can remove B. cereus spores nearly quantitatively from a phosphate buffer system at room or refrigeration temperature within 1 h (Table 1). Only 2 to 9%
of the original \textit{B. cereus} spores were in the sample solution after mixing the immunomagnetic beads with the spore suspension for 1 h. Colony counts indicated that \textasciitilde90\% of spores were removed from the 0.1\% BSA–PBS buffer system. Almost all of the captured spores were tightly bound to the immunomagnetic beads. Less than 1\% of the bead-bound spores were released from the matrix after three rounds of PBS washing. To determine their spore-binding ability in a food, pasteurized whole milk was used as a test system. The results given in Table 2 show that some of the spore-binding ability of immunomagnetic beads could be inhibited by whole milk. However, the immunomagnetic beads did specifically bind approximately 37\% of the \textit{B. cereus} spores in the presence of complex food components. These results indicated that immobilized streptavidin-conjugated single-chain antibody showed specific spore binding and removal ability in both buffer and whole-milk systems; however, the spore removal ability decreased to approximately 37 to 40\% in a whole milk system. Most of the milk lipids, approximately 4\% in whole milk, are present in 2- to 3-\mu m globules that are surrounded by a membrane (38). The presence of these fat particles may interfere with the effective contact of spores with the antigen binding site. However, the performance of immobilized \textit{Pseudomonas aeruginosa} recombinant antibody decreased from 95 to 75\% when the sample system was changed from PBS to fat-free milk (27). Thus, it is also possible that the soluble proteins in milk blocked some antigens on the spore or some antigen-binding sites on the surfaces of antibody-coated beads and thereby reduced the efficiency of specific binding. These data suggest that it is possible to use immobilized recombinant antibody fragments as a bioprocessing aid to concentrate organisms from food for microbiological evaluation.

This study has demonstrated that active anti-\textit{B. cereus} spore single-chain antibody conjugated to streptavidin can be expressed and recovered efficiently within 24 h. The recombinant fusion antibody functioned essentially in the same manner as the native antibody in immunoassays for pathogen detection and also exhibited the biotin binding characteristics of native streptavidin. Because the techniques to increase the productivity of soluble recombinant antibody (18) and scale-up and optimization of fermentation for mass production of single chain antibody (12, 23, 30) are available already, it may be possible to use this recombinant antibody in food processing and food testing or related industries. It could be applied to rapid immunoassay detection for bioselective concentration and potentially for removal of \textit{B. cereus} spores or other pathogens from liquid food systems to enhance food safety or to reduce the severity of required preservation processes.

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