Accumulation Time and Antioxidant Responses of Inorganic Arsenic on Brown Seaweed *Padina minor*

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ABSTRACT

Padina minor had a good ability to bind and accumulate arsenic (As). This research aimed to observe the As accumulation and detoxification time, also the antioxidant defense activities. Each gram of *P. minor* was culture in various As contaminate media for 7 days, then reculture in As-free media for 14 days. There was an acute effect on thali by decreasing its growth slowly till final day with small amount accumulation at concentration 25 µg iAs g^{-1}. Meanwhile, direct attenuation impact was due at 50 and 100 µg iAs g^{-1} with massive accumulation. The thali starts to recover after reculture in health environment for 14 days. Only As^{5+} was detected on the thali at day 7 and 21 were indicates internal oxidation of As^{3+} was done before 7 days. Various antioxidant activities such as decreasing polysaccharide and increasing activities of DPPH and flavonoid at high level were observed. Those indicated that 50 µg iAs g^{-1} was optimum iAs level on *P. minor*. The results indicate that *P. minor* has ability to oxidase and methylate with antioxidant role as defensive activities against iAs, but it also needs more than 14 days to recover in high level contamination.

Keywords: Arsenic, *Padina minor*, accumulation, methylation, antioxidant activity.

INTRODUCTION

Metal pollution is an endless problem for every biological aspect especially on aquatic ecosystem since recent decades. It could be due to natural production and waste water of anthropogenic activities (He et al., 2005). But increasing human population and industrial activities has major impact on last few years (Rocchetta et al., 2007). Thus, metal contamination on aquatic ecosystem and its effects on aquatic organisms are alluring topic to be observed and resolved.

Arsenic (As) is one of common toxic metal pollutant which producted by nature and anthropogenic waste water as inorganic arsenic (iAs) and organoarsenic (Nath et al., 2008). iAs has two different form, arsenite (As^{3+}) and arsenate (As^{5+}), while organic form commonly found as dimethylarsenic (DMA) and arseno-sugars (Francesconi and Kuehnelt, 2004). Most of the iAs is naturally found from rock and sedimentation process and also from anthropogenic activities such as mining, smelting and the processing of mineral ores. However, levels of As are higher in aquatic ecosystem than in most terrestrial area as it is relatively water-soluble (Rose, 2007).

The abundance As in aquatic environment could be translocate to living organism such as fish, seaweed and other aquatic plants before consumed by human. It could be serious problem for human health due to the effects of As breaking DNA-strain, inducing ROS, over-proliferation cell and eventually cancer (Vahter and Concha, 2001; Hughes et al., 2011). In this case, inorganic form has been known to be the most toxic of As form (IARC, 2004).

Small amount of iAs contents on fish was reported by Ysart et al. (2000) which mentioned only 1-3 % of iAs detected from 4.4 mg kg^{-1} total arsenic (tAs) in average on 94%

UK dietary fish. It is relatively lower than iAs content on some edible seaweed. Massive amount of iAs had been reports on edible seaweed including 71% in hijiki (Phaeophyceae) (Rose, 2007), 49% in *Cystoseira barbata* (Phaeophyceae) (Duncan et al., 2013) and 23% in *Codium vermilara* (Bryopsidophyceae) (Llorente-Mirandes et al., 2010). These contrasting differences on iAs content because fish could methylate iAs to be organic form like arsenobethaine or arseno-sugars in short time, but seaweed may needs more longer time.

Interestingly, *Padina* sp. (Phaeophyceae) has been reports with little iAs accumulation (Slejkovec et al., 2006; Duncan et al., 2013). These phenomenon is indicates that not all seaweed would accumulate As as inorganic form and also clarified that *Padina* sp. had a special ability to detoxified iAs. *Padina minor* Yamada is one of the common brown seaweed in Asia and distributed along the South coastline of Taiwan. Thus, *P. minor* could be the best option to develop as subject since its population in Taiwan and the reports on *Padina* sp.

On the other hand, different As methylation process in seaweed is due to several possibilities such as genetic ability, increasing reactive oxygen species (ROS) and antioxidant activities. The antioxidant activity has a role to chelate and detoxified metal ion (Noori, 2012). Production of antioxidant would bond, oxidized, and methylated the free iAs ion intrusion in the cell as detoxification mechanism. Thus, not only gene expression and ROS, antioxidant activity in seaweed also plays an important role on self-healing process in the case of metal contamination.

