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Phthalate Confers Protection Against UV-A Irradiation but is an Uncompetitive Inhibitor of Bovine Catalase at High Concentrations

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Solar ultraviolet water disinfection (SODIS) is a process in which contaminated water is exposed to sunlight in plastic bottles to create reactive oxygen species (ROS) including H_2O_2 to eliminate bacteria and other biological pathogens. However, the efficacy of this treatment is reduced by the presence of the enzyme catalase in bacteria which breaks down hydrogen peroxide (H_2O_2) and the presence of phthalate, a chemical commonly found in many plastics, which is believed to act as a UV filter by absorbing the UV-A wavelengths of sunlight. In this study, we asked whether the presence of phthalate protects the activity of catalase exposed to UV-A radiation. To measure catalase activity kinetically we used two assays: the H_2O_2 spectroscopy microassay and the Triton X-100 foam production assay. K_m and V_{max} were determined to quantitatively characterize the effects on enzyme activity. Phthalate was found not act as a UV-A filter to protect catalase from UV-A irradiation. However, phthalate is capable of protecting both the substrate-binding and catalytic ability of catalase at concentrations of 0.4 µg/L by other means yet to be identified. At higher concentrations of 50,000 µg/L, phthalate becomes an uncompetitive inhibitor of catalase that decreases catalytic activity by binding to the enzyme-substrate complex.

The tetrameric enzyme catalase is well known for its catalytic decomposition of H_2O_2 into water (H_2O) and oxygen (O_2), an important process in protecting cells from oxidative damage (1). As an anti-oxidant enzyme, catalase can prevent H_2O_2, a reactive oxygen species, from killing cells in oxidative decontamination treatments such as Solar Ultraviolet Water Disinfection (SODIS) (1). Disinfection of water by SODIS is based on two principles. The first is that UV-A, a 315 nm to 400 nm wavelength ultraviolet light spectrum, from sunlight reacts with dissolved oxygen to produce reactive oxygen specie (ROS) such as H_2O_2 to damage bacterial cells. The second is that the heat from the sunlight increases the water temperature above 52 Celsius; killing or at least inactivating bacteria (2).

While catalase is able to detoxify the hydrogen peroxide created by UV-A, the enzyme itself is vulnerable to

UV-A exposure leading to a multitude of detrimental effects including peptide aggregation, the dissociation of heme sites, oxidation, and the modification of charged properties which impede catalytic activity (3, 4). As an enzyme, the catalytic activity of catalase is also vulnerable to other changes in the chemical and physical environment. Phthalate is a phenolic plasticizer commonly present at 0.4 µg/L in plastic bottles used for SODIS, and has been proposed to enhance the germicidal activity of ROS by inhibiting catalase (1) though no mechanism has been clearly defined prior to this study. Conversely, a study by *Hsieh et al.* showed that the presence of phthalate instead protected catalase from UV-A damage. Hsieh *et al.* suggested that phthalate protects catalase from UV-A radiation by acting as a UV-filter and absorbing UV-A rays using the pi electrons on its aromatic ring structure (5). A second explanation is that phthalate could have acted as an enzyme modifier. Modifications can be transient or permanent and can include binding to and stabilizing the catalase monomer or tetramer; consequently protecting heme sites and vital peptide sequences in the enzyme structure from degradation and/or modification.

The purpose of this study was to assess if both or any of the two interpretations are valid explanations for the interaction between phthalate and catalase. An enzyme kinetics approach was used to characterize the nature of inhibition of bovine catalase, which is structurally and functionally similar to bacterial catalase, by UV-A radiation and to determine the effect of phthalate on the properties of the enzyme (6). A H_2O_2 spectrophotometric microassay measuring the concentration of hydrogen peroxide over time, and a Triton X-100 foam production assay measuring oxygen evolution, are two methods to determine catalase activity by observing substrate degradation and product formation respectively. They were used to determine the nature of catalase inhibition by UV-A and delineate the effect of phthalate on catalase under light, dark, and UV-A treated conditions and in the presence of phthalate. Measures of enzyme affinity for substrate (K_m) and maximum potential enzyme activity (V_{max}) will be compared across all conditions as a means of assessing damage or protection to catalase.

MATERIALS AND METHODS

Phthalate absorption. Three different concentrations of phthalate solution (0 µg/L, 0.4 µg/L, and 50,000 µg/L) were prepared by dissolving phthalic acid (potassium hydrogen phthalate from Sigma-Aldrich, catalogue # P–6758) in deionized water and scanned using a Beckman DU Series 500 spectrophotometer in a quartz cuvette from 240 to 400 nm to determine whether phthalate absorbs UV-A.

