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Mechanistic and toxicity studies of the photocatalytic oxidation of microcystin-LR

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Abstract

Cyanobacterial toxins present in drinking water sources pose a considerable threat to human health. Conventional water treatment systems have proven unreliable for the removal of these toxins and hence new techniques have been investigated. Previous work has shown that TiO_2 photocatalysis effectively destroys microcystin-LR in aqueous solutions, however, a variety of by-products were generated. In this paper, we report a mechanistic study of the photocatalytic destruction of microcystin-LR. In particular, the toxicity by-products of the process have been studied using both brine shrimp and protein phosphatase bioassays. © 2002 Published by Elsevier Science B.V.

Keywords: Microcystins; Protein phosphatase 1; Photocatalytic oxidation; TiO_2; Water treatment

1. Introduction

Cyanobacterial toxins produced and released by cyanobacteria in freshwater around the world are well documented [1,2]. Microcystins are the most common of the cyanobacterial toxins found in water, as well as being the ones most often responsible for poisoning animals and humans who come into contact with toxic blooms and contaminated water [3]. Acute exposure results in hepatic injury, which can in extreme cases prove fatal. One such incident occurred that resulted in the death of over 50 dialysis patients due to the use of microcystin-contaminated water in the treatment [4]. Chronic exposure due to the presence of microcystin in drinking water is thought to be a contributing factor in primary liver cancer (PLC) through the known tumour-promoting activities of these compounds [5]. It has been shown that the mode of action of the hepatotoxins is through inhibition of protein phosphatase 1, and 2A. These are the two classes of enzymes that act as 'molecular control switches' and regulate many processes inside animal and plant cells. At a molecular level, microcystins bind irreversibly to and inhibit the serine/threonine protein phosphatase 1 and 2A [6]. The binding complex of microcystin-LR and protein phosphatase 1 has been characterised with crystallographic analysis [7].

Since cyanobacterial toxins present in drinking water sources pose a considerable threat to human health, various treatments have been used to remove the toxins. However, it is believed that conventional water treatment systems have proven unreliable for the removal of these toxins from potable water [8,9].

Previous work has shown that TiO_2 photocatalysis effectively destroys microcystin-LR in aqueous solutions even at extremely high toxin concentrations, however, a variety of by-products were generated [10,11]. Further studies allowed the characterisation of some of the breakdown products and the assessment of their toxicity with brine shrimp bioassay [11].

In this study, protein phosphatase inhibition assay has been used to assess whether toxicity has been removed due to the degradation of microcystin and if further toxicity has been generated due to the breakdown products of microcystin in photocatalysis. This will enable relevant and specific toxicity information to be obtained about protein phosphatase inhibition, which is believed to cause tumour promotion and hepatotoxic toxicity.

2. Experimental details

2.1. Materials

Microcystin-LR was purified from a bloom of *Microcystis aeruginosa* using the procedure previously detailed [12].

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Titanium dioxide (Degussa P-25) and protein phosphatase 1 (Sigma, Pool, UK) were used as received. All solutions were prepared in Milli-Q water, and all other reagents used were analytical grade.

2.2. Photocatalysis

Aqueous solutions of microcystin-LR containing 1% (w/v) TiO_2 alone and 1% (w/v) TiO_2 plus 0.1% (w/w) H_2O_2 were illuminated in the presence of air with a 480 W xenon lamp (Uvalight Technology; spectral output 330–450 nm). The reactions were carried out in glass bottles with constant stirring. The distance from the UV lamp to surface of the test solution was 30 cm. The initial pH of the solution was 4 and the solution temperature on illumination equilibrated at 306 K. Prior to quantitative analysis by HPLC samples were centrifuged to remove TiO_2. Previous results had established that when H_2O_2/UV in the absence of TiO_2 did not result in any significant MC-LR destruction. Consequently, no step was taken to remove excess H_2O_2 in the test solution with TiO_2 plus H_2O_2.

2.3. Analysis

Treated samples were analysed by HPLC with photodiode array detection as previously detailed [11]. The eluent from HPLC was collected as fractions with an auto-sampler each minute after injection. Each of the fractions were further analysed with HPLC to confirm the isolation of detectable peaks of breakdown products of the photocatalytic process. Samples taken prior to HPLC separation and fractions collected from HPLC were all subjected to toxicity assay with or without dilution.

