Rapid Isolation of a Single-Chain Antibody against the Cyanobacterial Toxin Microcystin-LR by Phage Display and Its Use in the Immunoaffinity Concentration of Microcystins from Water

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A naïve (unimmunized) human semisynthetic phage display library was employed to isolate recombinant antibody fragments against the cyanobacterial hepatotoxins microcystin-LR. Selected antibody scFv genes were cloned into a soluble expression vector and expressed in Escherichia coli for characterization against purified microcystin-LR by competition enzyme-linked immunosorbent assay (ELISA). The most sensitive single-chain antibody (scAb) isolated was capable of detecting microcystin-LR at levels below the World Health Organization limit in drinking water (1 µg liter⁻¹) and cross-reacted with three other purified microcystin variants (microcystin-RR, -LW, and -LF) and the related cyanotoxin nodularin. Extracts of the cyanobacterium Microcystis aeruginosa were assayed by ELISA, and quantifications of microcystins in toxic samples showed good correlation with analysis by high-performance liquid chromatography. Immobilized scAb was also used to prepare immunoaffinity columns, which were assessed for the ability to concentrate microcystin-LR from water for subsequent analysis by high-performance liquid chromatography. Anti-microcystin-LR scAb was immobilized on columns via a hexahistidine tag, ensuring maximum exposure of antigen binding sites, and the performance of the columns was evaluated by directly applying 150 ml of distilled water spiked with 4 µg of purified microcystin-LR. The procedure was simple, and a recovery rate of 94% was achieved following elution in 1 ml of 100% methanol. Large-scale, low-cost production of anti-microcystin-LR scAb in E. coli is an exciting prospect for the development of biosensors and on-line monitoring systems for microcystins and will also facilitate a range of immunoaffinity applications for the cleanup and concentration of these toxins from environmental samples.

Cyanobacteria (blue-green algae) frequently form dense growths known as blooms in eutrophicated waters. The presence of these blooms in freshwater bodies can pose a significant threat to the health of humans and animals, as certain species of cyanobacteria are capable of producing toxins. The most frequently encountered group of cyanobacterial toxins is the microcystins, which are produced by several genera, including Microcystis, Planktothrix, and Anabaena (4). The microcystins consist of over 60 structurally related cyclic peptide hepatotoxins with the general structure cyclo-(D-alanine-X-D-cystins), which are composed of a general structure cyclo-(D-alanine-X-D-cystins) and a general structure cyclo-(D-alanine-X-D-cystins) that are known to play an important role in toxicity (9). The toxins are named according to the two variable L amino acids at positions X and Z; for example, microcystin-LR contains the amino acids leucine (L), and arginine (R) at these positions (4).

The toxicity of microcystins is mediated through inhibition of serine-threonine protein phosphatases 1 and 2A (23), which can cause both acute and chronic effects in mammals. Intoxication with high levels of microcystins can lead to hepatocyte necrosis and hemorrhage, with severe cases resulting in death (2). Long-term exposure to low concentrations of microcystins has also been implicated in tumor promotion (13). The contamination of water with microcystins has been linked to fatalities of livestock and wildlife throughout the world (4). Episodes of human poisoning have also been reported, including the deaths of over 50 patients at a hemodialysis clinic in Caruaru, Brazil, which were attributed to the presence of microcystins in the clinic’s water supply (33). Guideline values for drinking water were recently introduced by the World Health Organization (39), with a recommended limit of 1 µg of microcystin-LR equivalents per liter. There is therefore a need for sensitive and robust detection methods for determining the levels of microcystins in water.

Microcystins are routinely monitored using analytical techniques such as high-performance liquid chromatography (HPLC) and mass spectrometry (19, 20), but these can be slow and technically demanding and often require extensive sample processing prior to analysis. A promising alternative for microcystin detection is the use of immunological assays employing either polyclonal (6, 28) or monoclonal (32) antibodies. The enzyme-linked immunosorbent assay (ELISA) is quick to perform, inexpensive, requires minimum sample processing, and is capable of detecting microcystins within the levels set by the World Health Organization. In addition to their application in immunoassay formats, microcystin-specific antibodies are also useful tools for processing complex samples prior to toxin...
analysis. They have been used effectively in immunoaffinity chromatography to eliminate contaminants from biological samples (15) and to remove coeluting compounds when microcystins are concentrated from large water samples (16, 18), thus enabling identification of the toxins by HPLC.