Catalase treatment. A catalase solution was prepared by dissolving bovine liver catalase (Sigma C-30) in 0.05 M phosphate buffer (pH 7.0). Stock concentrations of catalase at 80 units/ ml and 1600 units/ ml were used in the H_2O_2 spectroscopy microassay and the Triton X-100 assay respectively. Catalase solutions were mixed with phthalate solutions to final phthalate

concentrations of 0 µg/L, 0.4 µg/L, and 50,000 µg/L in both assays. Each sample was then subjected to 6-hour incubation treatments termed: UV-A, light, and dark. With three phthalate concentrations each incubated at three different treatments, a total of 9 conditions were set up. "UV-A" treated samples were exposed to a Sylvania 20 W Blacklight-blue lamp (with a peak wavelength at 366 nm) at 11 cm away, "light" tubes were exposed to white fluorescent light at one meter away behind a UV-filter face shield (ANSI Z87 catalog # 6355-0001) and "dark" tubes were wrapped in aluminum foil.

H_2O_2 spectroscopy microassay to measure catalase activity. Hydrogen peroxide degradation was monitored as per the method of Li and Schellhorn (7). Briefly, catalase was added to a 96-well flat-bottom UV-transparent microplate (Greiner, catalogue # 655801). Hydrogen peroxide (35% (w/w) in H_2O, Sigma-Aldrich, catalogue # 349887) was serially diluted in 0.05 M phosphate buffer (pH 7.0) to final concentrations ranging between 62.5 mM to 0 mM was added to the UV-transparent microplate containing catalase. The plate was immediately placed in a spectrophotometer (Epoch^{TM} Microplate Spectrophotometer) and read every 6 seconds for 3 minutes at 22°C to monitor absorbance at a wavelength of 240 nm, which corresponds to the decomposition of hydrogen peroxide. A total of 3 trials were performed for each condition.

Triton X-100 foam production assay to measure catalase activity. The method of Iwase *et* al. was used to quantify the rate of oxygen formation as a measure of foam formation by the evolved gas bubbles (8). Briefly, 200 µl of the catalase solution and 200 µl of 1% Triton X-100 (Sigma-Aldrich) was transferred into Pyrex tubes. 35% hydrogen peroxide was serially diluted in 0.05 M phosphate buffer (pH 7.0) to final concentrations of 10.29 M, 8.82 M, 7.35 M, 5.88 M, 4.41 M, 2.94 M,1.47 M, 0.12 M and 0 M. 200 µl of hydrogen peroxide was added to the catalase tube and rapidly mixed. Foam formation was filmed for 2 minutes for each test condition.

Data Analysis. The videos of the foam formation in the Triton X-100 assay for catalase activity was analyzed using Dartfish 7 (version 7; Dartfish, [http://www.dartfish.com]) to determine the initial rate of oxygen formation. Foam height was divided by the total height of the tube to obtain a ratio that could be compared between different substrate concentrations and the rate of oxygen formation could be calculated by the change of foam height over time. For the 96 well microassay, the change of absorbance of hydrogen peroxide over time was used to obtain the rate of degradation of hydrogen peroxide. Both the rate of oxygen formation and the rate of degradation of hydrogen peroxide were used to plot Michaelis-Menten curves. Data were linearized using the Lineweaver-Burk and Eadie-Hofstee projections allowing calculations of V_{max} and K_m (8).

RESULTS

Phthalate does not absorb UV-A. The absorption spectra of phthalate was measured to determine if phthalate has the intrinsic capability to confer protection for catalase against UV-A. Results showed that phthalate did not absorb UV-A wavelengths ranging from 315 to 400 nm at concentrations of 0.4 µg/L and 50,000 µg/L. Thus, it does not act as a UV-A filter under our experimental conditions. It did however absorb at 306nm under supersaturated concentrations.

H_2O_2 spectrophotometric microassay (Spec assay) shows a decrease in K_m, V_{max} and V_{max}/K_m ratio due to UV-A exposure. The Spec assay tracked the changes in absorbance at 240 nm (A_{240}) to measure the decomposition of hydrogen peroxide and allowed for the determination of the initial reaction rate (first 6 seconds) in each sample. Eadie-Hofstee (EH) and Lineweaver-Burk (LWB) transformations of the data were performed to compare the kinetic parameter values obtained and data shows that EH values were notably more reliable as our LWB transformations often produced negative, invalid and skewed results.

In the absence of phthalate, this assay demonstrated that UV-A-irradiated samples had more than 6-fold decrease in V_{max}, more than 2-fold decrease in K_m and approximately 2-fold decrease in V_{max}/K_m of catalase when compared with the light and dark samples (Table 1). This shows that V_{max} decreased at a greater proportion than K_m under UV-A exposure. Light and dark samples however were relatively similar to each other in terms of all their kinetic parameters.

