Middle-East Journal of Scientific Research 19 (2): 139-143, 2014
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2014.19.2.13585

Glutamine Synthetase, Peroxidase and Protease as Indicators of the Ecological State of Higher Aquatic Plants

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Abstract: The contamination of water by heavy metals leads to conditions of ecological tension in the aquatic systems. In this work we consider to use the peroxidase, protease and glutamine synthetase of aquatic higher plants *Typha angustifolia* and *Lemna polórhyza* as indicators of ecological state of the aquatic biocenosis. In response to the 2.5 mg/l lead contamination the glutamine synthetase activity dropped down 10-fold already in 1 hour in the photosynthetic tissues of both plants with following restore up to 70-80% of initial activity values in 3 hours. Also strong inhibition of proteolytic activity without recovery in both *L. Polórhyza* and *T.angustifolia* leaves was detected after the lead introduction. By contrast, the peroxidase did not exhibit high sensitivity to the lead contamination and demonstrated 2-fold decreased activity. Taking together, these data demonstrate the possibility to evaluate the ecological state of higher aquatic plants by measuring the activity of the glutamine synthetase, peroxidase and protease in their photosynthetic tissues.

Key words: Lead contamination · Aquatic plants · Peroxidase · Protease · Glutamine synthetase

INTRODUCTION

In recent years, in many studies the enzymes are used as biomarkers of aquatic stress caused by pollutants [1, 2]. As a markers catalase, glutathione synthase and cytochrome oxidase are widely used. In this paper, we consider as indicators the peroxidase, protease and glutamine synthetase.

Glutamine synthetase (GS; EC 6.3.1.2, L-glutamate: ammonia ligase ADP forming) is a central enzyme of nitrogen flow in plants and catalyzes the ATP-dependent incorporation of ammonium (NH_4^+) to γ-carboxyl group of glutamate by glutamine formation. Ammonium assimilated by GS comes from a variety pathways, including direct revenues from the soil, the reduction of nitrates and nitrites, photorespiration, deamination of phenylalanine and the catabolite release of ammonia during the aging process. GSs are found in photosynthetic and non-photosynthetic tissues of higher plants and are oligomeric isoenzymes that are either in the cytosol or in chloroplasts [3]. In angiosperms, there are two major isoforms of GS, the cytosolic GS (GS1) and GS of chloroplasts (GS2). Ammonium assimilated into glutamine by GS2 in young leaves, is formed mainly due to nitrate reduction and photorespiration [4]. In turn, the cytosolic GS1 forms glutamine for intercellular transport of nitrogen. It assimilates ammonium incoming from the soil and is included in the biosynthesis of phenylpropanoids and remobilization of nitrogen [5, 6]. GS was shown to play an important role in the growth and development of plants [5]. As example, the transgenic poplar, having increased GS activity, have a high rate of vegetative growth [7], increased resistance to drought, oxidative stress as well as increased efficiency of nitrogen use [8]. Thus, the level of activity of GS in plants is the limiting factor in the growth and development.

Peroxidases are the enzymes that catalyze the oxidation of various organic compounds by the oxygen of hydrogen peroxide. Peroxidases are complex proteins and protect cells against reactive oxygen species (ROS). In the presence of H_2O_2, they are able to oxidize various substrates [9]. In higher plants over 40 different genes coding for peroxidases can be found, some isoforms of the enzymes can be produced by posttranslational

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modifications. Peroxidases are involved in the general response to stress as well as in many other physiological processes of plants, including respiration and photosynthesis, synthesis and degradation of the hormones and detoxification of reactive oxygen species formation [10, 11]. The guaiacol oxidizing peroxidases were used to assess the state of stress in rice [12], as well as in aquatic macrophytes - Sago pondweed (*Potamogeton pectinatus* L.) in response to cadmium [13].

Proteinases carry out in plant cells the selective cleavage of damaged or incorrectly folded proteins. Oxidative stress, the presence of heavy metals causes oxidation of cellular proteins which must be removed. As a consequence, the activity of proteases in the cells changes in response to stress [14]. It has been shown that cadmium and mercury causes an increase and lead, aluminum, nickel and conversely reduced proteolytic activity in sunflower cell. Thus, increased or decreased proteolytic activity can be used as an indicator of the stress for a plant [15].