ELISA formats have been used extensively in the detection of microcystins (6, 28, 32), and a number of diagnostic kits are now commercially available. However, application of these assays in routine monitoring programs has been restricted due to the difficulties in isolating reliable polyclonal antibodies from laboratory animals and the complexity and expense of producing monoclonal antibodies from hybridoma cell lines. Recombinant antibody technology provides a simpler and more cost-effective alternative for the isolation of microcystin-specific antibodies. Phage display technology (25) has facilitated the selection and enrichment of recombinant antibody fragments against a range of environmental haptons, including microcystins, from large antibody libraries (26). Antibody fragments selected against particular antigens can then be expressed in the bacterial host *Escherichia coli* in considerably larger amounts than whole antibodies produced by traditional methods. While representing <20% of the size of an intact antibody, fragments consisting of linked antibody heavy- and light-chain variable domains (scFv) have been shown to retain specificity and sensitivity for the target antigen (35). Furthermore, scFv fragments can be modified to increase their stability under nonphysiological conditions, including methanol (7, 35), which is used routinely to extract microcystins from cyanobacterial samples.

Here, we describe the isolation of recombinant phage-antibody clones against microcystin-LR from a naive human semi-synthetic phage display library. Selected phage antibodies were expressed as soluble single-chain antibody fragments (scAbs) and characterized by competition ELISA. The cross-reactivity of the most sensitive scAb clone was determined by ELISA of related toxins, and the detection and quantification of microcystins in cyanobacterial extracts was assessed by comparison with HPLC analysis. When immobilized, the scAb was capable of concentrating trace levels of microcystin-LR from large volumes of water prior to HPLC analysis, allowing the toxin to be identified and quantified.

**MATERIALS AND METHODS**

**Purification of microcystins and preparation of microcystin-LR conjugates.** Microcystin variants (microcystin-LR, -RR, -IL, and -LF) and nodulin were purified from lyophilized laboratory strains of cyanobacteria using flash chromatography (8, 21, 22).

Microcystin-LR was conjugated to both bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) according to published protocols (28). The methylglyoxalbisalnine residue of the toxin was first linked to 2-mercaptoethanolamine (Sigma Chemical Company, Poole, United Kingdom) (31) prior to the performance of a one-step gluteraldehyde coupling to a carrier protein (11). The microcystin-LR conjugates were analyzed for protein by the Bradford assay (3). Matrix-assisted laser desorption mass spectrometry was used to determine the hapten load per carrier protein for the microcystin-LR-BSA conjugate. This was found to be between 8 and 10 hapten per BSA molecule (26).

**Plasmids and bacterial strains.** The Griffin.1 library (Medical Research Council, Cambridge, United Kingdom) is a human-based V<sub>H</sub> and V<sub>L</sub> scFv phagemid library constructed from synthetic V<sub>H</sub> gene segments containing approximately 6.5 × 10<sup>8</sup> different phage antibodies (10). Antibody fragment expressions were carried out using the dicistronic expression vector pMS147. This vector is modified from pHELP1 and is inducible through IPTG (isopropyl-β-D-thiogalactopyranoside) (12). A human C<sub>H</sub> domain is situated immediately downstream of the scFv genes. This domain allows immunodetection and quantification of the expressed soluble polypeptide (called a scAb). The inclusion of a hexahistidine tag permits the purification of the scAb by immobilized metal ion chelate affinity chromatography (26). Vectors were transformed and subsequently expressed in *E. coli* strain XL-1 Blue (supE44, hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lacF' [proAB + lacZAM15 Tn10 (Tet)] (Stratagene)).

