Metabolism of Oxybenzone in Plants

Feiran Chen, Peter Schroder*

Helmholtz Zentrum München, GmbH, German Research Center for Environmental Health, Research Unit Microbiome Analysis, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany, feiran.chen@helmholtz-muenchen.de,

corresponding author. peter.schroeder@helmholtz-muenchen.de

Abstract

Oxybenzone (OBZ) is a common UV filter in personal care products, which enters aquatic systems mostly directly via recreational activities or indirectly through wastewater treatment plants discharges. It is an emerging contaminant due to its adverse impacts on aquatic ecosystems. To study the degradation capacity of OBZ in phytotreatment, the hairy root culture (*Armoracia rusticana*) and the wetland species *Cyperus alternifolius* were treated with OBZ. Depletion of spiked OBZ from the aqueous medium exceeded 70% after 120 h, and continuous uptake and accumulation in plant tissues was observed. Similar to its fate in mammalian cells OBZ could be activated into the phase I hydroxylated metabolite 2,4-dihydroxybenzophenone. Two subsequently appearing metabolites were identified as oxybenzone-glucoside and oxybenzone-(6-O-malonyl)-glucoside by LC-MS/MS. Formation of these metabolites increased over the experimental period. The identity of the metabolites was further confirmed by enzymatic synthesis, enzymatic and alkaline hydrolysis. To our knowledge this is the first time that OBZ metabolites are shown to occur in higher plant tissues, and that plant defense systems were elevated to counteract stress caused by exposure to OBZ. This study presents the huge potential of plants to eliminate OBZ, and the significance of phytoremediation to cope with environmentally critical UV-filters is discussed.

Keywords: sunscreen, phytoremediation, OBZ, glucoside, detoxification

1. Introduction

Personal care products including stimulants, fragrances, sunscreens, antimicrobials, and insect repellents are emerging contaminants, and have attracted much attention in recent years due to their presence in surface water and potential effects on ecosystems. Oxybenzone , also commonly known as Benzophenone-3, is an active ingredient in body care products protecting human skin from excess UV radiation. It has been added to 70% of the non-mineral sunscreens according to the database issued by Environmental Working Group (EWG, 2015). Centers for Disease Control and Prevention detected OBZ in the urine of 96.8% of U.S. residents (Calafat *et al*., 2008) and lower birth weight in baby girls was reported for mothers frequently exposed to OBZ (Wolff *et al*., 2008). This compound also appears to be an endocrine disrupter and has recently been considered as an emerging contaminant due to its ubiquity in lakes, municipal wastewater effluent and coastal areas (Balmer *et al*., 2005; Downs *et al*., 2016; Fent *et al*., 2010), posing adverse effects on aquatic organisms (Blüthgen *et al*., 2012; Downs *et al*., 2016). Since conventional sewage treatment processes are obviously not adequate for the removal of OBZ, it is necessary to apply alternative approaches to eliminate OBZ and mitigate its threat to aquatic organisms. Previous studies have investigated some removal processes of OBZ, for example, the oxidation of OBZ in water by ferrate (VI) with coexisting constituents or by laccase mediator system (Garcia *et al*., 2011; Yang and Ying, 2013), ozonation or ultrasound application (Gago-Ferrero *et al*., 2013; Zúñiga-Benítez *et al*., 2016). Although these advanced techniques report high effluent quality, the cost of installation and operation should be considered carefully before their implementation (Fent *et al*., 2006; Schröder *et al*., 2007). Plants and their associated microorganisms can be used for phytoremediation in constructed wetlands (CWs) and hydroponic setups (Schröder *et al*., 2007), and evidence for the role of plants for uptake and metabolism of target compounds is available. So far, studies on the transformation of OBZ are mainly limited in mammals, hence, in this study we investigate the metabolism of OBZ in plant tissues by using hairy roots cells of *Armoracia rusticana* (horseradish) as a model and *Cyperus alternifolius* as a potential candidate species. Structures of key metabolites are proposed and the formation of metabolites are presented as a function of time, and a recommendation on the phytoremediation potential is given.

2. Materials and methods

2.1 Hairy root exposure to OBZ

Horseradish (*A. rusticana* L.) hairy root culture (Nepovím *et al*., 2004) was grown in 100 mL full-strength Murashige&Skoog medium for 10 d. OBZ (Fluka, Germany) was added to the growth medium to reach a concentration of 100 µM, after 3 h, roots were washed twice with sterilized deionized water and transferred to fresh growth medium without OBZ. Samples were harvested at 2, 4, 6 and 24 h after transferring the cells, dried with lint tissue paper, frozen in liquid nitrogen and stored at - 20°C.

