

Full Length Research Paper

Expression, purification and characterization of a peroxidase from *Tamarix hispida*

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Peroxidase is one of the key enzymes of the cellular antioxidant defense system, which is mostly involved in the reduction of hydrogen peroxide. Here, a peroxidase gene, named ThPOD1 was isolated from a cDNA library, which was generated from root tissue of *Tamarix hispida* that was exposed to 0.4 M NaCl. The cDNA was subcloned in bacterial expression vector pET28a and expressed as N-terminal histidine tagged fusion protein in *Escherichia coli* (Rosetta gami). After induction with IPTG, a molecular weight of 42 kDa fusion protein was obtained and purified by Ni-NTA affinity chromatography. New Zealand white rabbit was immunized with the purified ThPOD1 protein and the antiserum was obtained. Western blot analyses showed that, the antibody reacted specifically to recombinant ThPOD1 protein. Therefore, the antibody was applied to detect expression of ThPOD1 protein in *T. hispida*, which was treated with 20% PEG and 100 μ M ABA, respectively. All treatments induced an increase in protein level of the ThPOD1 gene, especially the expression levels of ThPOD1 in roots were obviously higher than in leaves. This work established a good foundation for further study on the specific function of ThPOD1 under the condition of abiotic stress.

Key words: Peroxidase, prokaryotic expression, abiotic stress, *Tamarix hispida*.

INTRODUCTION

Molecular oxygen is essential for the existence of aerobic organisms including plants. Reactive oxygen species (ROS), which include the superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, are formed in all aerobic cells as by-products of normal metabolic processes (Asada, 1992). ROS are potentially harmful to the cell, as they can raise the level of oxidative damage through loss of cellular structure and function. However, cells possess antioxidants and antioxidative enzymes to interrupt the cascades of uncontrolled oxidation in cellular organelles (Lee et al., 2007). Among all the antioxidative

enzymes, peroxidase (POD) plays an important role in protecting organisms from being damaged by ROS both in standard conditions and under oxidative stress because they have an almost 1000-fold higher affinity for hydrogen peroxide when compared with catalases and their activities can be modified by different stress factors (Pekker et al., 2002; Foyer et al., 1997). Peroxidases have been extensively studied and show many attractive properties and potentially interesting applications in a number of field.

Peroxidases have been involved in a broad range of physiological processes such as lignification, senescence, auxin metabolism, the cross-linking of cell wall proteins, defense against pathogenic attack and a variety of abiotic stress tolerances (Hiraga et al., 2001; Passardi et al., 2005). Many workers have noted POD has a large family of isoenzymes in a variety of higher plants (Bakalovic et al., 2006). However, the inherent complexity of the physiological processes in which POD isoenzymes are involved makes understanding the

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Abbreviations: PCR, Polymerase chain reaction; ABA, abscisic acid; IPTG, isopropyl- β -D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

specific function of each of these enzymes rather difficult.

Therefore, we successfully cloned a peroxidase gene (named *ThPOD1*), which was generated from root tissue of *Tamarix hispida* that was exposed to 0.4 M NaCl (Li et al., 2009). In our previous studies, the transcript levels of the *ThPOD1* gene were shown to have been markedly increased under a variety of stress conditions, including cold, salt, drought and exogenous abscisic acid (Guo et al., 2010). These results indicated that, the *ThPOD1* gene may be involved in hydrogen peroxide-detoxification and thus, help to overcome the oxidative stress induced by abiotic and biotic stresses.

In this study, a full-length *ThPOD1* cDNA sequence demonstrated high expression in *E. coli* Rosetta-gami (DE3) and a highly specific and sensitive antibody against ThPOD1 was produced. The polyclonal antibody can detect the expression level of *ThPOD1* gene in *T. hispida* plants. These results provide a substantial base for understanding the detail function of peroxidase in abiotic stresses in future.