**Affinity selection of anti-microcystin-LR phage antibodies.** Griffin.1 library glycerol stock (100 μl) was inoculated into 50 ml of 2× TY broth (34) containing 1% glucose and 100 μg of ampicillin ml<sup>−1</sup> and incubated with shaking at 37°C to an optical density at 600 nm of 0.5 (1 to 2 h). M13 KO7 helper phage (Pharmacia) (1.4 × 10<sup>11</sup> PFU) were added to the 50-ml culture and incubated without shaking at 37°C for 30 min. The infected culture was centrifuged, and the resulting pellet was resuspended in 500 ml of 2× TY broth containing 100 μg of ampicillin ml<sup>−1</sup> and 25 μg of kanamycin ml<sup>−1</sup> and incubated overnight at 30°C. Phage-antibody particles were concentrated from the culture supernatant following centrifugation by precipitation with 100 ml of polyethylene glycol in 2.5 M NaCl (20% [wt/vol]) (10). Phage-antibodies rescued from the library were panned against a 75- by 12-mm immunotube (Griener Laborteknic, Gloucestershire, United Kingdom) coated with microcystin-LR-BSA (100 μg ml<sup>−1</sup>) in phosphate-buffered saline (PBS) as previously described (26). A total of five rounds of panning were carried out under the following conditions. In pan 2, microcystin-LR-KLH conjugate (100 μg ml<sup>−1</sup>) was used to coat the immunotube, while for pans 3 to 5, microcystin-LR-BSA (10 μg ml<sup>−1</sup>) was used. In pans 1, 2, and 3, bound phage were eluted from the immunotube using 100 mM triethylamine (24). The phage were eluted in pans 4 and 5 by incubation with constant turning for 1 h at room temperature with 2 ml of microcystin-LR dissolved in PBS. The concentrations of microcystin-LR used in these elutions were 1 μM in pan 4 and 1 μM in pan 5.

**Selection and screening of phage antibodies.** ELISA was employed to determine the binding of phage precipitated from each round of panning to microcystin-LR-BSA, microcystin-LR-KLH, and the carrier proteins BSA and KLH alone.

Phage-bottom 96-well Immunol 4 microtitre plates (Dynex, Sutton, United Kingdom) were coated with 100 μl of either of the conjugates or carrier proteins (10 μg ml<sup>−1</sup>) at 37°C for 1 h, followed by three washes with PBS. The plates were blocked for 2 h at 37°C with 200 μl of 2%Marvel-PBS (MPBS) per well and washed three times with PBS. Phage precipitated from each round of panning were added to the wells in 2% MPBS, and the plates were incubated for 1 h at room temperature. The plates were washed three times with PBS containing 0.1% Tween 20 (PBST; Sigma) and rinsed three times with PBS prior to the addition of 100 μl of anti-M13–peroxidase conjugate (Pharmacia Biotech, Uppsala, Sweden) per well well diluted 1/1,000. The plates were incubated at room temperature for 1 h, washed three times with PBS, and underwent addition of 100 μl of tetramethylbenzidine dihydrochloride solution (KPL Laboratories, Gaithersburg, Md.) to each well. The reaction was stopped with 50 μl of 1 M H<sub>2</sub>SO<sub>4</sub> per well, and the optical density was read at 450 nm using a microtiter plate reader.

Individual colonies from pans 3 to 5 were grown with shaking at 37°C in 100 μl of 2× TY broth containing 1% glucose and 100 μg of ampicillin ml<sup>−1</sup> in 96-well plates (Greiner) to an optical density at 600 nm of 0.5. The phage-antibody particles were rescued by infecting each well with 25 μl of M13 KO7 (10<sup>10</sup> PFU) and incubating the plates for 30 min standing followed by 1 h with shaking at 37°C. The plates were centrifuged for 10 min at 1,800 × g, and the pellets were resuspended in 200 μl of 2× TY broth containing 100 μg of ampicillin ml<sup>−1</sup> and 25 μg of kanamycin ml<sup>−1</sup> following incubation overnight at 30°C. The binding of individual phage supernatants to microcystin-LR-BSA and microcystin-LR-KLH and to BSA and KLH alone was determined by ELISA.

Phage-antibody clones found to bind to the microcystin-LR conjugates but not to BSA or KLH alone were then assessed for the ability to recognize free microcystin-LR by competition ELISA. This was carried out using the same method described above except that 50 μl of each phage supernatant was added to coated and blocked ELISA plate wells with 50 μl of 4% MPBS containing 1 μM microcystin-LR.

Antibody V<sub>H</sub> and V<sub>L</sub> genes from selected phage clones were amplified by PCR using scFv-specific primers. Fingerprinting of PCR products was achieved by carrying out two separate restriction digests of the PCR products. The heavy chains were digested with *Bst*NI (New England Biolabs, Hitchen, Hertfordshire, United Kingdom), and the light chains were digested in *Escherichia coli* strain XL-1 Blue (Stratagene, Mannheim, Germany). The digests were run out on a 3% agarose gel, and clones showing different banding patterns for the heavy- and light-chain
Expression, purification, and characterization of bacterial scAbs. Expressions of scAbs in transformed E. coli XL-1 Blue clones were carried out in Terrific Broth (37), as described previously (26).