2.2 Hydroponics study

Cyperus alternifolius of uniform size were placed in glass vessels containing Hoagland solution spiked with 5 µM, 25 µM and 50 µM OBZ, respectively. Three replicate vessels

were established for each exposure period (i.e., 24, 72 and 120 h) at each concentration, control plants (without OBZ) and control assays (without plants) were set up under the same conditions. The nutrient solutions were collected and analyzed to determine the OBZ concentration for each exposure time. In addition, root, stem and leaf samples were collected and frozen at -80 °C for further measurements.

2.3 Sample preparation and LC-MS analysis

Plant material were ground and extracted with 1.5 mL H_2O/Acetonitrile (30/70, v/v). After 5 min ultrasonication and 30 min centrifugation at 13,000 ×g. Supernatants were purified with 3 cm^3 60 mg Oasis HLB solid phase extraction (SPE) columns. Growth medium was filtered with PVDF syringe filters prior to analysis. LC-MS analysis was performed with a HPLC system (Varian ProStar 210) coupled to an ion trap mass spectrometer (Varian 500-MS). A Phenomenex HYDRO-RP column (C18, polar endcapped; particle size 4 µm; 50 mm × 2.0 mm) was applied for separation of analytes using H_2O with 0.1% formic acid as mobile phase A, acetonitrile with 0.1% formic acid as mobile phase B with following gradient: 0–2 min 97% Buffer A; 2–10 min 95% Buffer B; 10–12 min 95% Buffer B; 12–12.5 min 97% Buffer A; 12.5–17 min 97% A. The flow rate was 0.3 mL/min. Concentration of OBZ was determined by an external standard calibration curve. The HPLC eluent was introduced to the mass spectrometer using a pneumatically assisted electrospray source (positive mode). The interface was adjusted to the following conditions: capillary voltage, 63 V; needle voltage, 4500 V; drying gas temperature, 300 °C. MS/MS spectra were obtained by collision-induced dissociation using nitrogen as the collision gas. LC-TOF-MS experiments were conducted on an Ultimate 3000 LC system (ThermoFisher) coupled to an ultra-high resolution Maxis 4g plus TOF mass spectrometer (Bruker) equipped with an electrospray source. The LC conditions were identical as above. The TOF-MS was operated in the positive polarity mode with active focus under the following conditions: Capillary voltage, 5500 V; nitrogen dry gas temperature, 225 °C; dry gas flow, 10 L/min; nebulizer pressure, 2 bar.

2.4 Extraction of O-glucosyltransferase

3 g of hairy roots were homogenized and extracted with 100 mM sodium phosphate-buffer pH 6.5 containing 10 mM DTE, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 1% PVP K90 at 4°C for 30 min. After centrifugation at 15,000 ×g for 30 min at 4°C, proteins in the supernatant were precipitated progressively by addition of ammonium sulphate to 40% and 75% saturation and centrifuged at 18,500 ×g for 30 min at 4°C respectively. Consequently, the pellets were resuspended in 2.5 mL 200 mM Tris/HCl buffer with 2 mM $MgCl_2$ and 1 mM DTE, pH 7.3. Proteins were desalted by chromatography through PD 10 columns (GE Healthcare, UK) and stored at – 80°C before use.

2.5 Enzymatic synthesis and alkaline hydrolysis

Enzymatic formation of the glucosides was performed by incubating 100 µM OBZ with 2 mM uridine diphosphate glucose, 1.25 mM 4-NPG, 1.25 mM salicin in 200 mM Tris/HCl buffer, pH 7.5. The reaction was started by adding 100 µL glucosyltransferase containing enzyme extract to yield a final volume of 200 µL, incubated for 1 h at 30°C (Messner *et al*., 2003). Experiments without enzyme served as control. The reaction was stopped by precipitating the protein with 10 µL concentrated phosphoric acid and centrifuged at 13,000 ×g for 2 min. Enzymatic hydrolysis was started by incubating metabolite extracts (prepared after SPE, freeze-dried and re-dissolved in 400 µL of 50 mM sodium phosphate buffer, pH 5.0) with 300 U of commercial β-D-glucosidase at 36 °C for 2 h. Control experiments were free of glucosidase. Reaction was stopped by applying samples to the SPE columns (Huber *et al*., 2012). Alkaline hydrolysis was performed by incubating plant extracts after SPE with 0.1 N NaOH for 6 h at room temperature, and samples without NaOH were controls (Petroutsos *et al*., 2007). All the samples were analyzed by LC-MS.

