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Investigation of *in vitro* salt stress on peroxidase enzyme of *Amsonia orientalis* and purification of peroxidase from non-stressed and salt-stressed plants

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In this study, we focused on *in vitro* salt stress on peroxidase (POD) enzyme activity change of *Amsonia orientalis*. The plant was subjected to 25, 50, 75, 100, 125 and 150 mM NaCl salinity for 30 days. Change of POD activity was observed by spectrophotometric and activity staining assays. Our findings indicated that POD activity didn't dramatically change especially when plant was exposed to high salinity. The specific activity of POD of non-stressed plant was calculated as 0.74 (U/mg), while the highest specific activity was seen at 50 mM NaCl and the lowest specific activity at 150 mM NaCl as 0.87 (U/mg) and 0.54 (U/mg), respectively. For further analysis of salt stress effect on POD activity, the enzyme was purified from non-stressed and 50 mM salt-stressed *A. orientalis.* Similar purification profiles were observed for non-stressed and 50 mM salt-stressed plant at 4.570-fold purification with 8% yield and at 4.10-fold purification with 7% yield. The molecular weights of purified enzymes from both extracts were determined by electrophoretic methods as 59 kDa. These results suggest that POD is a strong defensive enzyme against salinity due to non-change of its activity especially in 50 mM NaCl by monitoring POD activity in crude and purified extracts.

Keywords: Peroxidase (POD), *Amsonia orientalis*, Salt stress, Purification

INTRODUCTION

Salinity is an abiotic stress that inhibits biological and physiological parameters of plants. Salinity stress alters general metabolic processes and enzymatic activities and leads to molecular damage to important metabolic pathways of plants like photosynthesis by causing increased production of reactive oxygen species [1]. Reactive oxygen species (ROS: singlet oxygen (O_2), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-)) are regarded as the main sources of damage to cells under biotic and abiotic stresses [2]. Toxicity effect of reactive oxygen species is highly efficient on antioxidant defense systems, including both nonenzymic (ascorbate, glutathione, alpha-tocopherol) and enzymic (catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) peroxidases (POD)) constituents in plant cells [3]. Antioxidant enzymes, which act as detoxifiers due to their ROS scavenging activities, are important defensive biomolecules of plants. Peroxidase (POD: EC 1.11.1.7) [4], which converts H_2O_2 to water, is an important antioxidant enzyme involved in oxidative protection. POD, an iron heme protein, catalyzes the reduction of H_2O_2 with a concurrent oxidation of the substrate, mostly located in the cell wall and involved in oxidation of phenol compounds in the synthesis of lignin [5]. *Amsonia orientalis* Decne. (syn. *Rhazya orientalis* (Decne.) A. DC.) which belongs to Apocynaceae family, is commonly known as "Blue star" with its profuse pale-blue flowers. It is a medicinal and ornamental plant having natural distribution only in northwest Turkey and northeast Greece [6]. Within the Bern Convention (1979) [7], the European Council placed the plant in the list of the plant species that must be conserved on European scale. Also, the plant is listed in the category of "Critically Endangered" (CR) in the "Red Data Book of Turkish Plants" [8]. This data, by themselves, bring doubts about the plant's ability of resistance to several stress factors. Thus, samples from Balıkesir population of the plant were taken to *ex situ* conservation to protect the plant from stress factors [9] and the plant's genetic diversity among the remaining populations was determined [10]. Also, as a conservation strategy, *in vitro* studies were conducted and mass production of the plant was achieved [11,12]. Yet, it is not clear that the decrease in the number of individuals or in the distribution area of the plant is caused by any environmental stress factors. Therefore, it is thought that understanding of stress-related plant reactions would shed light on the plant's ability to survive in case of exposure to such stresses. For such purposes, *in vitro* plant studies provide reliable data by using controlled, non-season-dependent and repeatable experiments. In this study, we focused on the investigation of the

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peroxidase enzyme activity of *A. orientalis* under *in vitro* salinity stress, and the purification of the plant peroxidase in order to assess the reliability of activity data and finally to understand the plant's defensive ability and mechanism to response to salinity stress, and to develop more efficient conservation strategies.