MATERIALS AND METHODS

Construction of expression vector pET28a(+)-ThPOD

To clone *ThPOD1* into an expression vector, its open reading frame was amplified with gene-specific primers containing *Bam*H I and *Xho* I sites (underlined) (upstream primer: 5'-ATCGGGATCCCAAGCACAAAAAGCCTACCGCCG-3', downstream primer: 5'-CGATCTCGAGTGTATTCTGACCGCCTTCCATACTC-3') and cloned into pET28a(+). The cycling conditions were as follows: 94°C for 2 min; followed by 29 cycles of 94°C for 30 s, 56°C for 30 s and at 72°C for 60 s, with a final extension at 72°C for 7 min. The amplified product was gel purified and digested by *Bam*H I and *Xho* I, then cloned into the corresponding restriction sites of the pET28a(+) (Novagen, USA) vector. *E. coli* JM109 was transformed with the recombinant vector by heat shock and cultured at 37°C on LB agar with kanamycin (100 µg/ml) for selection of transformed clones. The recombinant plasmids were named pET28a(+)-*ThPOD*. The expression construct was checked for inframe fusion by restriction enzyme digestion and DNA sequencing.

Induction and expression of recombinant proteins

After sequencing to ensure inframe insertion, the recombinant plasmid was transformed into *E. coli* Rosetta gami and incubated in LB medium at 37°C with shaking at 220 rpm. When absorbance at 600 nm reached 0.6, expression of the recombinant ThPOD1 protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). One milliliter samples were taken every hour, up to 7 h after induction and the induced bacterial cells were pelleted by centrifugation at 10,000 g for 5 min, resuspended in 100 µl of 1×SDS loading buffer, boiled for 5 min and analyzed by SDS-PAGE. The *E. coli* Rosetta gami transformed with the pET28a(+) vector was used as a control group.

Purification of ThPOD1 and polyclonal antibody preparation

The recombinant ThPOD1 protein was purified using His-Bind column kits as described by the manufacturer (Novagen, Madison, WI). The protein concentration was analyzed using the Bradford

technique and total bacterial protein was resolved on 12% SDS-PAGE. The ThPOD1 fusion protein was cut out of the gel, ground into powder and lysed in physiological salt solution. The solution was mixed with Freund's complete adjuvant at a ThPOD1 concentration of 1 to 2 mg/ml and an adult rabbit was subcutaneously injected with 0.5 ml every week for a total of four weeks. About 35 days later, the rabbit was sacrificed and the polyclonal antibody was obtained from the total blood.

Western blotting analysis

To identify the expression of ThPOD1 protein, roots and leaves extracts of *T. hispida* seedlings were lysed in electrophoresis sample buffer. Protein concentrations were determined using Bradford protein assay (Biorad). Each sample, equivalent to 10 mg total protein was run on 12% SDS-PAGE and subsequently, transferred to PVDF membrane (Millipore). The blotting membrane was blocked with 5% dry milk for 1 h and then, the membrane was incubated at TBST buffer containing 1.0% milk with antiserum (1:2000) from rabbit at 4°C overnight. After washing three times for about 30 min, the membrane was further incubated for 1 h with 1:5000 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (Sino-American Biotechnology Company). After three times of washing about 10 min each in TBST buffer detection was performed using ECL staining. The specificity of polyclonal antibody was evaluated by western blotting analysis.

Abiotic stress tolerance assay in *T. hispida*

T. hispida seedlings were planted in a mixture of turfy peat and sand (2:1 v/v) in a greenhouse with 75% relative humidity and a constant temperature of 24°C. In order to detect expression of ThPOD1 protein under various conditions, 3 month old *T. hispida* seedlings were treated with 20% polyethylene glycol (PEG) and 100 µM abscisic acid (ABA). Leaf and root tissues of the seedlings were harvested at 0, 6, 24, 48 and 72 h after each treatment and immediately stored in liquid nitrogen and stored at -70°C for protein isolation. Total protein samples were extracted from control and different treated *T. hispida* tissues following the method of Lu et al., 2008.

