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.

Key words: 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 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]. 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 Ltd.; spectral output 330-450nm). The reactions were carried out in glass bottles with constant stirring. The distance from the UV lamp to surface of the test solution was 30cm. 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 50mM Tris-HCl, 1.0g L^{-1} BSA, 1.0mM $MnCl_2$ and 2.0mM dithiothreitol, pH 7.4. p-Nitrophenyl phosphate (5mM) was prepared in buffer

containing 50mM Tris-HCl, 20mM $MgCl_2$, 0.2mM $MnCl_2$ and 0.5g L^{-1} BSA, pH8.1. 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 25µl of test solution to 25µl 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 minutes followed by addition 200µl of p-nitrophenyl phosphate solution (substrate). The plate was incubated at 37^{o}C during which the reaction occurred. The rate of production of p-nitrophenol was measured at 4 minute intervals for 60 minutes at 405nm on a Dynatech MR 5000 Reader. A dose dependent 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 (Figure 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 31.3-125 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 (1mg mL^{-1}) used for quantitative analysis of photocatalysis and break down products toxicity assay was extremely high. This enabled directly 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 Table1 shows 85.6% of microcystin-LR was destroyed within the first 5 minutes of photocatalysis with 97.2% of the toxin destroyed in 20 minutes. No microcystin was detectable after 30 minutes 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 minutes with total disappearance by 10 minutes photocatalysis time. These observations were similar with those previously reported [16].

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

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 (Figure.3.). In this case there was a rapid reduction in PP1 inhibition within 5 minutes photocatalysis time with a complete disappearance in 20 minutes.

Interestingly, the inhibition of PP1 slightly increased in reaction products collected at 45 and 60 minutes with a inhibition about 10-20% (Figure.3). Since the disappearance of toxicity occurred in 20 minutes, the inhibition of 45 and 60 minute 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 IC50, a critical level for bio-toxicity, and dilution of the original solution resulted in the reduction and disappearance of enzyme inhibition (Figure 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 PP 1 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 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 minutes was isolated with HPLC by injection of large volume (200μl). Fractions from eluent were collected every minute

after injection. All the fractions then subjected, along with pre-HPLC parent solutions, to PP1 assay. Figures 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 (1mg mL^{-1}, one million-fold higher than the limit recommended by WHO 1μg L^{-1}). The destruction 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 (1μg mL^{-1}) as detailed in Figure 7. 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 minutes photocatalysis, no significant toxicity could be detected from the reaction solutions (IC50> 50μg 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 PP-1 inhibition. PP-1 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 PP-1 inhibition of treated water. This is a significant finding as the reduction of PP1 inhibition may reduce levels of primary liver cancer (PLC).

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Legends for figures and tables.

Table 1. Decomposition of microcystin-LR with photocatalysis

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

Table 3. Toxicity of breakdown products of microcystin-LR against brine shrimp (Initial concentration of MC-LR 200ug/ml)

Figure 1. Standard Inhibition curve of microcystin-LR against protein phosphatase 1 (PP1) read at 40min 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 3 observations, and the vertical bars indicate the standard deviation of the mean

Figure 2. Destruction and PP1 inhibition of microcystin-LR (photocatalysis with TiO_2). Each point plotted for PP1 inhibition is the mean of 3 observations. The concentration of PP1 in the test was 4 μg mL^{-1}.

Figure 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 3 observations. The concentration of PP1 in the test was 4 μg mL^{-1}

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

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

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

Figure 7. PP1 Inhibition of breakdown products produced by 1μg/ml of MC-LR with photocatalysis

Table 1.

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

Table 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

Table 3.

Exposure time (min.)	LC50 ($\mu g\ mL^{-1}$)	
	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