MATERIALS AND METHODS

Plant material and disinfection

Healthy shoots, 10 cm in length, were collected from 8-year-old field-grown individuals of *Amsonia orientalis* before flowering in May 2013 and all leaves were cut off. The shoots were washed under tap water for 15 min, and then cut into 1-2 cm long segments that had at least one node (single node explants). Explants were disinfected by dipping in 70% ethyl alcohol (EtOH) for 2 min and then in 1% (v/v) sodium hypochlorite (NaOCl) for 12 min. Disinfected explants were rinsed with sterilized water three times in order to remove NaOCl residues. Excess water on the explants was taken by sterile filter papers before inoculations.

Culture establishment, explant preparation and in vitro salt treatment

Disinfected single node explants were inoculated vertically in culture vessels containing 40 mL of Murashige and Skoog's (1962) medium (MS), [10], supplemented with 1.0 mg l^{-1} BAP. At the end of the incubation period, following the excision of emerged shoots from explants and cutting them into single node explants, these were sub-cultured on the same fresh medium. Through repeating this process, subcultures were regularly obtained on the same medium at 30 d intervals until a desired number of single node explants was reached. Single node explants were then transferred to a fresh MS medium containing 0, 25, 50, 75, 100, 125 and 150 mM NaCl and incubated for 30 d under the same conditions.

Culture medium and conditions

All variants of MS medium were supplemented with 30 g l^{-1} of sucrose and 7 g l^{-1} of plant agar. The pH of the plant culture media was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving. Disinfection treatments and inoculations were carried out aseptically in a laminar air flow cabinet. Cultures were maintained at a temperature of 23±1°C in a plant growth chamber with a 16/8 h light/dark photoperiod under an illumination of 80 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by cool-white fluorescent lights.

Preparation of crude extract

Non-treated (2.92 g) and 25, 50, 75, 100, 125 and 150 mM salt-stressed plants (1.5, 2.08, 1.57, 2.85, 1.54 and 2.63 g, respectively) were homogenized with a mortar in a 20-fold extraction buffer of 50 mM sodium phosphate (pH 7.0). The homogenate was filtered and then centrifuged at 14000 *g* for 15 min at +4 °C (Sigma 4-16 K). The supernatant was collected and used as crude extract for analysis of POD activity. Protein concentration was determined according to Bradford method with bovine serum albumin as standard [13].

Determination of POD activity

POD activity was determined using the pyrogallol oxidation method [14] in a 2 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM pyrogallol, 100 µL suitably diluted enzyme and 5 mM H_2O_2. The increase in absorbance was recorded at 425 nm within 5 min after enzyme extract was added and the reaction was stopped with 1mL of 2.5 N H_2SO_4. Enzyme activity was determined using the extinction coefficient at 425 of 2640 $M^{-1}cm^{-1}$ for pyrogallol [15], where one unit of enzyme activity corresponds to the formation of one milligram of purpurogallin per 5 min [16].

SDS-PAGE and activity staining analysis of antioxidant enzymes

The SDS-PAGE was carried out using Bio-Rad Mini-Protean system with 5% stacking and 12% separating gels prepared according to Laemli *et al.* [17]. Sample proteins were dissolved in loading buffer containing a reducing agent and heated to 95 °C for 7 min. SeeBlue® Plus2 Pre-stained Protein Standard (LC5925) was used as molecular weight marker. Electrophoresis was carried out for 10 min at 300 V, followed by2-3 h run at 400 V. Gels were stained using silver stain [18] and photographed.
Electrophoretic analysis of POD was also performed by non-denaturing 12% separating and 5% stacking gels at 4 °C, 100 V for 10 min, then at 120 V for 60 min using Biorad mini protean II electrophoresis system. POD was stained according to the following method: gel activity was detected using incubating gels in 10 mM pyrogallol and 5 mM H_2O_2 in 50 mM pH 7 phosphate buffer at room temperature until appearance of the orange-brown bands, measured against achromatic background.