RESULTS

Cloning and expression of *ThPOD*

The gene encoding ThPOD1 was amplified by polymerase chain reaction (PCR) using ThPOD1-specific primers and *T. hispida* root cDNA library as a template and cloned into the expression vector pET28a(+) in order to allow expression as a fusion protein in *E. coli* Rosetta-gami (DE3) (data not shown). SDS-PAGE analysis of the whole lysate from IPTG-induced cells, when compared with that from the non-induced control (Figure 1, lane 4) showed an additional band with the expected molecular weight of recombinant ThPOD1 protein (42 kDa; Figure 1, lane 5). Moreover, the expression levels of recombinant ThPOD1 protein increased with the increasing of induction time (Figure 1).

Soluble analysis and purification of recombinant protein

Cells from the induced culture were suspended in PBS

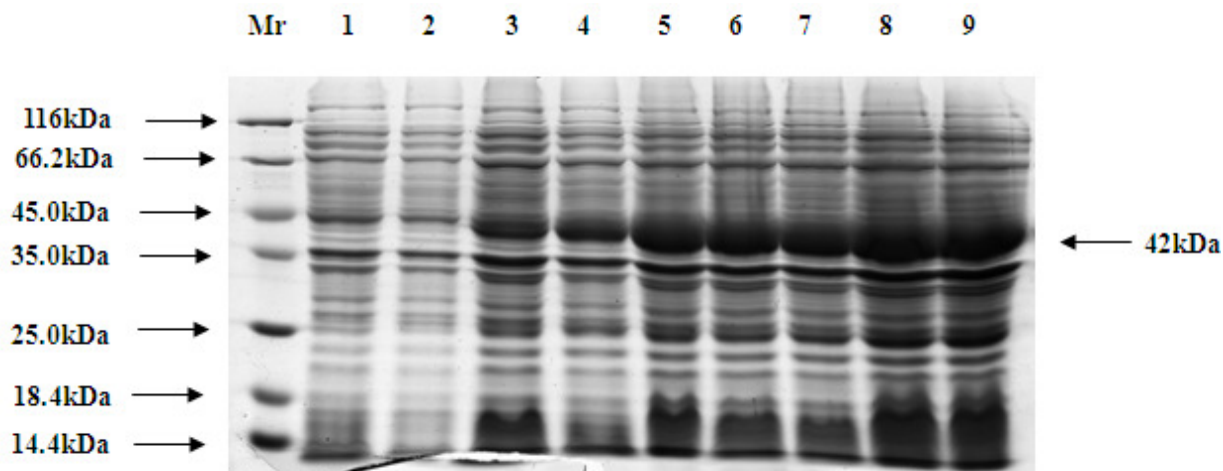


Figure 1. SDS-PAGE (12%) analysis of recombinant ThPOD1 protein expression in *E. coli* Rosetta gami (Coomassie blue staining). Lane 1 (M), Protein marker; lane 2 (L1), whole cell lysate of Rosetta gami *E. coli* cells containing the empty vector pET28a(+) without IPTG induction; lane 3 (L2), whole cell lysate of non-induced Rosetta gami *E. coli* cells containing the plasmid pET28a(+)-ThPOD1; lane 4 to 10 (L3-9), whole cell lysate of Rosetta gami *E. coli* cells containing the plasmid pET28a(+)-ThPOD1 with IPTG induction for 1, 2, 3, 4, 5, 6 and 7 h, respectively. The bands corresponding to the products of ThPOD1 are indicated by an arrow (approximately 42 kDa).