Spec assay shows two distinct effects at two different phthalate concentrations. At 0.4 µg/L, phthalate increased both the V_{max} and K_m values of catalase by more than 2-fold for the UV-A condition. At this phthalate concentration, there was no effect on light and dark conditions. At 50,000 µg/L, phthalate increased both V_{max} and K_m values of catalase for the UV-A sample but at a lesser extent (~1.5-fold). However, phthalate at this concentration appears to decrease V_{max} and K_m values of catalase by half for the light and dark conditions. In summary, phthalate at 0.4 µg/L appears to promote catalase activity for UV-A samples only while phthalate at 50,000 µg/L decreases catalase activity for light and dark samples.

Triton X-100 foam production assay (Triton assay) shows a parabola-like trend on a Michaelis-Menten plot. The Triton assay measured the production of Triton X-100 foam as a representation of the volume of oxygen produced by the catalase reaction and allowed us to obtain an initial reaction rate (first 3 seconds). UV-treated catalase conditions showed a marked reduction in enzyme activity compared to light and dark samples (Fig 1). The light and dark plots share a similar trend up until H_2O_2 concentrations of around 3 M. Above 3M H_2O_2, the light samples display lower reaction velocities than the dark samples. All samples eventually decrease in velocity as H_2O_2 concentrations exceed 7 M, exhibiting a parabola-like trend. The V_{max} is achieved at H_2O_2 concentrations between 4 M and 9 M. The pH of the reaction ranges between 6.4 and 7.0. At the peak rate, the pH was approximately 6.7.

Triton assay shows a decrease in K_m,V_{max} and V_{max}/K_m ratio (Table 2). In the absence of phthalate, this assay demonstrated that UV-A-irradiated samples had more than 10-fold decrease in V_{max}, more than 4-fold decrease in K_m and more than 4-fold decrease in V_{max}/K_m of catalase when compared with the light and dark samples. This shows that V_{max} decreased at a greater proportion than K_m under UV-A exposure, similar to the trend seen in the Spec assay. Light and dark samples were relatively similar to each other in terms of all their kinetic parameters, similar to the Spec assay.

TABLE 1 Effect of combinations of light and phthalate on the K_m and V_{max} of bovine catalase in the H_2O_2 spectrophotometric microassay

		K_m (mM)		V_{max} (ΔmAU_{240} s^{-1})		V_{max}/K_m (ΔmAU_{240} s^{-1} mM^{-1})	
Experimental Condition	[Phthalate] (µg/L)	EH	LWB	EH	LWB	EH	LWB
UV	0	137 ± 13	105 ± 6	37 ± 3	29 ± 2	0.27 ± 0.04	0.28 ± 0.34
UV	0.4	417 ± 62	296 ± 7	85 ± 2	61 ± 2	0.20 ± 0.03	0.21 ± 0.36
UV	50,000	250 ± 60	242 ± 95	52 ± 13	51 ± 19	0.21 ± 0.10	0.21 ± 0.03
Light	0	652 ± 96	503	375 ± 31	65	0.57 ± 0.13	0.57
Light	0.4	606 ± 140	-	360 ± 22	-	0.60 ± 0.29	-
Light	50,000	298 ± 84	109 ± 4	180 ± 28	74 ± 2	0.68 ± 0.30	0.68 ± 0.04
Dark	0	339 ± 66	300	214 ± 42	191	0.63 ± 0.25	0.63
Dark	0.4	363 ± 60	-	225 ± 21	-	0.62 ± 0.16	-
Dark	50,000	116 ± 42	116 ± 10	87 ± 15	87 ± 6	0.75 ± 0.40	0.75 ± 0.12

EH and LWB represent parameters derived from the Eadie-Hofstee and Lineweaver-Burk projections, respectively. Numerical suffixes after each experimental condition denote different levels of phthalate co-incubated with the catalase, expressed in µg/L. In cases where there is no error value, only one trial was accepted in the calculation of that parameter.