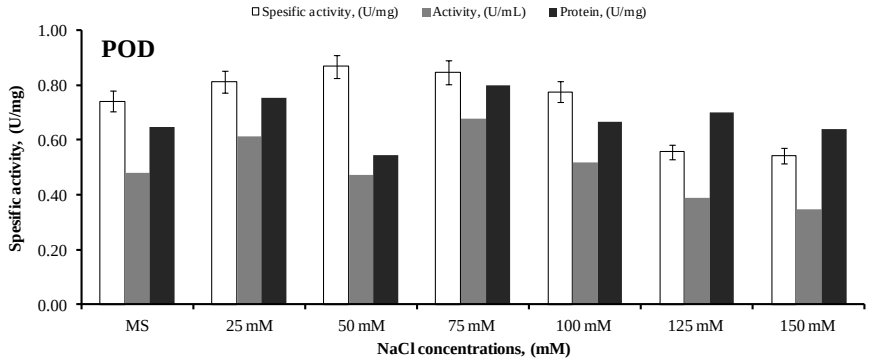


Fig. 1. Effect of increasing NaCl concentration on POD specific activity, activity and total protein amount of *A. orientalis*. Values are the mean ± SE for three observations. Vertical bars indicate mean±SE of three replicates.

Purification of peroxidase from non-treated and 50 mM salt-stressed A. orientalis

Separation of POD from other proteins in the supernatant was achieved by a three-step procedure including ammonium sulfate precipitation, anion exchange and gel filtration chromatography. The crude enzyme extract was brought to 90% saturation with ammonium sulfate under continuous stirring. After 7 h, the solution was centrifuged at 14000 *g* for 15 min. The precipitate was dissolved in a minimum volume of 50 mM phosphate buffer (pH 7.0) and dialyzed overnight in the same buffer. The concentrated dialysate was loaded on a DEAE-Sepharose column (2×30 cm) previously equilibrated with 100 mM NaCl in 50 mM phosphate buffer (pH 7.0) and as weluted at 0.5 mL/min with the same buffer. The fractions were monitored for protein amount and POD activity. POD active fractions were then pooled and concentrated. Concentration was processed in a 5 kDa cut-off membrane (OMEGA membrane disc, OM005025) with Millipore stirred cell (10 mL working volume and 25 mm diameter).

Partially purified and concentrated POD enzyme (1.5 mL) obtained by ion exchange chromatography was subjected to gel filtration chromatography using Sephacryl S-200 column (GE Healthcare, Uppsala, Sweden). The glass column with an internal diameter of 1 cm was packed to the height of 30 cm. Sample was loaded onto the column previously equilibrated with 50 mM phosphate buffer (pH 7.0) and was eluted at 0.2 mL/min with the same buffer. 2 mL fractions were collected and analyzed for protein content and enzyme activity.

RESULTS AND DISCUSSION

Effect of salt stress on POD activity

According to former reports *A. orientalis* prefers sandy-loamy soils with no salt, slightly alkaline, mid-calcareous, poor in organic material, very rich in iron and magnesium [9]. In this study, we investigated the effect of salt stress on the antioxidant enzyme POD. The change of enzyme activity is given in Fig. 1.