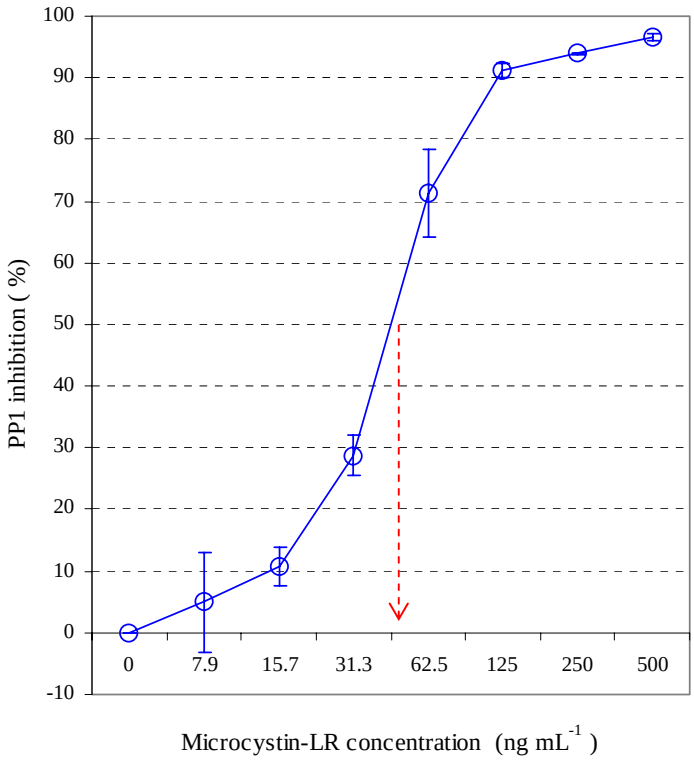

100
90
80
70
60
50
40
30
20
10
0
-10
PP1 inhibition (%)
0
7.9
15.7
31.3
62.5
125
250
500
Microcystin-LR concentration (ng mL-1)

Figure 1.

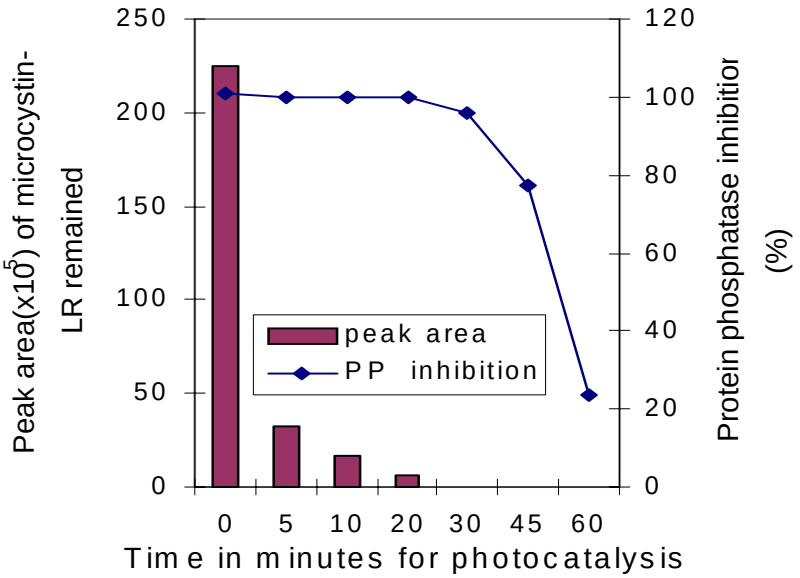

250
200
150
100
50
0
120
100
80
60
40
20
0
0 5 10 20 30 45 60
Time in minutes for photocatalysis
peak area
PP inhibition
Peak area(x10^5) of microcystin-LR remained
Protein phosphatase inhibition (%)

Figure 2.

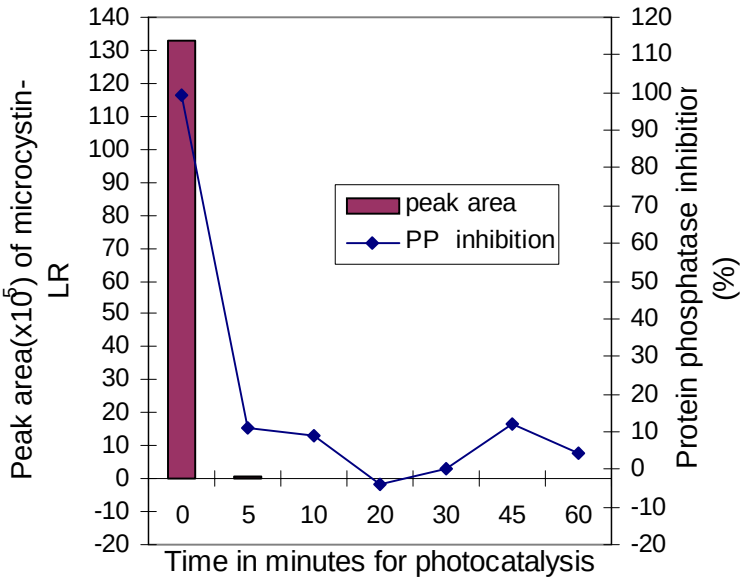

Peak area(x10^5) of microcystin-LR
Protein phosphatase inhibition (%)
peak area
PP inhibition
Time in minutes for photocatalysis
0
5
10
20
30
45
60

Figure 3.