Protein phosphatase inhibition assay was performed using a modification of previously reported colorimetric procedures [13–15]. Protein phosphatase 1 (PP1) was diluted with buffer containing 50 mM Tris–HCl, 1.0 g l^{-1} BSA, 1.0 mM $MnCl_2$ and 2.0 mM dithiothreitol, pH 7.4. *p*-Nitrophenyl phosphate (5 mM) was prepared in buffer containing 50 mM Tris–HCl, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5 g l^{-1} BSA, pH 8.1. All buffers were freshly prepared before use. Microcystin-LR and test samples were prepared with Milli-Q water.

The assay was conducted by addition of 25 μl of test solution to 25 μl of PP1 solution in a 96-well polystyrene microtitre plate. After a few seconds gentle shaking, the microtitre plate was kept in room temperature for 5 min followed by addition of 200 μl of *p*-nitrophenyl phosphate solution (substrate). The plate was incubated at 37 °C during which the reaction occurred. The rate of production of *p*-nitrophenol was measured at 4 min intervals for 60 min at 405 nm on a Dynatech MR 5000 Reader. A dose dependent

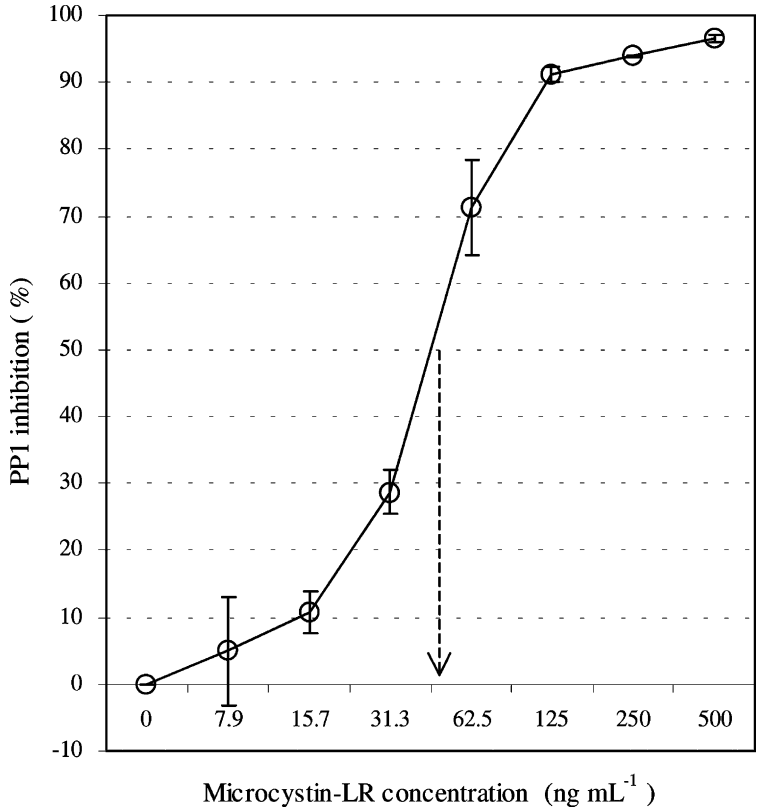

Fig. 1. Standard inhibition curve of microcystin-LR against protein phosphatase 1 (PP1) read at 40 min of reaction. Arrow shows the estimated IC_{50} (about 47 ng ml^{-1}). The concentration of PP1 in the test was 4 μg ml^{-1}. Each point plotted is the mean of three observations, and the vertical bars indicate the standard deviation of the mean.

kinetic activity of protein phosphatase 1 (PP1) against substrate (*p*-nitrophenyl phosphate) was established to assess the enzyme activity prior to sample test. A standard inhibition curve of microcystin-LR was constructed by measuring the percentage inhibition of enzyme activity against a negative control of Milli-Q water. All enzyme assays were performed in triplicate.

3. Results and discussion

The standard curve for the PP1 inhibition of MC-LR (Fig. 1) shows 100% inhibition of the enzyme occurring at toxin concentrations over 500 ng ml^{-1}, with a detection limit around 30 ng ml^{-1} (20% inhibition). The linear region of the curve appeared between 31.3 and 125 ng ml^{-1} microcystin-LR. From the curve, the IC_{50} level was determined to be around 47 ng ml^{-1}.

The concentration of microcystin-LR (1 mg ml^{-1}) used for quantitative analysis of photocatalysis and breakdown products toxicity assay was extremely high. This enabled direct analysis of the toxin and reaction products by both HPLC and toxicity assay without multiple processing that would be necessary to quantify the much lower levels found in the environment. Such high toxin concentrations, however, were rapidly degraded on photocatalysis. The results in Table 1 shows 85.6% of microcystin-LR was destroyed within the first 5 min of photocatalysis with 97.2% of the toxin destroyed in 20 min. No microcystin was detectable after 30 min photocatalysis. The addition of 0.1% H_2O_2 to the photocatalytic system significantly enhanced the destruction of microcystin-LR (Table 1). In this case 99.6% of the toxin was destroyed within 5 min with total disappearance by 10 min photocatalysis time. These observations were similar with those previously reported [16].