The expressed scAbs were purified via the hexahistidine tail by Ni\(^{2+}\) charged immobilized metal ion chelate affinity chromatography (30), using Fast Flow Sepharose (Pharmacia). The purified scAbs were dialyzed against PBS and stored at \(-20^\circ\)C. Antibody fragment concentration was quantified by capture ELISA of the human Cx domain (27).

Each of the expressed scAb clones was characterized for binding to free microcystin-LR by using indirect competition ELISA. Microtiter plates were coated with microcystin-LR–BSA, washed, and blocked as before. Equal volumes of a subsaturating concentration of each scAb (1 nM) and serial doubling dilutions of microcystin-LR in sterile PBS were incubated for 1 h at 4\(^\circ\)C in 1.5-ml Eppendorf tubes. Control tubes were prepared in which scAb was mixed with sterile PBS. Following incubation, 100 \(\mu\)l of each solution was added to triplicate microtiter wells, and the plates were incubated at room temperature for 1 h. The plates were washed three times with PBST before 100 \(\mu\)l per well of sterile PBS. Following incubation, 100 \(\mu\)l of each toxin were prepared in sterile PBS, and ELISA was carried out as described above.

Cross-reactivity of anti-microcystin-LR scAb and detection of microcystins in extracts of cyanobacterial strains. Indirect competition ELISA was employed to determine the ability of the most sensitive anti-microcystin-LR scAb clone (3A8) to detect three other purified microcystin variants (microcystin-RR, -LW, and -LF) and the related cyanobacterial hepatotoxin nodularin. Doubling dilutions of each toxin were prepared in sterile PBS, and ELISA was carried out as described above.

Cyanobacterial extracts were prepared from three lyophilized strains of Microcystis aeruginosa, PCC 7820 (Pasteur Culture Collection, Paris, France), Sci-crocystis aeruginosa extracts of cyanobacterial strains.

Preparation of anti-microcystin-LR immunoaffinity gel. The anti-microcystin-LR immunoaffinity gel was prepared by coupling 30 \(\mu\)g of 3A8 scAb to 0.5 ml of Ni\(^{2+}\) charged Fast Flow Sepharose for 2 h at room temperature with gentle rotation in a polystyrene column (Bio-Rad, Hercules, Calif.). The columns were washed several times with 5 ml of sterile PBS to remove unbound scAb prior to use. Binding of scAb to the Ni\(^{2+}\) charged Sepharose support was confirmed by capture ELISA of the column eluates. The binding capacity of the gel was determined by resuspending it in 1 ml of an aqueous solution of microcystin-LR at a concentration of 0.7 \(\mu\)g ml\(^{-1}\). The solution was used to immobilize microcystin-LR was changed from BSA to microcystin-LR–BSA and microcystin-LR–KLH conjugates (Fig. 1). The binding of phage-antibody populations to both KLH and BSA alone remained low throughout. In pan 2, the carrier protein used to immobilize microcystin-LR was changed from BSA to KLH in order to minimize the enrichment of phage-antibodies to these proteins. Alternating the carrier proteins between pans 1 and 2 would account for the reduction in binding to microcystin-LR conjugates observed in pan 2 (Fig. 1). Enrichment of phage-antibodies against microcystin-LR conjugates increased in pan 3, where the selection stringency was increased by lowering the microcystin-LR–BSA concentration used to coat the immunotube from 100 to 10 \(\mu\)g ml\(^{-1}\).

Individual phage-antibody clones were selected from pans 3 to 5 for the ability to bind microcystin-LR conjugates in the presence and absence of 1 \(\mu\)M free microcystin-LR. Of the original 134 clones selected for binding to microcystin-LR conjugates, 26 were chosen for fingerprinting based on the ability to recognize free antigen. The selected clones showed between 18 and 78\% reduction in binding to microcystin-LR conjugates in the presence of 1 \(\mu\)M free microcystin-LR.

Fingerprinting of antibody V\(_{H}\) and V\(_{L}\) genes from selected clones were sequenced and assembled. Of the clones sequenced, 3A8 was chosen for further analysis.