Considering the toxic effects of iAs and its massive accumulation on seaweed, the present study aimed to evaluate the accumulation time and the antioxidant responses of *P. minor* since *Padina* sp. had a special ability to detoxified iAs. This study can serve to support the ecological function of *P. minor* as biomonitor of iAs contamination.

MATERIAL AND METHODS

Seaweed preparation.

P. minor thali was collects during the summer season in July 2016 from Kenting, Taiwan. The thali was clean with seawater and categorized based on the condition and size. The selective thali was acclimatized for 7 days in 30‰ seawater on 25° C under 80 µmol photons m^{-2} s^{-1} irradiance for 12:12 light cycle with PES enhancement solution at the Laboratory of Algae Physiological Ecology, National Pingtung University of Science and Technology, Taiwan.

Laboratory experiment.

Three iAs concentrations were set up on 25, 50 and 100 µg As L^{-1}, which compiled from equal concentration of arsenite (As^{3+}) and arsenate (As^{5+}) (High-Purity Standards, USA) (v/v) and kept under 4° C till used. Meanwhile, the experiment was conducts by culturing selective thali (± 1.0 g) for a liter of 30‰ seawater in 25 ± 1° C with 80 ± 5 µmol photons m^{-2} s^{-1} at 12:12 photocycle with tiny aeration and challenged against various concentration of iAs. Thali was removed from the contaminate media and reculture for 14 days more in the arsenic-free media to observe their responses against arsenic toxicity. Seawater media was replaced every 7 days with six replicates for each groups.

Specific growth rates (SGR).

The specific growth rates of thali were determined according to Wong and Chang (2000) using the equation SGR = 100 [ln (W_t / W_0)] / (t), where SGR is the specific growth rate (% day^{-1}), (W_t) and (W_0) were represent as final and initial fresh weight of thali (g), and (t) is the time period of experiment (d).

Arsenic intakes analysis.

Inductively coupled plasma emission mass spectrophotometer (ICP/MS) (Thermo Fisher, USA) was used to determine iAs contents in thali and seawater medium following methods by Choi et al. (2011). The accumulation of iAs in thali were measured by extracted 100 mg of powder in 50% methanol with 1% HNO_3 (HSE Chemicals, UK). While total arsenic (tAs) was measured by atomic absorption spectrophotometer (AAS) (Hitachi ZA3000, Japan) with graphite furnace methods by macerated 100 mg powder in 10 mL HNO_3 at 90^o C over a period of 14 h in dry bath. The fortification level of each sample was 50 ng As L^{-1} as limit of detection (LOD).

Antioxidant activity assay.

Antioxidants were test on poly saccharide, DPPH scavenge, flavonoid and iron chelation activities at day 7 and day 21. Polysaccharide was extract by 0.05 ml of 5% phenol (Panreac, Spain) followed by 0.5 ml 95.5% sulfuric acid (HSE Chemicals, UK) and measured at 490 nm after 25 minutes with glucose as standard solutions. The DPPH radical scavenging activity was determined after 30 minutes incubation with 0.05 ml of 1 mM DPPH solution at 517 nm (Yen and Chen, 1995). Total flavonoids were measured by aluminium chloride colorimetric assays. Each samples were mixed with 0.04 ml of 10% $AlCl_3.6H_2O$ (Panreac, Spain), 0.04 ml 1M CH_3COOK (Panreac, Spain), and 1.12 ml distilled water respectively. The value was determined at 415 nm after 40 minutes incubation with quercetin (Sigma-Aldrich, USA) as standard solution. While, iron chelation activities was determined by methanol extraction followed by 2 mM $FeCl_2$ (Panreac, Spain) and 5 mM ferrozine (Sigma-Aldrich, USA), then measured at 562 nm (Kuda et al., 2005).

Statistical analysis.

Data were analyzed by one-way Analysis of Variance (ANOVA) using SPSS 16.0 software. Homogeneity of variance was performed by Duncan's multiple range test with mean ± SD and $p<0.05$ ($n=3$). Statistical comparisons were performed to evaluate the variations on SGR, iAs and tAs intake, and antioxidant activities.

RESULTS AND DISCUSSION

Toxicity and biotransformation of arsenic.