The toxicity of the decomposition products of the photocatalytic process is shown in Figs. 2 and 3. Although there was a rapid disappearance of the microcystin on photocatalysis (Fig. 2) the PP1 inhibition only slightly decreased up to 20 min reaction time. The inhibition, however, rapidly reduced after 30 min photocatalysis, while about 20% inhibition remained after 60 min.

Table 1
Decomposition of microcystin-LR with photocatalysis

Time (min)	Microcystin-LR decomposed (μg ml^{-1})		Decomposition rate (%)	
	TiO_2*	TiO_2/H_2O_2	TiO_2*	TiO_2/H_2O_2
0	1000	1000	0	0
5	144.163	3.9	85.6	99.6
10	74.318	0	92.6	100
20	28.006	0	97.2	100
30	1.447	0	99.9	100
45	0.231	0	100	100
60	0.141	0	100	100

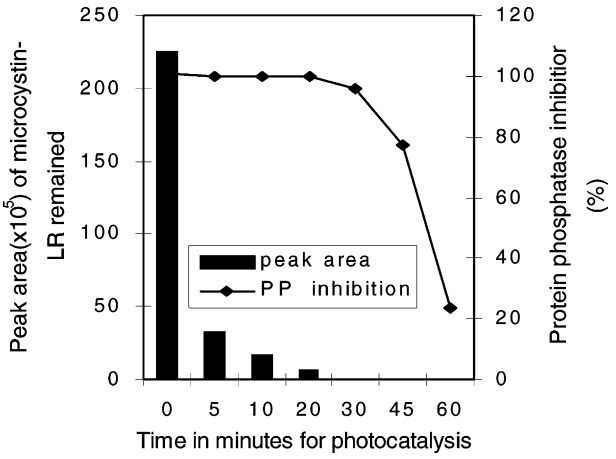

Fig. 2. Destruction and PP1 inhibition of microcystin-LR (photocatalysis with TiO_2). Each point plotted for PP1 inhibition is the mean of three observations. The concentration of PP1 in the test was 4 μg ml^{-1}.

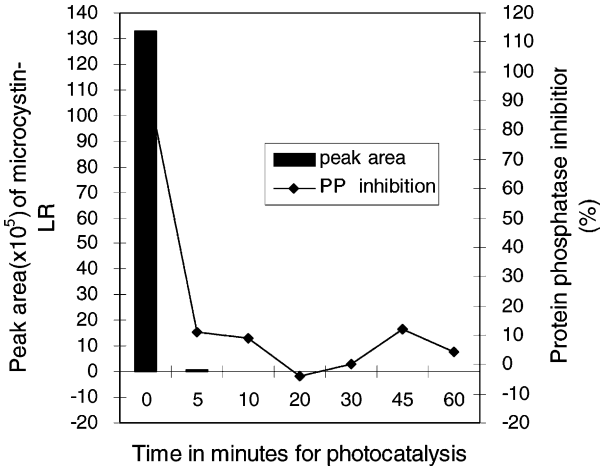

Fig. 3. Destruction and PP1 inhibition of microcystin-LR (photocatalysis with TiO_2 plus H_2O_2). Each point plotted for PP1 inhibition is the mean of three observations. The concentration of PP1 in the test was 4 μg ml^{-1}.

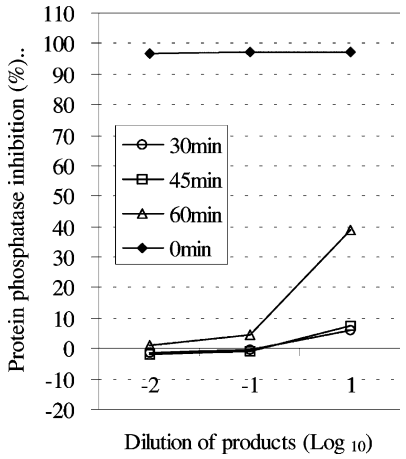

Fig. 4. Inhibition of reaction products with dilutions. Legend shows the time treated with TiO_2/H_2O_2.