The aim of the work was to elucidate the aquatic plants enzymes responding in short time (3 h) to the lead introduction for using as a indicators of environment contamination.

MATERIALS AND METHODS

Experiments were performed under laboratory conditions (22°C) in 30 L tanks with natural water and plants of *Typha angustifolia* or *Lemna polórhyza* taken from Sredniy Kaban lake (Kazan, Republic of Tatarstan, Russia). As a pollutant a lead acetate was added to the water until the final concentration 2.5 mg/l (10-fold exceeding of the maximum permissible concentration). The leaves of *T. angustifolia* (40 cm from the end) and several plants of *L. ðîlórhyza* were taken before the pollutant injection and after 1, 2 and 3 hours after the injection.

Sample Preparation: The leaves were homogenized by rubbing with a glass in cold buffer DB (50 mM KCl, 50 mM NaCl, 2 mM MgCl2, 50 mM Tris-HCl, 1 mM PMSF pH 7.4). The lysate was centrifuged for 15 min at 13000 × g to remove the debris and the protein concentration was measured according to [16].

The Glutamine Synthetase Activity: was defined by modified protocol of [17, 18]. The assay mixture for the biosynthetic reaction consisted of 100 mM imidazole-HCl (pH 7.0), 125 mM hydroxylamine-HCl, 20 mM $MgCl_2$•$6H_2O$, 10 mM ATP and 100 µl of cell extract. The mixture was preheated at 30°C for 5 min. The reaction was initiated by adding 50 µl of 500 mM glutamate, giving a final assay volume of 500 µl. The reaction was stopped after a 60-min incubation by the additon of 1 ml of stop mix (5.5% $FeCl_3$ • $6H_2O$ [w/v], 2% trichloroacetic acid [w/v] and 0.78% HCl) and centrifugation for 5 min at 10.000 × g to remove the precipitated proteins. The Fe (III)-complex of the reaction product γ-glutamylhydroxamate was measured spectrophotometrically by absorbance at 540 nm. GS specific activity is calculated as nMol of γ-glutamylhydroxamate produced min^{-1} milligram of $protein^{-1}$. A reaction mixture without glutamate served as the blank.

The Peroxidase Activity: was measured on the chromogenic substrate guaiacol [19]. The reaction mixture (1 ml) consisted of 25 mM Na-acetate HCl pH 5.0, 8.26 mM guaiacol (Sigma), 8.8 mM H_2O_2. To a preheated at 30°C freshly prepared mixture (not later than 10 minutes before the experiment) 5-100 µl of the cell extract were added, vortexed and measured the absorbance at a wavelength of 470 nm every 20 sec. A reaction mixture without cell extract served as the blank. Activity values were averaged from the values when the time dependence was linear. Peroxidase specific activity is calculated as nMol of guaiacol oxidized min^{-1} milligram of protein . (^{1}An extinction coefficient for guaiacol was 26.6).

The Proteolytic Activity: was defined on the chromogenic substrate azocasein [20]. To 200 µl of the cell extract 400 µl of azocasein (1% in 50 mM Tris HCl pH 7.5) was added and incubated for 2 h at 30°C. The reaction was stopped by addition of 800 µl of TCA (5%) with following incubation for 5 minutes at -20°C and centrifugation for 5 min at 10.000 × *g* for 3 min to remove unhydrolysed azocasein. To the 1200 µl of the supernatant 240 µl of 4N NaOH was added to neutralize the acid and the absorbance was measured at 410 nm in a 1 cm cuvette. A sample without incubation served as a blank. Proteinase specific activity is calculated as nMol of azocasein digested min^{-1} milligram of $protein^{-1}$.

RESULTS AND DISCUSSION

The Glutamine Synthetase Activity after the Lead Treatment: Lead is a metallic pollutant coming from industrial wastes, combustion of fossil fuels and use of agrochemicals. We investigated the response of GS activity in photosynthetic tissues of *T. angustifolia* and *L. ðîlórhyza* to the lead introduction into the growth water. Samples were harvested after 1, 2 and 3 hours after the lead injection and the enzymatic activities were measured as described in materials and methods.