2.6 Protein extraction and antioxidative enzymes assay

The extraction of soluble protein was carried out according to the method described by Schröder *et al*. (Schröder *et al*., 2005). All enzyme assays were conducted in a 96-well spectrophotometer (Spectra max Plus 384). Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined as the change from epinephrine to adrenochrome at 480 nm for 5 min. One unit of SOD activity is defined as the amount of protein required for 50% inhibition of adrenochrome formation (Polle *et al*., 1989). Catalase (CAT, EC 1.11.1.6) activity was measured by the decrease in absorption at 240 nm (ε 0.036 $mM^{-1}cm^{-1}$) due to the consumption of H_2O_2 (Verma and Dubey, 2003). Peroxidase (POX, EC 1.11.1) activity was assayed at 420 nm (ε 26.6 $mM^{-1}cm^{-1}$) using Guajacol (25.05 mM per reaction) as a substrate (Diekmann *et al*., 2004). Ascorbate peroxidase (APOX, EC 1.11.1.11) activity was measured according to the method described previously (Lyubenova *et al*., 2015) with modifications. Each reaction contained 1mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and enzyme extract in 50 mM KH_2PO_4/K_2HPO_4 buffer, and the activity was determined at 290 nm (ε 2.8 $mM^{-1}cm^{-1}$).

3. Results and discussion

3.1 Removal of OBZ from hydroponic medium

Hydroponic experiments were performed to evaluate the ability of *C. alternifolius* to remove OBZ. Generally, removal of xenobiotics in hydroponic systems has been be attributed to abiotic and biotic processes (Yan *et al*., 2016; Zhang *et al*., 2016). For OBZ, photodegradation and evaporization were not expected to occur due to the photostability of OBZ towards UV irradiation and low volatility of this compound (Liu *et al*., 2011). In the experiments without plants, OBZ concentrations remained constant over the incubation time indicating that the adsorption on vessels was negligible. Therefore, biotic processes accounted for the main removal process. The OBZ concentration in the medium dropped dramatically after 120 h, and more than 73.9±9.1% of OBZ were removed from media for all three different initial concentrations (Figure 1).

3.2 Identification of metabolites

Horseradish hairy root culture provides us a simple and fast approach to investigate the potential of plants to metabolize OBZ. The first insight of OBZ pathway obtained after hairy roots exposed to OBZ for 3 h (Figure 2). MS measurement detected a polar metabolite with its

pseudo-molecular ion at m/z 413.1218 $[M+Na]^+$. It was identified as oxybenzone-glucoside (OBZ-Glu) which corresponded to the molecular formula $C_{20}H_{22}O_8Na$ (413.1207). Fragmentation of OBZ-Glu in MS/MS generated two major fragments at m/z 185 and m/z 251 $[M+Na]^+$, corresponding to the dehydroglucose and OBZ. Incubation of samples containing the products of m/z 413 with β-D-glucosidase resulted in the disappearance of OBZ-Glu signal, while samples without glucosidase still showed the peak of OBZ-Glu. Incubation of OBZ with glucosyltransferase extracted from hairy roots synthesized OBZ-Glu in vitro. Another metabolite was detected at m/z 499.1223 $[M+Na]^+$, with a formula confirmed as $C_{23}H_{24}O_{11}Na$ (499.1221). It was identified as oxybenzone-(6-O-malonyl)-glucoside (OBZ-Mal-Glu), which was 86 units larger than that of OBZ-Glu, suggesting an additional malonyl group. Fragmentation of m/z 499 in MS/MS resulted in a major fragment of m/z 455, this loss of 44 Da is attributed to the decarboxylation of malonic acid during fragmentation of a malonylglucopyranoside (Kazuma *et al*., 2003; Petroutsos *et al*., 2008). MS/MS also generated fragements of m/z 413 $[M\text{-malonyl}+Na]^+$ and m/z 251 $[M\text{-malonyl-dehydroglucose}+Na]^+$. Incubation with NaOH has hydrolyzed OBZ-Mal-Glu completely and OBZ-Glu was released from this reaction. Therefore, OBZ-Mal-Glu was formed in a secondary conjugation step with malonic acid conjufgated on the OBZ-Glu. After transferring the culture to OBZ free media, the transformation of OBZ to the above mentioned metabolites proceeded actively, and the amount of metabolites increased over time with OBZ-Mal-Glu tend to be the dominant metabolite after 24 h.