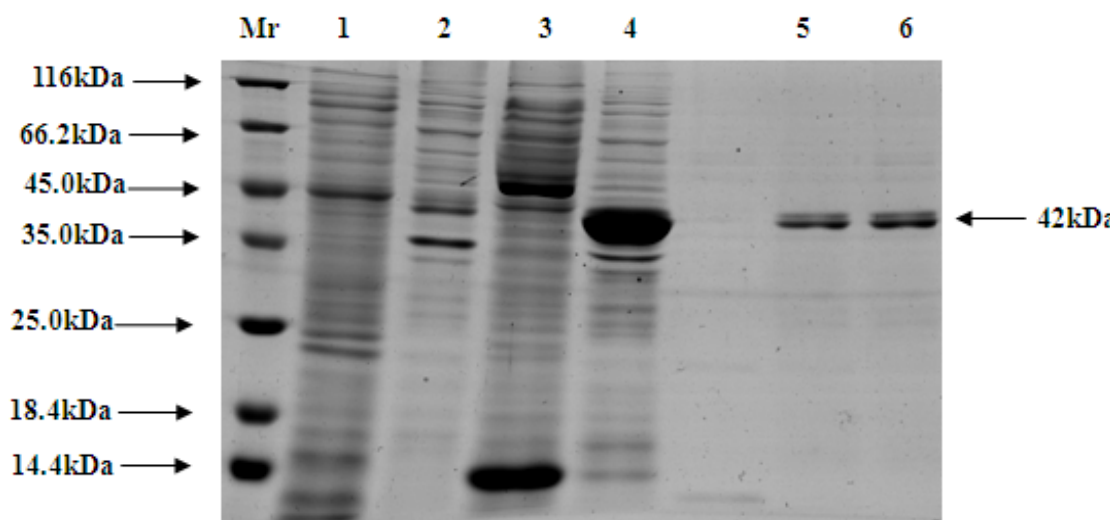


Figure 2. SDS-PAGE analysis of recombinant ThPOD1 protein after ultrasonic fragmentation and purified protein. Lane 1 (Mr), Protein marker; lane 2 (L1), whole cell lysate of *E. coli* cells containing the empty vector pET28a without IPTG induction; lane 3 (L2), whole cell lysate of induced *E. coli* cells containing the plasmid pET28a; lane 4 (L3), the soluble fractions of *E. coli* cells containing the pET28a-ThPOD1 with IPTG induction; lane 5 (L4), the insoluble fractions of *E. coli* cells containing the plasmid pET28a-ThPOD1 with IPTG induction; lane 6,7 (L5,6), Purified protein. The bands corresponding to the products of ThPOD1 are indicated by an arrow (approximately 42 kDa).

buffer, sonicated, clarified and analyzed by SDS-PAGE (Figure 2). The recombinant ThPOD1 protein remained associated to the insoluble fraction after cellular disruption, most likely as inclusion bodies as demonstrated in Figure 2 (lane 4). To recuperate the recombinant protein, the pellet was washed with a mildly denaturing buffer (1.5% sodium lauryl sulfate) (SLS), to

remove contaminating soluble cytoplasmic proteins and then with highly denaturing (8 M urea) to disrupt the inclusion bodies. This treatment resulted in dissolution of the aggregated recombinant protein, which could then, be applied to the His-Bind column. The soluble fraction was purified using His-Bind column kits as described by the manufacturer and produced single bands on SDS-PAGE

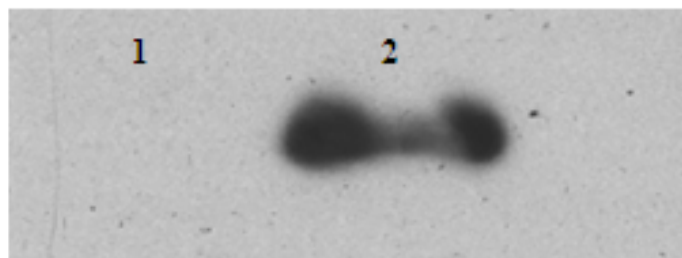


Figure 3. Western blot analysis using specific antibody against the purified ThPOD1 protein. Lane 1 (N) negative control that is, protein sample from *E. coli* cells containing empty vector (pET28a); lane 2, sample from *E. coli* cells containing pET28a-ThPOD1. The bands corresponding to the products of ThPOD1 are indicated by an arrow (approximately 42 kDa).