Selection of anti-microcystin-LR phage-antibodies and characterization of bacterial scAbs. ELISA of phage-antibodies rescued from the Griffin.1 library prior to affinity selection (pan 0) and from each subsequent pan indicated an enrichment of phage-antibodies which bound to microcystin-LR–BSA and microcystin-LR–KLH conjugates (Fig. 1). The binding of phage-antibody populations to both KLH and BSA alone remained low throughout. In pan 1, the carrier protein used to immobilize microcystin-LR was changed from BSA to KLH in order to minimize the enrichment of phage-antibodies to these proteins. Alternating the carrier proteins between pans 1 and 2 would account for the reduction in binding to microcystin-LR conjugates observed in pan 2 (Fig. 1). Enrichment of phage-antibodies against microcystin-LR conjugates increased in pan 3, where the selection stringency was increased by lowering the microcystin-LR–BSA concentration used to coat the immunotube from 100 to 10 \(\mu\)g ml\(^{-1}\).
phage clones indicated six different banding patterns (results not shown). These six unique scFv genes were subcloned into the soluble expression vector pIMS147 (12) for expression in *E. coli* XL-1 Blue cells.

The binding of the six expressed scAb clones to free antigen was determined by competition ELISA using a subsaturating concentration of scAb (with respect to microcystin-LR–BSA binding) incubated with a range of free microcystin-LR concentrations. All six scAbs bound to free microcystin-LR, with concentrations required to inhibit 50% of binding (IC50s) ranging from 4.5 nM to 1 μM (Table 1). Subsequent experiments were carried out using the most sensitive scAb clone, 3A8. The detection limit (IC20) of this scAb was determined to be 0.8 nM (Fig. 2).

**Cross-reactivity of anti-microcystin-LR scAbs.** The scAb clone 3A8 was then used in competition ELISA to assay three other microcystin variants, microcystin-RR, -LW, and -LF, and nodularin. The 3A8 scAb recognized all four toxins with IC50s of 0.2 nM for microcystin-RR, 12 nM for nodularin, and 200 nM for microcystin-LW and -LF (Fig. 3). The relative cross-reactivities of scAb 3A8 for these four toxins with respect to microcystin-LR were 2,250 (microcystin-RR), 38 (nodularin), and 2% (microcystin-LW and microcystin-LF).

The ability of scAb 3A8 to detect microcystins in cyanobacterial extracts by ELISA was then determined (Fig. 4), and the results were compared with those of HPLC analysis. The IC50s of the *M. aeruginosa* PCC 7820 and Sciento extracts were 1.3 and 9.5 mg of cells liter⁻¹, respectively. ELISA and HPLC failed to detect any microcystins in the extract of the nontoxic strain *M. aeruginosa* CYA 43. Toxin concentrations in *M. aeruginosa* PCC 7820 and Sciento extracts were then estimated as microcystin-LR equivalents by comparison with purified toxin. The ELISA method detected the equivalent of 284 (±21) μg of microcystin-LR ml⁻³ in the *M. aeruginosa* PCC 7820 extract and 140 (±31) μg ml⁻³ in the Sciento extract. The microcystin concentrations of these extracts determined by HPLC analysis were 300 and 151 μg ml⁻¹, respectively.

**Evaluation of anti-microcystin-LR immunoaffinity columns.** The binding capacity of an anti-microcystin-LR immunoaffinity column prepared with scAb 3A8 was determined by loading 20 1-ml volumes of glass distilled water each containing 0.7 g of microcystin-LR ml⁻¹. HPLC analysis of each of the 20 eluates collected indicated that 100% binding of microcystin-LR occurred in the first three loads and breakthrough of the toxin occurred in the fourth load (Fig. 5). Saturation of the immunoaffinity column was achieved after 13 loads, and complete recovery of 0.7 μg of toxin was achieved in each of the final 7 loads. The maximum load of the column was calculated as 6.24 g of microcystin-LR. Elution of the immunoaffinity column in four 1-ml fractions of 100% methanol recovered 6.17 g of microcystin-LR in the first 1 ml of methanol, representing approximately 99% of the total toxin loaded.

To ensure that retention of microcystin-LR by the immunoaffinity column was due to specific binding of the toxin by the 3A8 scAb and not to nonspecific interactions between the toxin

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**TABLE 1.** Sensitivities of purified scAbs isolated from the Griffin.1 phage display library against purified microcystin-LR

<table>
<thead>
<tr>
<th>Clone</th>
<th>IC50 (nM)</th>
<th>Limit of detection (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A8</td>
<td>4.5</td>
<td>0.8</td>
</tr>
<tr>
<td>5B12</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>5B6</td>
<td>45</td>
<td>7</td>
</tr>
<tr>
<td>4C1</td>
<td>200</td>
<td>45</td>
</tr>
<tr>
<td>4B6</td>
<td>300</td>
<td>90</td>
</tr>
<tr>
<td>3C10</td>
<td>1,000</td>
<td>250</td>
</tr>
</tbody>
</table>

*a For free microcystin-LR; Values are the means of triplicate determinations.