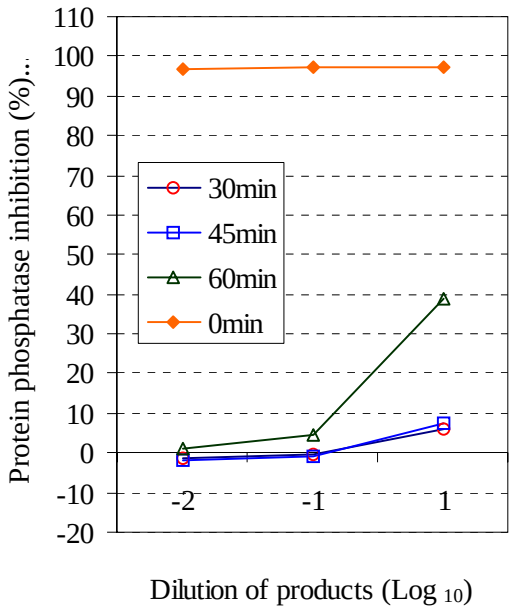

110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
Protein phosphatase inhibition (%)...
30min
45min
60min
0min
-2
-1
1
Dilution of products (Log 10)

Figure 4.

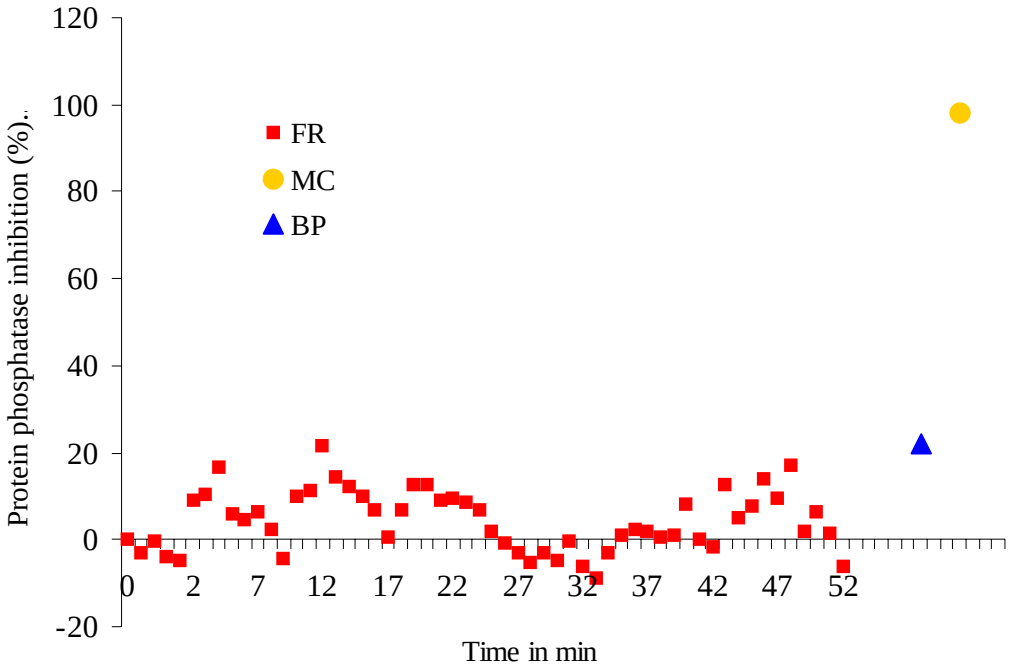

120
100
80
60
40
20
0
-20
Protein phosphatase inhibition (%)..
FR
MC
BP
0
2
7
12
17
22
27
32
37
42
47
52
Time in min

Figure 5.