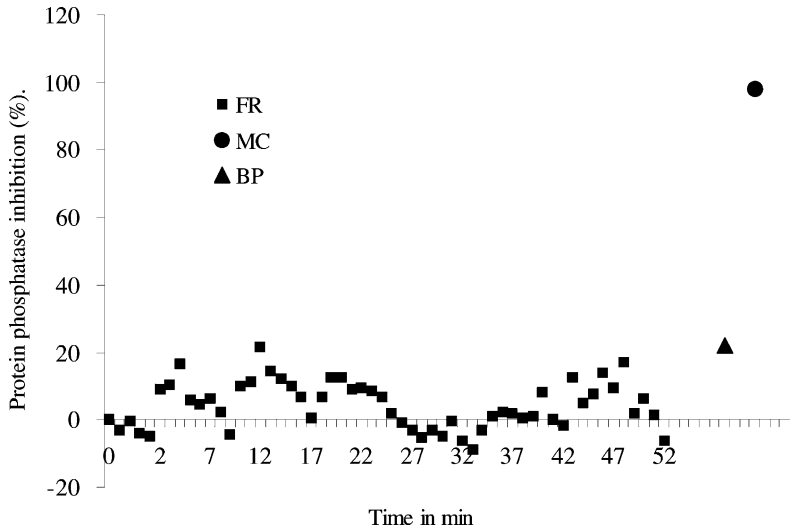

Fig. 5. Protein phosphatase inhibition of fractions collected from HPLC eluent for by-products of microcystin-LR photocatalysis in 30 min with TiO_2. FR: fractions collected from HPLC eluent in minutes; MC: microcystin-LR 1 mg ml^{-1}; BP: 30 min by-products before HPLC fractionation.

In the system where H_2O_2 was added not only was the destruction of microcystin much faster but also the toxicity of the treated solution dropped more quickly (Fig. 3). In this case there was a rapid reduction in PP1 inhibition within 5 min photocatalysis time with a complete disappearance in 20 min.

Interestingly, the inhibition of PP1 slightly increased in reaction products collected at 45 and 60 min with an inhibition about 10–20% (Fig. 3). Since the disappearance of toxicity occurred in 20 min, the inhibition of 45 and 60 min products would be unlikely due to residual microcystin-LR but may possibly be caused by carbonyl acids and similar molecules generated as degradation products. Such molecules might produce some mild non-specific inhibition against the enzyme. To support this assumption, the reaction products were diluted and tested again for PP1 inhibition. At the same time their pH was measured. As expected, the inhibition of original solution did not reach IC_{50}, a critical level for bio-toxicity, and dilution of the original solution resulted in the reduction and disappearance of enzyme inhibition (Fig. 4). In contrast, PP1 inhibition of microcystin-LR (at 0 min) was not reduced at all with the same dilution. This suggested that the mechanism of PP1 inhibition resulting from photocatalysis products was different from that of microcystin-LR. Table 2 revealed that the 60 min reaction solution (original) of photocatalysis (TiO_2/H_2O_2) had a pH of 4.5. This original solution plus equal volume of enzyme assay buffer (pH 7.4) changed the pH to 4.6. A 1% original solution plus enzyme assay buffer had a pH of 6.8. The optimum pH for PP1 reaction should be pH 7.4, pH levels below

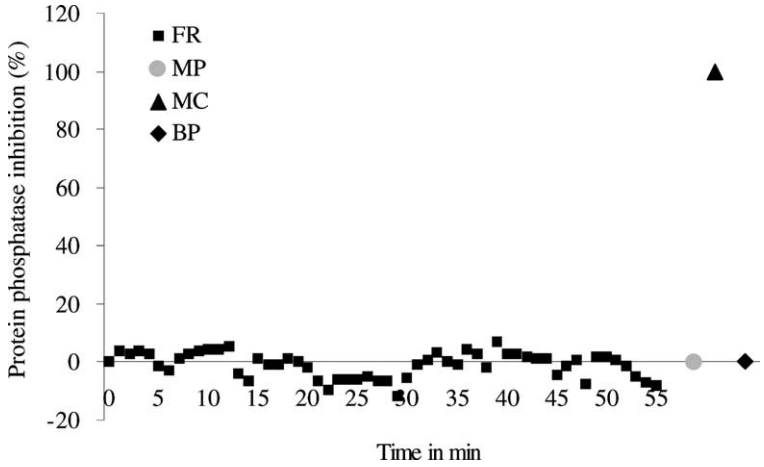

Fig. 6. Protein phosphatase inhibition of fractions collected from HPLC eluent for by-products of microcystin-LR photocatalysis in 30 min with TiO_2 plus H_2O_2. FR: fractions collected from HPLC eluent in minutes; MP: HPLC mobile phase before passing column; MC: microcystin-LR 1 mg ml^{-1}; BP: 30 min by-products before HPLC fractionation.