3.3 Transformation of OBZ in *C. alternifolius*

In OBZ treated *C. alternifolius*, in addition to OBZ-Glu and OBZ-Mal-Glu, a hydroxylated OBZ was detected with a mass transition of $[M+H]^+$ m/z 215-137. This metabolite was confirmed as phase I product 2,4-dihydroxybenzophenone (DHB) by comparing the retention time and fragmentation to the authentic DHB. DHB is formed via dealkylation of the methoxy side chain at the para-position of OBZ, generally mediated by cytochrome P450 monooxygenase enzymes (Okereke *et al*., 1994). This phase I activation is in consistent with that found for OBZ destruction in mammalian liver (Wang and Kannan, 2013). OBZ-Glu and OBZ-Mal-Glu formed without phase I activations, probably because the already existing hydroxyl group suitable for phase II transformation. Phase II metabolites are characterized as stable, more water soluble and less toxic than parent compound (Coleman *et al*., 1997; Zamek-Gliszczynski *et al*., 2006). Malonylation of glucoconjugate acts as a signal for transport into the vacuole, promoting efficient sequestration of these products (Sandermann, 1992; Taguchi *et al*., 2010). All three metabolites occurred both in roots and shoots, DHB concentration in leaf was significantly higher (11.2-39.3 folds) than that of in roots over the exposure time. The amount of OBZ-Glu and OBZ-Mal-Glu was higher in roots than in shoots. Still, the amount increased with exposure time in all treatments. Due to the lack of reference materials of OBZ-Glu and OBZ-Mal, their concentrations are given in peak area as arbitrary units (Figure 3).

3.4 Plant stress response after OBZ exposure

To investigate the phytotoxicity of OBZ on *C.alternofolius*, activities of antioxidative enzymes including SOD, CAT, APOX and POX were measured because their role as first line of defence against the oxidative stress caused by exposure to xenobiotics (Mittler, 2002). Generally, significant induction of enzyme activities were observed after exposure to OBZ for 72 h (Table 1). Highly induced activities were detected for CAT (25 μM OBZ, 72 h) and POX (50 μM, 120h) in roots. The enhancement was generally more distingguished in roots than in leaves, and higher induction occurred at higher OBZ concentrations. 5μM OBZ, which is comparable to the maximum amount of OBZ detected in environment, generally resulted in less stress compared to the other higher concentrations. The induced enzyme activities indicate their role in defense and adaptation against stress resulting from OBZ exposure.

4. Conclusion & Outlook

This study proved that the presence of plants improve the removal of OBZ from water and provided insights in the pathway of OBZ in plants. Horseradish hairy roots experiment presented the main metabolites of OBZ, and hydroponic study showed the great potential of *C. alternifolius* in removal of OBZ. Being aware of the contamination by different types of UV filters, an exposure of hairy roots with a mixture of pollutants-OBZ and the typical non-organic sunscreen titanium dioxide is under investigation. This ongoing study will reveal the influence of mixture pollutants on the removal and transformation of OBZ and at same time will provide implications for elimination of titanium dioxide nanoparticles by plants.

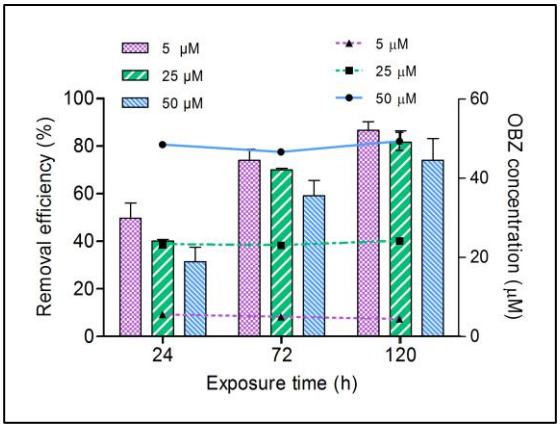

Figure 1 Removal effeciency of OBZ (5, 25 and 50 μM) from hydroponic culture medium by *C.alternifolius* (left Y axis). OBZ concentration (μM) in medium without plants over incubation time, no statistical difference among exposure time for each concentration according to ANOVA at $p < 0.05$ (right Y axis). Error bars indicate standard deviation (n=3).