Different concentration iAs has a different effect on the growth of *P. minor* (**Figure 1**). An acute effect of iAs on *P. minor* was shown after contaminated with 25 µg As L^{-1}. It was indicated by declining growth values by 0.13% day^{-1} at day 14 and 0.18% day^{-1} at day 21 after removed from arsenic media. In contrast, appreciable concentrations of iAs (50 and 100 µg L^{-1}) were directly attenuated the thalis at day 7 by -1.2 and -1.3 % day^{-1}, respectively. However, the condition of thali seems recover by increasing growth values to 0.6 and 0.5% day^{-1} for 50 and 100 µg As L^{-1} respectively at day 21 in arsenic-free media. Similar condition had been reported by Bahar et al. (2013) in microalgae *Scenedesmus* sp. It could survive and grew in low arsenic contamination. Those anomalies were indicated that *P. minor* and *Scenedesmus* sp. could tolerate the toxicity of low level iAs, but not at higher concentration.

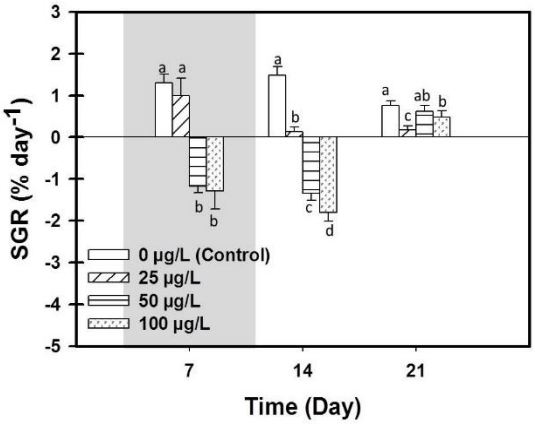

Figure 1. Growth rates of P. minor after exposed with various iAs over a period of 7 days. All data are presents as mean ± SD with $p<0.05$ compared with each control group.

There was a tendency to enhanced influx of arsenic into the thali after elevation of iAs external concentration (**Table 1**). Interestingly, only As^{5+} was detected on *P. minor* even iAs was contented with equal amounts of both As^{3+} and As^{5+}. The thalis seems unsaturated with arsenic in low concentration which detects only 2.3 µg g^{-1} as As^{5+} at day 7. But the arsenic influx were significantly increased by 8.5 and 14.4 µg g^{-1} as As^{5+} at concentration 50 and 100 µg As L^{-1} at day 7 respectively. The absence of As^{3+} in the thali shows that most of As^{3+} had been oxidized to become As^{5+} before 7 days. Otherwise, Klump (1980) reported that *Fucus spiralis* was kept both As^{3+} and As^{5+} inside the thali and could methylated As^{3+} without oxidation process. The different metabolism of iAs between both brown seaweed may happens due to different types of metallothionein (Davis et al., 2003), methylation process (Briat et al., 2010), or antioxidant responses.

After 14 days reculture in arsenic-free media, thalis had been methylated As^{5+} to become organic form. It was evinced by decreasing As^{5+} contents in the thali and no iAs was detected in culture media. Moreover, the organoarsenic had been excreted back to media since reducing tAs contents at the same time. Thus, *P. minor* could be classified as phytodegradation by the ability to recover and reduced the toxicity of arsenic (Mirza et al., 2014).

Table 1. Inorganic and total arsenic intakes on *P. minor* at day 7 and 21.

Initial As conc. (µg iAs L^{-1})	As concentration (µg As g^{-1})					
	Day 7			Day 21		
	As^{3+}	As^{5+}	tAs	As^{3+}	As^{5+}	tAs
0	n.d	0.09 ± 0.12$^{\#}$	1.98 ± 0.27$^{\#}$	n.d	0.06 ± 0.04$^{\#}$	1.97 ± 0.16$^{\#}$
25	n.d	2.29 ± 0.27	3.65 ± 0.75	n.d	0.39 ± 0.1	3.24 ± 0.36
50	n.d	8.48 ± 1.39*	10.01 ± 0.33*	n.d	0.45 ± 0.26	3.47 ± 0.51
100	n.d	14.37 ± 0.87*	14.84 ± 0.22*	n.d	0.77 ± 0.3*	5.2 ± 0.98*

Values indicate the means ± SD (*n*=3) with statistical different (*) between iAs treatment group with control group at same time. ($^{\#}$) means control group with no iAs addition; *n.d*, not detected; 50 ng As L^{-1} was applied as LOD and LOQ.

Antioxidant activities.