Table 2
pH measurement of reaction solution of photocatalysis (TiO_2/H_2O_2)

Sample	pH
A (original solution of photocatalysis)	4.5
A plus equal volume of buffer, pH 7.4	4.6
1% A plus equal volume of buffer, pH 7.4	6.8

this will result in a certain level of enzyme inhibition. This evidence further supported above elucidation on the cause of mild inhibition from 45, and 60 min reaction products.

The degradation solution following photocatalysis at 30 min was isolated with HPLC by injection of large volume (200 μl). Fractions from eluent were collected every minute after injection. All the fractions then subjected, along with pre-HPLC parent solutions, to PP1 assay. Figs. 5 and 6 show that none of these fractions had any significant inhibition against PP1.

As previously described, the concentration of microcystin-LR used in this study was significantly higher than that found in the natural environment and therefore poses a potential problem in potable water supplies (1 mg ml^{-1}, 1 million-fold higher than the limit recommended by WHO 1 μg l^{-1}). The destruction of such high concentrations of microcystin would generate correspondingly high concentrations of breakdown products. If such high concentration of breakdown products failed to have any PP1 inhibition, then the breakdown products resulting from the same photocatalysis of microcystin-LR contaminated natural water would have a lower potential PP1 inhibition risk. Further evidence to support this assumption came from PP1 assay on a solution containing a much lower toxin concentration (1 μg ml^{-1}) as detailed in Fig. 7.

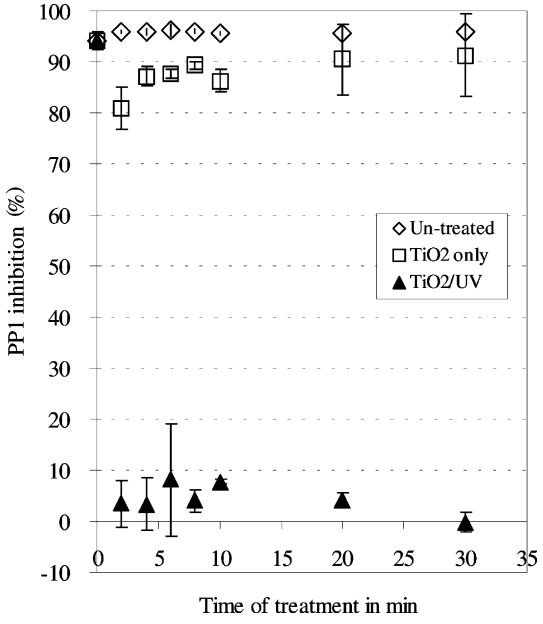

Fig. 7. PP1 inhibition of breakdown products produced by 1 μg ml^{-1} of MC-LR with photocatalysis.

Table 3
Toxicity of breakdown products of microcystin-LR against brine shrimp (initial concentration of MC-LR 200 μg ml^{-1})

Exposure time (min)	IC_{50} (μg ml^{-1})	
	TiO_2	TiO_2/H_2O_2
0	2.0	3.0
2	10.8	10.0
4	17.3	16.5
6	27.5	29.5
8	41.3	>50
10	>50	>50
20	>50	>50
30	>50	>50

Although the concentration of microcystin was still much higher than occurring in natural environment, the reaction solution that contained breakdown products failed to show any inhibition against PP1 after 2 min with UV/TiO_2 treatment.

In addition to the specific toxicity represented by PP1 inhibition, a general bio-toxicity of breakdown products was also evaluated with brine shrimp assay. Table 3 shows that after 6–8 min photocatalysis, no significant toxicity could be detected from the reaction solutions ($IC_{50} > 50$ μg ml^{-1}). These results were in agreement with those obtained using the PP1 assay.

4. Conclusion

The effectiveness of TiO_2 photocatalysis for the removal of microcystin-LR from water has been established. Not only does the process rapidly remove the toxin but also the by-products appear to be non-toxic. The photocatalytic process has also significantly reduced the PP1 inhibition. PP1 inhibition is potentially one of the most serious harmful effects to humans who may consume water contaminated by microcystins. Many traditional water treatment processes are less effective at reducing this hazard or the repeatability of the process is difficult to replicate. The TiO_2 system produced very repeatable results that will provide confidence in the technique as a process for microcystin removal. The addition of H_2O_2 not only enhances the destruction of the toxin but also rapidly increases the reduction of PP1 inhibition of treated water. This is a significant finding as the reduction of PP1 inhibition may reduce levels of PLC.

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