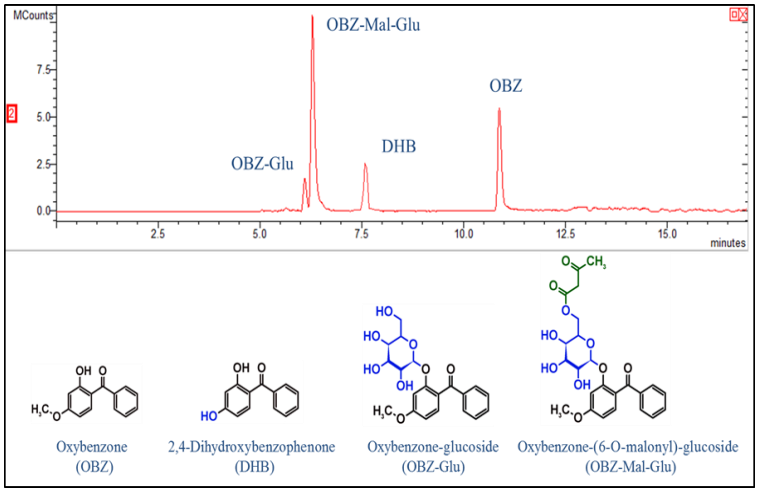

Figure 2 LC-MS/MS Chromatogram of samples containing OBZ, OBZ-glu, OBZ-Mal-Glu and DHB, obtained at product ion m/z 151 or m/z 137(*SIM* mode). The scheme below proposes structures of the metabolites detected in planta.

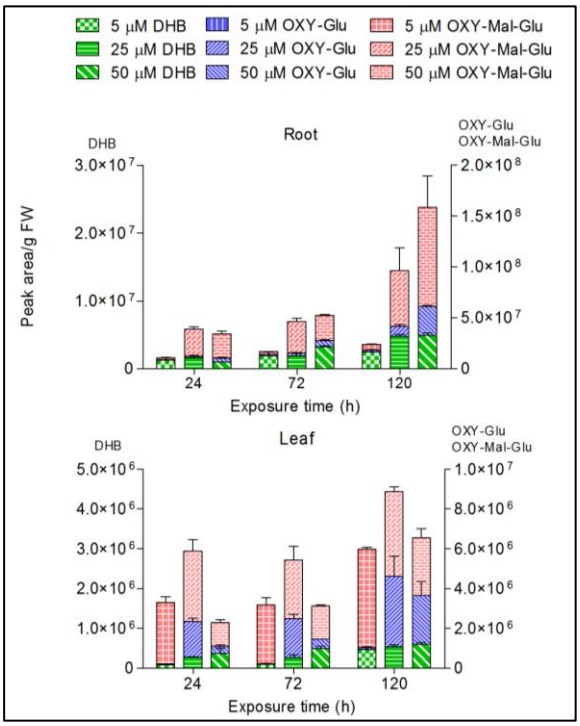

Figure 3 Formation of OBZ metabolites: DHB, OBZ-Glu & OBZ-Mal-Glu in roots, leaves of *C.alternifolius* after 24, 72 and 120 h incubation. Values are mean of three parallel individuals, error bars indicate SD.

Table 1 Activities of antioxidative enzymes in root (RT) and leaf (LF) tissues of *C. alternifolius* exposed to 5, 25 and 50 µM of OBZ for 24, 72 and 120 h. Enzyme activities are depicted relative to controls without OBZ treatment. Data are means of three replicates

	SOD activities (% of control)						**CAT** activities (% of control)					
	5 µM		25 µM		50 µM		5 µM		25 µM		50 µM	
	RT	LF	RT	LF	RT	LF	RT	LF	RT	LF	RT	LF
24	94.78	109.86	71.42	92.54	109.29	83.06	138.41	85.40	210.90	90.71	203.04	92.89
72	159.08	113.07	148.89	171.72	161.02	197.46	134.49	97.46	239.20	80.26	185.82	78.29
120	116.98	117.87	175.55	164.52	174.05	181.73	139.76	134.98	165.49	131.60	175.41	134.11
	APOX activities (% of control)						**POX** activities (% of control)					
	5 µM		25 µM		50 µM		5 µM		25 µM		50 µM	
	RT	LF	RT	LF	RT	LF	RT	LF	RT	LF	RT	LF
24	115.71	112.74	99.43	85.75	128.62	79.01	81.02	109.15	62.99	111.60	63.54	103.24
72	150.31	118.96	161.14	124.76	190.20	117.13	137.89	136.15	125.09	166.30	151.29	165.27
120	173.24	137.89	200.50	182.38	216.96	187.38	172.79	124.73	174.08	105.07	238.30	178.60

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