When compared to control; increasing NaCl concentrations didn't change the POD activity until 150 mM NaCl, but according to protein profile (Fig. 1) the lowest protein content was observed at 50 mM NaCl-stressed plant. The specific activity of an enzyme is the activity of an enzyme per mg of total protein (expressed in U/mg). It is the amount of product formed by an enzyme in a given time interval under given conditions per mg of total proteins. Compared to control, the specific activity increased to 109%, 117%, 114% and 105% at the 25mM, 50 mM, 75 mM and 100 mM NaCl concentrations, respectively. But at the high salt concentrations (125 mM and 150 mM) the specific activity of peroxidase decreased to 75%. The highest specific activity was observed at the 50 mM NaCl concentration. Peroxidases are not only having an important effect on the decomposition of H_2O_2 but also on lignification, plant growth, salt and heavy metal stress [19,20]. The highest specific activity for POD at 50 mM NaCl indicated the highest decomposition capacity of H_2O_2. 50 mM NaCl concentration might be considered as threshold salt concentration for the plant to protect itself against salt stress. Decreasing of specific activity of peroxidase at the increasing NaCl concentrations might be an indicator of reduced defense ability to salinity of *A. orientalis*. The increased or unchanged antioxidant enzyme activity under salt stress may be related to the tolerance to salt stress [21]. In tolerant plant species, POD activity was found to be higher, enabling plants to protect themselves against the oxidative stress whereas such activity was not observed in sensitive plants [22]. In this study, the POD activity remained unchanged in *A. orientalis*. However, specific activity of POD in *A. orientalis* explants

increased at 50 mM NaCl, then decreased at higher salt concentrations (Fig. 1), so 50 mM NaCl treatment indicated that POD might play a role in *A. orientalis* defense against salt. Induced POD activity by salt stress increased for cotton [22], alfalfa [19], *Jatropica curcas* L. [20], but decreased in *Cucumis sativus* L. [23] and remained constant under rising NaCl levels was reported [22].

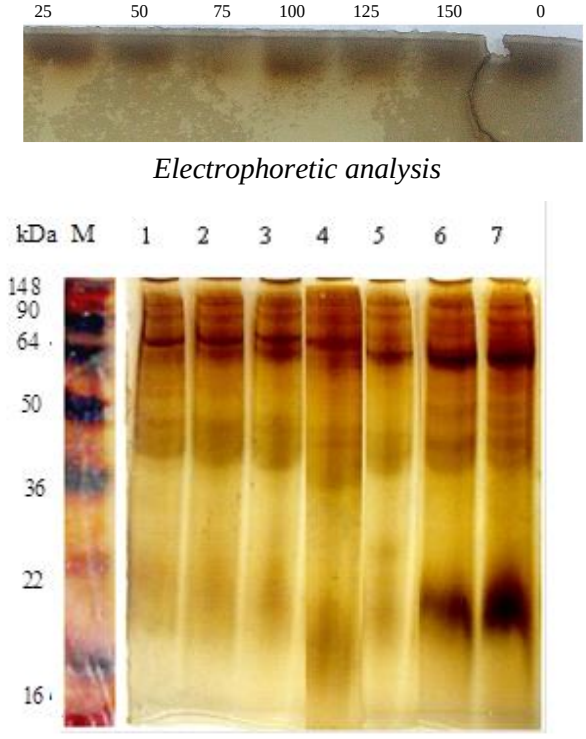


Fig. 2. (A) Staining of POD activity on Native PAGE. Each well was loaded with 15 µg protein. Numbers on the gels indicate the NaCl concentrations. (B) SDS-PAGE analysis with silver staining of total protein of NaCl treated *A. orientalis*. Lanes M, C, 1, 2, 3, 4, 5, 6, 7 from left to right represent marker and proteins extracted from control, 25, 50, 75, 100, 125 and 150 mM NaCl-treated plants. For each lane,15 µg protein was loaded.

POD activity of *A.orientalis* under salt stress was also determined by activity staining. Although more than one POD isoenzyme was reported [24], for this plant single POD isoenzyme was visualized on the gel (Fig. 2A). Intensities of POD activity on gel were not different for both salt-treated and non-treated plant. This result was also supported by spectrophotometric analysis of POD activity suggesting that there was no dramatic decrease in enzyme activity in response to increasing NaCl concentrations. This would contribute to the defense mechanism of *A.orientalis* against higher salt treatments.