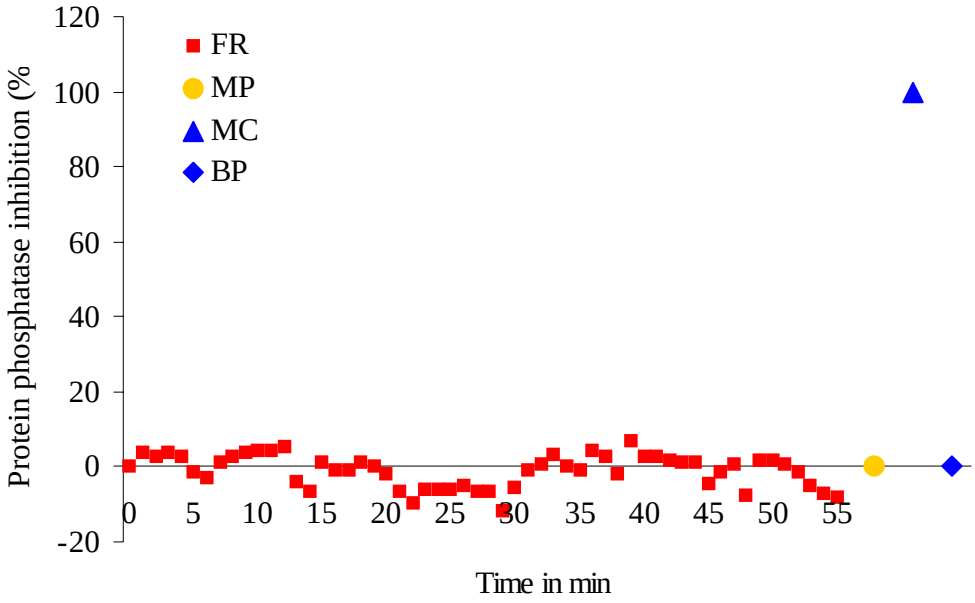
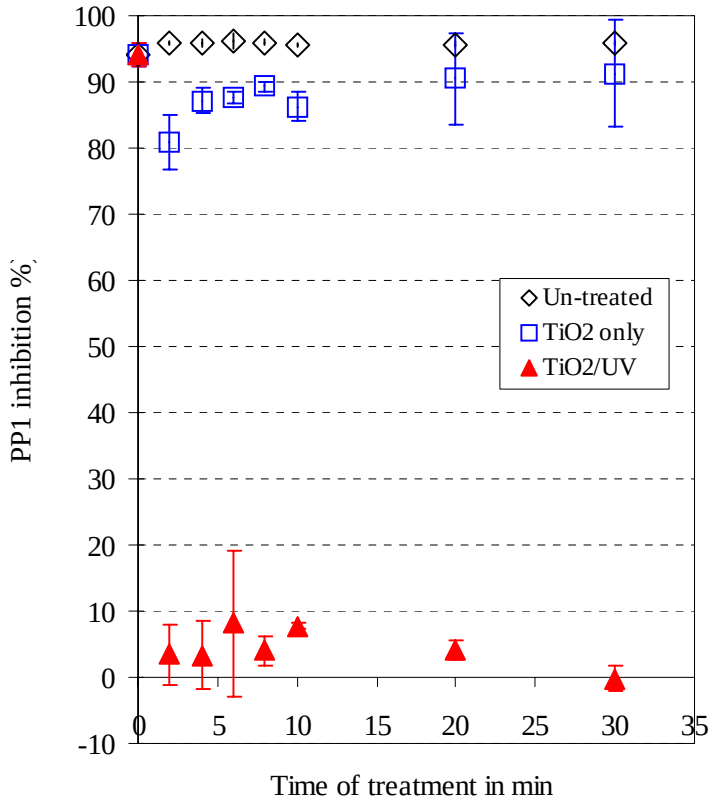

120
100
80
60
40
20
0
-20
Protein phosphatase inhibition (%
FR
MP
MC
BP
0
5
10
15
20
25
30
35
40
45
50
55
Time in min

Figure 6.


100
90
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50
40
30
20
10
0
-10
0
5
10
15
20
25
30
35
PP1 inhibition %`
Time of treatment in min
Un-treated
TiO2 only
TiO2/UV

Figure 7.