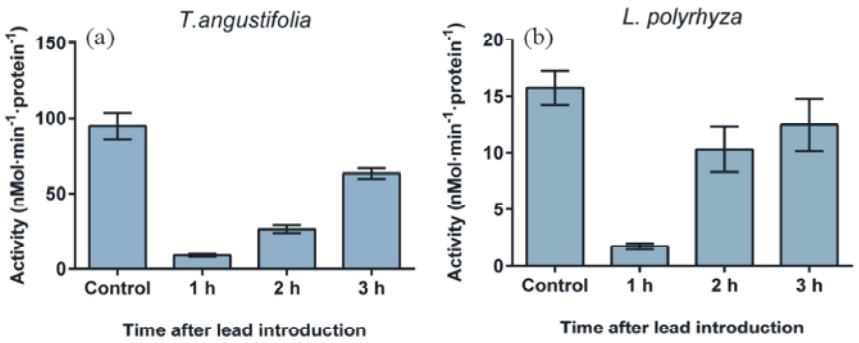

Fig. 1: The activity of glutamine synthetases in the photosynthetic tissues of *T. angustifolia* (A) and *L. ðîlórhyza* (B) after the lead injection up to final concentration 2.5 mg•l^{-1} (10-fold exceeding of the maximum permissible concentration). The biosynthetic activity of GS was quantified by measuring of the reaction product γ-glutamylhydroxamate Fe (III)-complex.

Already after 1 hour of lead exposition, the glutamine synthetase activity was 10 fold lower in the photosynthetic tissues of both plants (Figure 1). Nevertheless, in 3 hours the activity was recovered up to 70-80% of initial values. Notable, in the *L. Polórhyza* the activity was recovered faster; already in 2 h it has reached 70% and 80% in 3 h. In our assays the cytosolic glutamine synthetase (GS1) activity was primarily measured since the most chloroplasts were intact after leaves rubbing (almost no chlorophyll was detectable by spectroscopy in a cell lysate, not shown) and were removed during centrifugation. Since GS1 forms glutamine for intercellular transport of nitrogen [6], its repression should lead to the nitrogen starvation of the plant and suppression of nitrogen metabolism.

The mechanism of glutamine synthetase repression is discussible. Since glutamine synthetase is a metal-dependent enzyme (it requires Mg^{2+} and Mn^{2+} ions), its activity repression can be caused by the substitution of Mg^{2+} and Mn^{2+} by the lead as was shown for many other enzymes [for review, see 21, 22]. From the other side this is hard to believe that lead is able to penetrate quickly into the plants and inhibit the enzymes directly. More possible, this activity reduction is due to the plants strategies to cope with lead toxicity, including reduced uptake into the cell, constructive metabolism decreasing and generation of reactive oxygen species (ROS) [21, 22]. Since GS1 assimilates ammonium incoming from the soil [5], probably, the lead stress damages the root cells membrane and impairs uptake of essential elements from the environment [21].

The Guaiacol Oxidizing Peroxidases Activity after the Lead Treatment: The lead stress leads also to the ROS overproduction as a general stress response [21]. Under these conditions peroxidases are normally activated to protect the cell from the damage. Previosly, guaiacol oxidizing peroxidases were shown to be activated in maize in response to stress [12]. By contrary, the exposition to the lead resulted in 2-fold decrease of guaiacol oxidizing peroxidase activity in *T. angustifolia* leaves (Figure 2a). By contrast to the glutamine synthetase, the peroxidase activity was not restored in 3 h, being even reducing by the time. We attribute this effect with a direst inhibition of the peroxidases by lead penetrated to the plant and, probably, substitution the iron in the catalytic center. The inhibition of peroxidases should greatly reduce the adaptive capacity of the plant and result in a lot of events of damage of DNA, membrane and other compounds.

In *L. ðîlórhyza* the activity drops to 15% of initial activity, but then was almost completely restored in 3 h (Figure 2b). Probably, the fully aquatic *L. polórhyza* adapts it metabolism to the pollutant more quickly compared with *T. angustifolia*.