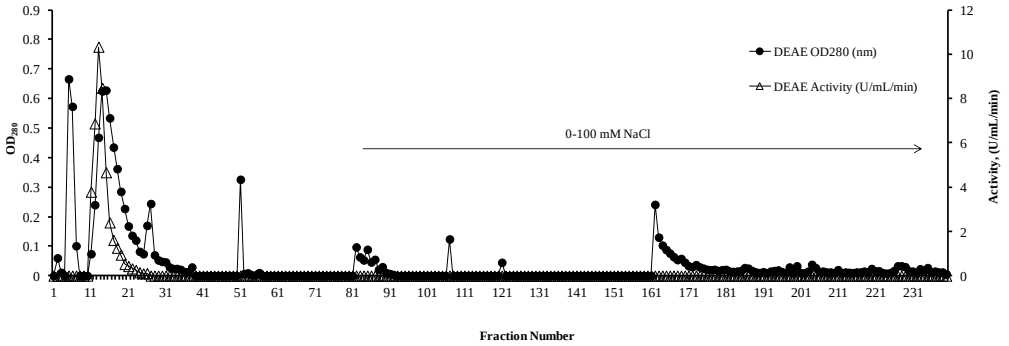
Amounts of total proteins in non-treated and salt-treated extracts of *A. orientalis* were analyzed by SDS-PAGE (Fig. 2B). As visualized from SDS-PAGE intensity, 59 kDa molecular weight protein decreased in amount as a result of 100 mM, 125 mM and 150 mM NaCl treatments (Fig. 2B). Intensity of decrease was dependent on NaCl concentration. But it was also found that the sharpness of the 64 kDa protein band increased at 100 mM, 125 mM and 150 mM NaCl treatment and seemed to be proportional to the NaCl concentration. Moreover, the highest NaCl concentrations (125 mM and 150 mM) exhibited prominently increased protein bands of 19 kDa. These results indicate that a plant grown under salt stress shows either induction (64 and 19 kDa) or repression (81 kDa) in the synthesis of some polypeptides.

Purification of peroxidase from non-stressed and 50 mM salt-stressed A. orientalis

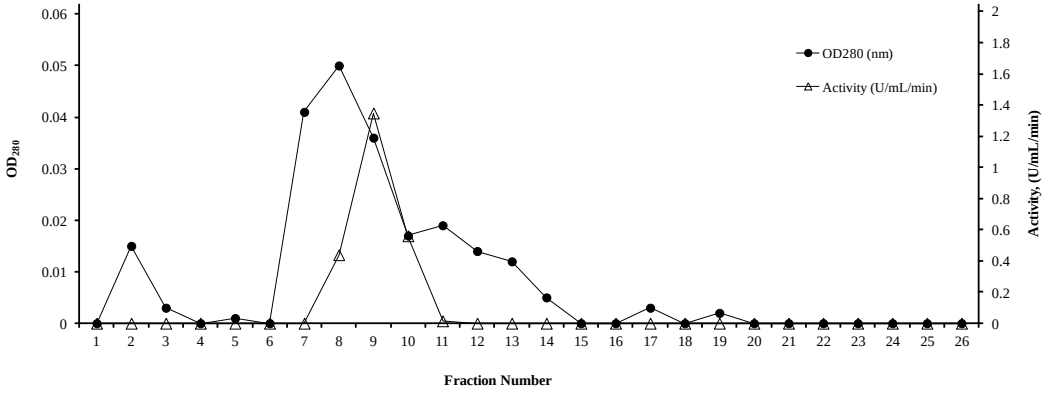
Peroxidases have broad industrial applications, especially in medicine, food and water treatment [25]. There are various reports about purification of peroxidase from different plants using chromatographic techniques [25-27]. As the highest specific activity was observed at 50 mM NaCl-treated *A. orientalis*, we purified peroxidase from 50 mM NaCl salinity and control plant using three simple steps including ammonium sulfate precipitation, anion exchange chromatography with DEAE-sepharose and gel permeation chromatography on Sephacryl-S 200. The purification profile is summarized in Table 1. 4.57-fold and 4.00-fold purification with 8% and 7% yield was achieved for control and 50 mM salt-stressed *A.orientalis*, respectively.