Different antioxidant responds had been shown after polluted by various concentration of iAs (**Table 2**). Polysaccharide content is one of reactable compound against metal pollution. Andrade et al. (2010) reports that polysaccharides contents had been more synthesizes to entraps Cd and Pb in the cell wall as defensive mechanism of *P. gymnospora*. However, polysaccharide production after treatment was significantly lower than control group. In control group, *P. minor* could produces around 717.5 mg L^{-1} at day 7 then increased to 1007.3 mg L^{-1} polysaccharide at day 21. But it was significantly decreased after treats with various iAs level till less than 40 % compared to control group at the same observation time. The different responses on *P. minor* and *P. gymnospora* may due caused As had smaller molecular weight than Cd and Pb. Thus, it leads high accumulation of iAs in thali caused As was infiltrate by cell wall and directly accumulate in the vacuole.

Table 2. Antioxidant activities on P. minor at day 7 and 21 after treatments.

iAs contamination (µg As L^{-1})	Time (day)	
	7	21
	Polysaccharide (mg L^{-1})	
Control	717.54 ± 34.38	1007.33 ± 20.31
25	188.53 ± 10.27*	182.63 ± 22.3*
50	275.38 ± 35.72*	280.15 ± 33.44*
100	166.65 ± 29.04*	168.65 ± 30.28*
	DPPH radical scavenge (%)	
Control	76.96 ± 1.79	82.08 ± 2.4
25	68.59 ± 2.42*	80.18 ± 1.25
50	69.42 ± 3.3*	90.71 ± 1.71*
100	64.2 ± 3.6*	78.2 ± 4.03
	Flavonoid (mg g^{-1})	
Control	23.99 ± 6.04	328.78 ± 29.79
25	70.14 ± 22.82	515.9 ± 95.38*
50	598.07 ± 53.87*	926.79 ± 32.56*
100	189.67 ± 45.03*	331.62 ± 72.56
	Iron Chelation (%)	
Control	12.76 ± 2.38	5.3 ± 2.01
25	2.46 ± 1.63*	4.81 ± 2.65
50	4.1 ± 1.42*	3.28 ± 2.08
100	4.18 ± 1.98*	4.51 ± 1.51

Values indicate the means ± SD (*n*=3) with statistical different (*) between iAs treatment groups with control group at same time.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is used to investigate radical scavenging activity. In DPPH radical scavenging assay, the activity after various As treatments were shows only around 68.59, 69.42 and 64.2% on 25, 50 and 100 µg iAs L^{-1} respectively, it was relatively lower than control group at day 7. But it was increased to 80.18, 90.71, and 78.2% at day 21 after removed from 25, 50, and 100 µg iAs L^{-1} contaminate media, respectively. Increasing activity of *P. minor* extract on DPPH free radical indicates that it was potent to scavenge free iAs as results as its defense function. High activity of DPPH in thali is due to high fucoxanthin production on brown seaweed as defensive metabolism against free radical intrusion (Airanthi et al., 2011). Babu et al. (2013) also mentioned that higher DPPH scavenging activity on triphala (a traditional Ayurvedic herbal formulation) than vitamin C indicates its potential to scavenge radical ion. Considering by those assumption, thali may intensify the methylation As^{5+} to organoarsenic process till the last day.

Flavonoid is known that it can bind to biologic polymers such as enzymes, DNA, metal ion chelator and free radicals scavenge as antioxidant parameters (Brandy, 1992). The flavonoid content was significantly increased after exposure. It happens because active metal can stimulate

more flavonoid synthesize to chelate free iAs inside the thali (Van Acker et al., 1998). However, the 50 µg As L^{-1} is optimum contamination levels for *P. minor* which is appeared by flavonoid activities. This condition was due to decreasing flavonoid contents at 100 µg As L^{-1} in both day 7 and 21. While, iron chelation activity was shows no different between control group and iAs treatment groups. Van Acker et al. (1998) also mentioned that iron chelation activity does not have a crucial role in microsomal lipid peroxidation. This phenomenon is indicates that iAs detoxification probably happens directly on vacuole methylation process.

CONCLUSION

The result of this study is demonstrate that *P. minor* has an ability to oxidized As^{3+} become As^{5+} before 7 days then methylate it to organic form. High growth rate value was indicated *P. minor* could tolerance low level of As pollution. While, activity of antioxidant parameters such as increasing DPPH scavenge and more flavonoid production at day 21 had been shown the important role of antioxidant to detoxify As. Thus, the presence of *P. minor* in ecosystem is necessary to reduce iAs in the marine environment, also as biofilter and biomonitor agent for As contamination.

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