The Proteolytic Activity after the Lead Treatment: At the last, we established the proteolytic enzymes pool activity after the lead introduction. The lead resulted in a drastical inhibition of proteolytic activity in both *L. polórhyza* and *T.angustifolia* leaves (Figure 3). The activity did not recover in 3 h of observation, being almost on the identification limit. The same lead effect on proteases was previously reported for the sunflower cells [14]. Since the most of plant proteases are reported to be metalloproteases [reviewed in 23], we propose that the lead displaces metal ion in the enzymes active center. Proteinases in plants are involved in the degradation of damaged proteins, therefore, the lack of proteolytic activity can lead to the loss of control over the proteins quality, accumulation of degraded proteins, proteins with impaired function.

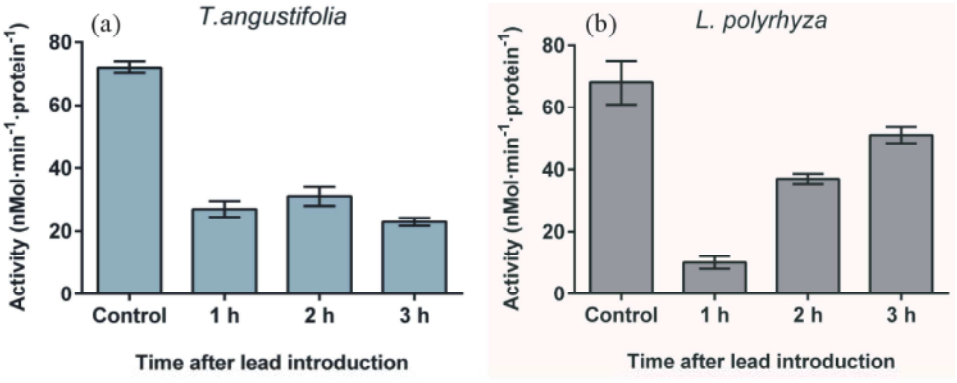

Fig. 2: The activity of peroxidases in the photosynthetic tissues of *T. angustifolia* (A) and *L. ðîlórhyza* (B) after the lead injection up to final concentration 2.5 mg•l^{-1} (10-fold exceeding of the maximum permissible concentration). The activity was measured by guaiacol oxidation by the plant cell extract in presence of H_2O_2.

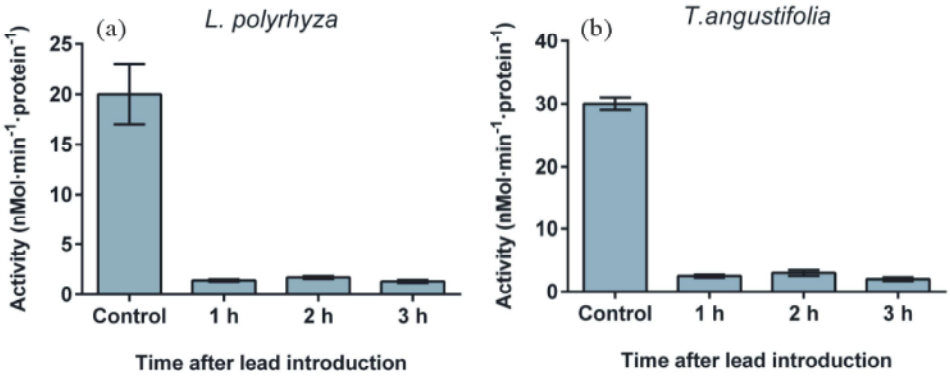

Fig. 3: The activity of proteinases in the photosynthetic tissues of *T. angustifolia* (A) and *L. ðîlórhyza* (B) after the lead injection up to final concentration 2.5 mg•l^{-1} (10-fold exceeding of the maximum permissible concentration). The activity was measured by azocasein hydrolysis.

Taking together, our data demonstrate the possibility to evaluate the ecological state of higher aquatic plants by measuring the activity of the glutamine synthetase, peroxidase and protease in their photosynthetic tissues. While the glutamine synthetase activity reflects significantly the recent volley contamination by heavy metals occurred in several hours, the proteolytic activity may correspond to the long standing contamination. The peroxidase activity did not demonstrate high sensitivity to the lead contamination. Nevertheless, its activity can be used as a control to avoid the pathogens invasion effects on the enzymatic systems of plant.

ACKNOWLEDGEMENTS

This research was supported by Ministry of Education and Science of the Russian Federation and by the subsidy of the Russian Government tosupport the Program of competitive growth of Kazan Federal University.

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