The percent ammonium sulfate was obtained as experimental effect of saturation (30-90%) on the first step of purification process. All fractions were assayed for peroxidase activity and nearly all peroxidase was observed in dialyzed 90% ammonium sulfate saturation while peroxidase activity of supernatant of this fraction equalled zero. Concentrated enzyme with 90% ammonium sulfate saturation was loaded on a DEAE-sepharose column. After anion exchange chromatography, peroxidase was purified 3.57- and 2-fold with 42% and 19% yield for control and 50 mM salt-stressed *A.orientalis*, respectively. This small increase for the number of purifications is probably due to a large amount of proteins non-bound on column material (Fig. 3A). The pooled and concentrated fractions of this step were loaded onto Sephacryl S-200 column for further purification (Fig. 3B). The

final purification and yield of peroxidase increased to 4.6 and 8%, 4.1 and 7% for control and 50 mM salt stressed *A. orientalis*, respectively.



(A)



(B)

Fig. 3. (A) Typical elution profile of DEAE fractions of *A. orientalis*. Elution was achieved at a flow rate of 0.5 mL/min with 3-mL fractions. Fractions 1-80 were pooled with 50 mM phosphate buffer (pH 7.0), 81-160 were pooled with 50 mM phosphate buffer (pH 7.0) + 50 mM NaCl, and 161-240 were pooled with 50 mM phosphate buffer (pH 7.0) + 100 mM NaCl. (B) Gel permeation column fractions. The column was equilibrated with 50 mM phosphate buffer (pH 7.0), at a flow rate of 0.2 mL/min and with 2-mL fractions.

Table 1. Purification profile of non-stressed and 50 mM salt-stressed *A. orientalis*

Sample	Volume (ml)	Activity (U)	Protein cont. (mg)	S.A (U/mg)	Purif. Fold	Act. Rec. (%)
Crude extract of non-stressed *A. orieantalis* explant	20.0	95.80	13.00	7.37	1.00	100.00
$(NH_4)_2SO_4$ precipitation	4.7	84.38	8.02	10.52	1.43	88.10
DEAE column	7.5	40.00	1.52	26.32	3.57	42.00
Sephacryl S-200 column	6.0	8.09	0.24	33.71	4.57	8.45
Crude extract of 50 mM salt stressed *A. Orientalis*	20.0	100.76	4.57	22.03	1.00	100.00
$(NH_4)_2SO_4$ precipitation	4.8	65.40	2.06	31.77	1.44	64.91
DEAE column	6.9	19.22	0.44	44.08	2.00	19.07
Sephacryl S-200 column	5.0	6.74	0.07	90.28	4.10	6.69

SDS-PAGE (Fig 4) results showed that these steps were sufficient for purification of enzyme, because all contaminated proteins in the crude extract and pooled fractions of DEAE-sepharose column were not observed in the final enzyme extract. Similar steps were performed by Yadav *et al*. [20] for purification of peroxidase from banana stem juice and Tzika and Sotiorudos [28] from olives.

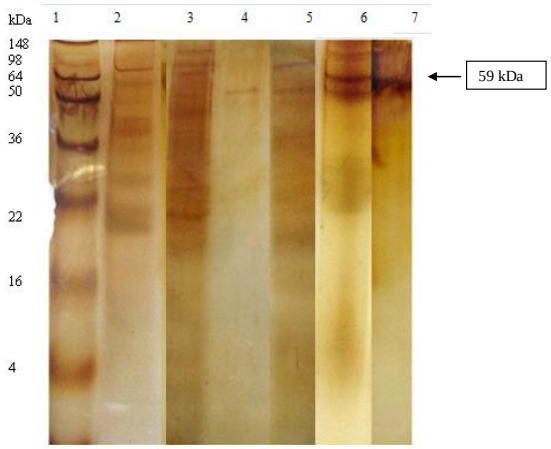


Fig. 4. Silver staining of SDS-PAGE gel. Electrophoresis was carried out at 100 mV for 10 min and at 120 mV for 120 min on 12% polyacrylamide gel system. For 1, 2, 3, 4, 5, 6 and 7 lanes; 15 µg protein; for 6 and 7 lanes 60 µg protein was applied. Lane 1: protein molecular mass marker; lane 2: crude extract of 50 mM NaCl -treated plant; lane 3: crude extract of control plant, lane 4: pooled DEAE fractions of control, lane 5: pooled DEAE fractions of 50 mM NaCl stressed enzyme, lane 6: purified and 4-fold concentrated 50 mM NaCl -treated plant, lane 7: purified and 4-fold concentrated control plant.