*b Limits of detection represent the IC20s of microcystin-LR.

FIG. 2. Indirect competition ELISA determining the ability of scAb clone 3A8 to recognize free microcystin-LR. The dashed line represents the IC50 of free microcystin-LR, and the dotted line represents the detection limit of the assay (the IC20 of free microcystin-LR). The points are the means of three determinations and are a representative set from repeated experiments. The error bars represent sample standard errors.
and the Ni\(^{2+}\) charged Sepharose support, a control column was prepared using the same procedure but without the 3A8 scAb. None of the microcystin-LR loaded onto this control column was retained.

**Application of immunoaffinity columns to concentrate microcystin-LR from water.** The potential of 3A8 immunoaffinity columns to concentrate microcystin-LR from large sample volumes was determined using 150 ml of distilled water spiked with 4 \(\mu\)g of the purified toxin. The concentration of microcystin-LR in these samples (27 ng ml\(^{-1}\)) fell below the limit of quantification offered by HPLC analysis (400 ng ml\(^{-1}\)) (Fig. 6a). Application of triplicate samples to scAb 3A8 immunoaffinity columns followed by elution in 1 ml of 100% methanol recovered 94% (\(\pm\)3.6%) of the microcystin-LR and allowed identification and quantification of the toxin in concentrated samples by HPLC analysis (Fig. 6b).

**DISCUSSION**

The selection of recombinant antibody fragments from naïve phage display libraries offers a straightforward and inexpensive

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**FIG. 3.** Indirect competition ELISA determining the ability of scAb clone 3A8 to recognize microcystin-RR (●), nodularin (▲), microcystin-LW (■), and microcystin LF (○). The dashed line represents the IC\(_{50}\) of free microcystin variant. The points are the means of three determinations and are a representative set from repeated experiments. The error bars represent sample standard errors.

**FIG. 4.** Indirect competition ELISA determining the ability of scAb 3A8 to detect microcystins in cyanobacterial extracts from three strains of *M. aeruginosa*, 7820 (●), Sciento (○), and CYA 43 (■). The dashed line represents the IC\(_{50}\) of extract. The points are the means of two determinations and are a representative set from repeated experiments. The error bars represent sample standard errors.
alternative to the methods currently used for producing polyclonal and monoclonal antibodies specific for microcystins. We have already reported the application of an anti-microcystin-LR scAb cloned from a synthetic naïve phage display library (the Tomlinson I library) (26) which detected microcystin-LR, -RR, -LW, and -LF in the micromolar range. In this study, use of the Griffin.1 library allowed us to isolate a scAb which is over 800 times more sensitive for microcystin-LR and is capable of detecting the toxin below the guideline level set by the World Health Organization (1 µg liter⁻¹) (39). To our knowledge, the scAb used in this study (3A8) is the most sensitive recombinant antibody currently available for the detection of microcystins.

The improved sensitivity of antibody fragments isolated from the Griffin.1 library compared to those from the Tomlinson I library may be associated with the length of the third complementary determining region (CDR3) in the heavy-chain variable domain, which is thought to play an important role in anti-hapten binding (14). Recent studies have suggested that while shorter heavy-chain CDR3s tend to form planar binding sites suitable for binding large proteins, longer heavy-chain CDR3s are capable of forming anti-hapten binding pockets (5, 36). The heavy-chain CDR3 in the Tomlinson I library is conserved at seven amino acids, while the Griffin.1 library incorporates variable heavy-chain CDR3 lengths. Panning of the Griffin.1 library against microcystin-LR conjugates enabled the isolation of a more sensitive anti-microcystin-LR clone (3A8), which was found to have 10 amino acid residues at this position (36).