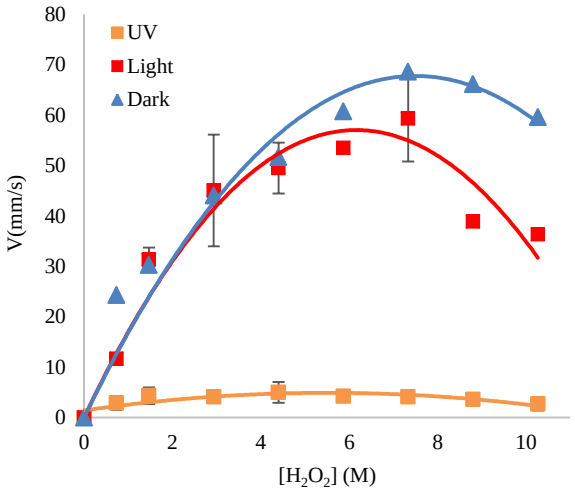

FIG 1 The effect of exposing bovine catalase to different light conditions on the enzyme kinetics in the Triton X-100 foam production assay

Triton assay shows addition of phthalate has no notable effect on kinetic parameters (Table 2). In the presence of phthalate, the K_m of UV-A-irradiated catalase appears to increase minimally at 0.4 µg/L and 50,000 µg/L concentrations of phthalate. However, V_{max} did not appear to have any change. In the dark sample, the K_m appears to decrease 2-fold at 50,000 µg/L but other parameters could not be analyzed confidently as they had overlapping errors. In addition, most of the light samples' data generated negative kinetic values during data transformation and had to be omitted, making comparisons between all samples impossible.

DISCUSSION

This study looked at two possible mechanisms in which phthalate could have conferred protection to bovine catalase from UV-A irradiation. The first hypothesis speculated that phthalate absorbed at the UV-A range of 315 nm to 400 nm and thus, reduced the intensity of UV-A which was exposed to catalase. The second hypothesis was that phthalate interacted directly with catalase as an enzyme modifier. The effects of this interaction would be reflected in changes in V_{max} and K_m values which represent the catalytic and substrate-binding ability of an enzyme, respectively.

Our experimental phthalate concentrations showed no absorbance at UV-A wavelengths. Supersaturated levels of phthalate did however absorb at the shorter UV-B wavelength of 306 nm. Thus, even at equimolar concentrations to catalase, phthalate could not act as a filter to absorb UV-A light and protect catalase. Although previous studies have suggested that phthalate is similar in structure to other phenolic compounds and contain delocalized pi electrons as part of a conjugated system, the extent of conjugation is closely related to the energy of absorption (1, 10). In highly conjugated systems, the energy difference between molecular orbitals is diminished and the electrons are excited by longer wavelengths (10). Phthalate, in its pure form, contains two esters that branch off from adjacent carbons in an aromatic ring. Thus, we have reason to believe that phthalate has too few degrees of conjugation to display significant absorption at UV-A wavelengths.

Since phthalate does not act as a UV-A filter, the other possibility was that phthalate was interacting directly with catalase as an enzyme modifier. In order to understand the mechanism of protection, it was first necessary to understand the nature of UV-A damage to catalase by looking at the enzyme kinetic values, V_{max} and K_m, which reflect the catalytic and substrate-binding ability of an enzyme respectively. The H_2O_2 spectrophotometric microassay (Spec assay) and Triton X-100 foam production assay (Triton assay) measure

TABLE 2 Effect of combinations of light and phthalate on the K_m and V_{max} of bovine catalase in the Triton X-100 foam production assay

		K_m (M)		V_{max} (mm s^{-1})		V_{max}/K_m (mm s^{-1} M^{-1})	
Experimental Condition	[Phthalate] (µg/L)	EH	LWB	EH	LWB	EH	LWB
UV	0	0.8 ± 0.3	1.6 ± 0.5	3 ± 1	5 ± 2	2 ± 1	4 ± 2
UV	0.4	1.6 ± 0.2	1.4 ± 1.1	3 ± 1	4 ± 1	1 ± 1	3 ± 1
UV	50,000	2.3 ± 0.8	2.4 ± 1.8	4 ± 1	6 ± 1	3 ± 1	4 ± 2
Light	0	4.5 ± 1.7	1.9 ± 0.4	45 ± 4	43 ± 4	14 ± 8	22 ± 2
Light	0.4	4.8 ± 2.3	5.8 ± 0.9	59 ± 13	85 ± 52	14 ± 1	13 ± 7
Light	50,000	2.3 ± 0.2	-	41 ± 7	-	21 ± 3	-
Dark	0	4.8 ± 0.7	2.8 ± 0.2	48 ± 13	36 ± 12	9 ± 2	11 ± 1
Dark	0.4	-	-	-	-	-	-
Dark	50,000	-	-	-	-	-	-

EH and LWB represent parameters derived from the Eadie-Hofstee and Lineweaver-Burk projections, respectively. Numerical suffixes after each experimental condition denote different levels of phthalate co-incubated with the catalase, expressed in µg/L. In cases where there is no error, only one trial was accepted in the calculation of that parameter.

substrate (H_2O_2) degradation and product (O_2) formation respectively as measures of enzyme rate.