In spite of their higher purification number (8.37 and 52), the yield of purified enzyme was nearly 10 and 25 times lower (0.73% and 0.29%) than our results. Purification of recombinant plant peroxidase from *Pichia pastoris* was achieved by Spadiut *et al.* [27] and they reported 4.0 fold purification of the enzyme with 50% yield by anion exchange chromatography and 2.0 fold with 100% yield by size exclusion chromatography. Besides, same steps were carried out by Thogsook and Barrett [29] for purification of peroxidase from broccoli; according to their results these steps were not sufficient for the purified enzyme. But in our study peroxidase was purified with a single band on SDS-PAGE (Fig. 4). Molecular weight of peroxidase from *A.orientalis* was 59 kDa as determined by the SDS-PAGE (Fig. 4). Peroxidase was purified with TPP method by our group and this result is in good agreement with the previously finding of Yuzugullu *et al.* [30]. Peroxidases with various molecular weight have been reported in the range of 30-150 kDa. 86 kDa and 79 kDa peroxidase from *Pseudomonas* sp. and *Leptogium saturninum* were reported [26,31].

The purification of peroxidase from *A. orientalis* with relatively higher yield was introduced first in the current study, which may contribute to further industrial applications following its proper cultivation and reintegration in its natural habitats. Also, planned *ex situ* conservation studies on the plant would make us include a detailed salinity test of the soils.

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Conflict of interest: *The authors declare that there are no conflicts of interest.*

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ИЗСЛЕДВАНЕ НА *in vitro* СОЛЕВИЯ СТРЕС ВЪРХУ ПЕРОКСИДАЗНИЯ ЕНЗИМ НА *AMSONIA ORIENTALIS* И ПРЕЧИСТВАНЕТО НА ПЕРОКСИДАЗА ОТ НЕСТРЕСИРАНИ И СТРЕСИРАНИ СЪС СОЛ РАСТЕНИЯ

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(Резюме)

В това изследване се фокусирахме върху влиянието на *in vitro* солевия стрес върху промяната на ензимната активност на пероксидазата (POD) на *Amsonia orientalis*. Растението беше подложено на 25, 50, 75, 100, 125 и 150 mM соленост (NaCl) за 30 дни. Промяната на активността на POD беше проследена спектрофотометрично чрез оцветяване на активността. Нашите изследвания показват, че активността на POD не се променя драстично, особено когато растението е изложено на висока соленост. Специфичната активност на POD на нетретирано растение е изчислена като 0.74 (U/mg), докато най-високата специфична активност се наблюдава при 50 mM NaCl, а най-ниската специфична активност при 150 mM NaCl като 0.87 (U/mg) и 0.54 (U/mg). За по-нататъшен анализ на влиянието на солевия стрес върху активността на POD, ензимът от нетретирани и третирани с 50 mM NaCl *A. orientalis* беше пречистен. Подобен профил на пречистване беше наблюдаван при нестресирани и стресирани с 50 mM NaCl растения, като 4.57-кратно пречистване дава 8% добив, а 4.10-кратно пречистване - 7% добив. Молекулното тегло на пречистените ензими от двата екстракта беше определен чрез електрофоретични методи като 59 kDa. Тези резултати показват, че POD е силен защитен ензим срещу соленост, което се дължи на непроменената му активност, по-специално при 50 mM NaCl, при наблюдение на активността на POD в суров и пречистен екстракт.