ELISA of purified microcystins and nodularin using scAb 3A8 revealed that while it recognized all of the toxins assayed, cross-reactivity was variable. The scAb appeared to bind most strongly to microcystin-RR, followed by microcystin-LR and nodularin. However, 3A8 was over 40 times less sensitive for microcystin-LW and -LF. This may be due to the method employed to couple microcystin-LR to BSA and KLH for immobilization during panning. The conjugation of microcystin-LR to carrier proteins via N-methyldehydroalanine (Fig. 1) is believed to expose the arginine residue located adjacent to Adda at position 4 of the peptide ring (29, 40). Like Adda, the arginine side chain in microcystin-LR is flexible (1), and its exposure may have biased the selection of antibody fragments during panning. The ability of scAb 3A8 to recognize arginine side chains, which are present in microcystin-LR and -RR and nodularin but not in microcystin-LW and -LF, may account for

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FIG. 5. Determination of the maximum load of the anti-microcystin-LR immunoaffinity column prepared with 30 µg of scAb 3A8 bound to 0.5 ml of Ni²⁺ charged Fast Flow Sepharose. The graph shows the percentage of unbound microcystin-LR collected after 0.7 µg ml⁻¹ was loaded in distilled water, as determined by HPLC.

FIG. 6. HPLC chromatograms of a 150-ml sample of distilled water spiked with 4 µg of purified microcystin-LR before (a) and after (b) concentration on a scAb 3A8 immunoaffinity column.
its variable cross-reactivity. However, this did not appear to have any effect on the detection and quantification by ELISA of microcystins in toxic extracts of *M. aeruginosa*, despite the differences in the toxin profiles of these extracts. HPLC analysis of the 7820 extract revealed microcystin-LR, -LY, -LW, and -LF, while the Sciento extract was found to contain microcystin-LR and -RR. Determinations of microcystins in these extracts as microcystin-LR equivalents by ELISA were only slightly underestimated compared with HPLC analysis. The scAb was also capable of distinguishing between toxic and nontoxic strains of *M. aeruginosa*, as no microcystins were detected in the CYA 43 extract by either HPLC or ELISA. Interestingly, one of the other scAb clones isolated during panning (5B6 [Table 1]) was found to be more sensitive for microcystin-LW and -LF than scAb 3A8 (results not shown) despite being 10 times less sensitive for microcystin-LR. It may be possible to optimize the cross-reactivity of the current ELISA format for natural samples by using a combination of these scAbs.

This study also demonstrated the potential of anti-microcystin scAbs in the immunoaffinity concentration of microcystins from large volumes of water (Fig. 6). HPLC analysis of microcystins in biological and environmental samples, such as plant and animal tissues or lake water, often requires the removal of contaminating sample matrix effects. In most monitoring studies, samples are processed using solid-phase extraction with octadecyl silanized cartridges before analysis by HPLC (15, 16, 38). However, this method is not sufficiently selective to eliminate coeluting compounds, which can interfere with the accurate identification and quantification of microcystins in natural samples. Immunoaffinity chromatography has shown considerable promise in the concentration and cleanup of microcystins from natural samples prior to HPLC analysis (15, 16, 38). However, current methods employing either polyclonal or monoclonal antibodies are extremely limited due to the time and expense required to produce the large amounts of antibodies necessary for preparing immunoaffinity columns. To resolve this problem, complex methodologies have recently been developed to enable the columns to be reused (17). In the present study, retention of microcystin-LR by immobilized scAb was found to decrease significantly when the columns were used more than once (data not shown). While elution in 100% methanol facilitated maximum recovery of bound toxin, it is likely to have had an adverse effect on scAb stability. Alternative elution methods could be investigated to determine whether column regeneration is possible. It may also be possible to stabilize the scAb through introduction of an interdomain disulfide bond, which has previously been shown to improve the performance of recombinant antibody fragments in methanol (7, 35). However, as it is possible to express large quantities of recombinant scAbs in *E. coli* at relatively low cost, the development of reusable columns may not be necessary. A further advantage of the columns developed in this study is that immobilization of scAb via the hexahistidine tag ensures maximum exposure of antigen binding sites. This enabled over 6 µg of microcystin-LR to be retained on columns prepared using only 30 µg of purified scAb. It is also worth noting that while previously reported methods require large-volume water samples to be concentrated to 5 to 15 ml prior to immunoaffinity chromatography (18, 38), the columns developed here allowed 150 ml of distilled water to be directly applied to the scAb immunoaffinity column without affecting retention. Although our scAb immunoaffinity columns have yet to be tested using natural water samples or bloom material, performance may be improved by using combinations of scAbs or by immobilizing larger quantities. As the scAb isolated during this study can be expressed rapidly and inexpensively in *E. coli*, it will enable us to optimize these columns for the concentration and cleanup of microcystins, providing a powerful tool for the analysis of these toxins in a variety of sample matrices.

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