In the absence of phthalate, both reaction assays showed that the catalytic ability of catalase was inhibited and its enzyme-substrate affinity was increased when irradiated with UV-A; represented by a marked decrease in V_{max}, K_m and the ratio of V_{max}/K_m of catalase. The decrease in all three parameters is an unexpected and generally less described phenomenon as most interpretations of enzyme inhibition depend on one of the parameters being a relative constant in the presence of an inhibitor. In this case, however, we observed that with UV-A exposure, the V_{max} decreases at a greater proportion than the K_m. This suggests that the more likely explanation for the catalase inhibition is damage to the catalytic ability of catalase instead of increased enzyme-substrate affinity. Another way to interpret this is that UV-A cannot be classified as a single-effect enzyme inhibitor as it affects many faculties of the enzyme. This is not a surprising outcome as UV-A is an energy form that causes a wide variety of damage and not a physical compound such as a chemical or protein that directly binds to catalase at specific sites to cause inhibition. Furthermore, we considered the possibility that UV-A may have interacted with other components in the purchased bovine catalase powder which was not pure. These components could have subsequently led to the changes in kinetic parameters observed.

The addition of phthalate to catalase incubated with UV-A had two distinct effects at two different concentrations.

At 0.4 µg/L, phthalate increased both the V_{max} and K_m values of catalase for only the UV-A condition, suggesting that phthalate protects catalase from UV-A irradiation by protecting its catalytic and substrate-binding abilities. There was no effect on light and dark conditions, showing that the protective effect was dependent on UV-A irradiation. At 50,000 µg/L, phthalate also increased both V_{max} and K_m values of catalase for the UV-A sample but at a lesser extent. Interestingly, phthalate at this concentration appears to decrease V_{max} and K_m values of catalase for the light and dark conditions. This decrease in V_{max} and K_m, while maintaining a constant V_{max}/K_m ratio, is a characteristic of uncompetitive enzyme inhibitors (11). In summary, phthalate appears to protect catalase from UV-A exposure but becomes an uncompetitive inhibitor at higher concentrations.

For the Triton assay, concentrations of hydrogen peroxide above 7 M H_2O_2 appear to inhibit catalase (Fig 1). Substrate inhibition was not observed in the Spec assay because the assay used much lower H2O2 concentrations than the Triton X-100 assay. One explanation for the inhibition is that excessive hydrogen peroxide can damage the enzyme via oxidation of certain peptide residues and bonds, subsequently altering its binding and catalytic activity (12). Another possibility is the change in pH associated with the addition of high concentrations of hydrogen peroxide. The addition of H2O2 into phosphate buffer caused the pH of the reaction to decrease from 7.0 to 6.4 at the highest H2O2 concentration. A lowered pH has an effect on enzyme surface charge and fold structure, possibly reducing its activity (12). The latter possibility however was not probable as the pH change to 6.4 was too minor to significantly affect enzyme activity (12).

In conclusion, our results support the second hypothesis that phthalate directly interacts with catalase as an enzyme modifier to protect it from UV-A. The character of phthalate however, depends on its concentration and condition as it is an uncompetitive enzyme activator at low concentrations under UV-A

conditions and an uncompetitive enzyme inhibitor at high concentrations.

FUTURE DIRECTIONS

The true nature of phthalate's binding to catalase is unknown. As it is not a protein, it would not be easy to tag both molecules for an affinity test. However, phthalic acid is an acid well known to form esters with other molecules and can be labeled to perform an affinity test. If phthalic acid is shown to bind sufficiently to catalase, the next step logical step would be to use an analysis technique such as Electron Paramagnetic Resonance to crystallize and examine the structure catalase in complex with phthalic acid. This technique has been shown to work on analyzing formic acid binding within the active sites of *Helicobacter pylori* catalase. However, if phthalic acid is only binding transiently on the exterior surface of catalase, the results of crystallization may not reflect the true nature of phthalic binding (14).

Another observation during the experiment was that phthalate did not absorb in UV-A wavelengths but instead, it had a strong peak at 306nm which is in the UV-B range. Ultraviolet lights tend to emit at a broad UV spectrum which includes wavelengths in the UV-B and UV-C range. Thus, one possibility is that the protection conferred by phthalate was due to absorption at 306nm wavelengths and not at our lamp's 366nm peak wavelength. In order to distinguish between the two, future experiments should use light filters or a coherent light laser to isolate purely UV-A and UV-B wavelengths to incubate the catalase samples and to analyze the same kinetic parameters.

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