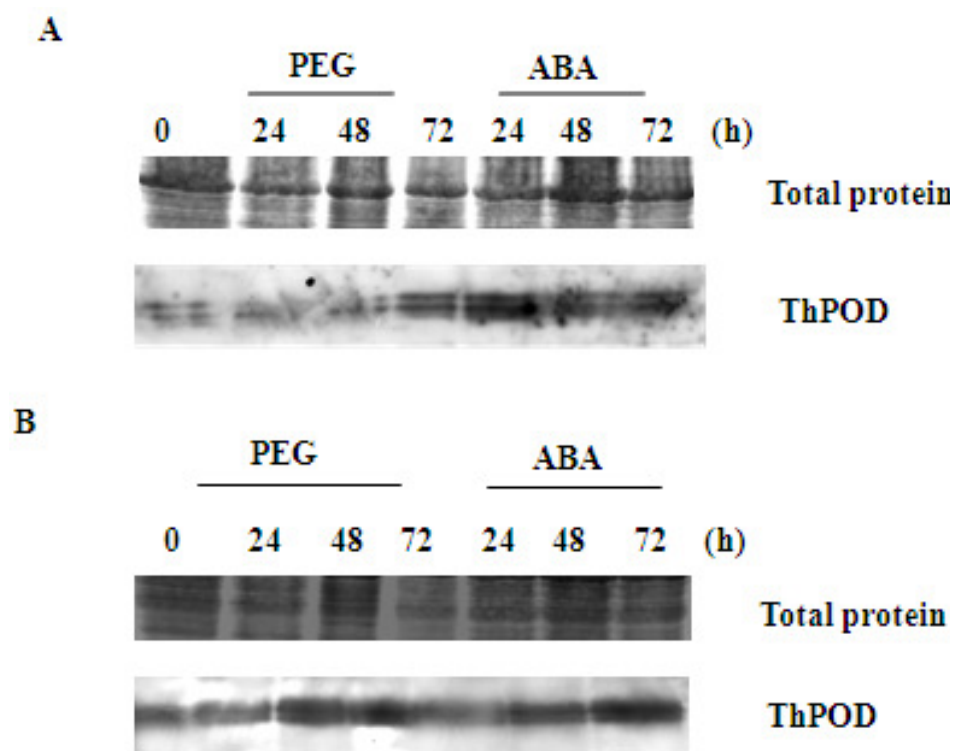


Figure 4. Fluctuation of the ThPOD1 protein level under PEG (20%) and ABA (100 μ M) treatment in *T. hispida*. A, In *T. hispida* leaves; B, in *T. hispida* roots. Total protein was extracted from *T. hispida* leaf and root tissues at time points indicated. The antibody against ThPOD1 was produced by immunizing rabbit and used at a dilution of 1:2000. The dilution of secondary antibody was used at 1:5000. About 30 μ g of protein was analyzed by the dye-binding assay.

(Figure 2, lanes 5, 6).

To determine whether the recombinant proteins were successfully translated, immunoblotting was conducted using anti-ThPOD1 antibodies. ThPOD1 were detected at band sizes of ~42 kDa, which are the expected molecular weights of the translated products of ThPOD1 cDNA (Figure 3).

Expression of ThPOD in *T. hispida*

The expression of ThPOD1 was observed under ABA and drought (20% PEG) stresses in different *T. hispida* tissues (Figure 4). The 20% PEG and 100 μ M ABA treatment induced an increase in protein level of the ThPOD1, especially the expression level of ThPOD1 in

roots which were obviously higher than in leaves. The result of ThPOD1 expression analysis was in accordance with result of real-time quantitative PCR data analysis (Guo et al., 2010). The expression level increase of the ThPOD1 in leaves could be observed with the ABA and PEG treatments after 48 and 72 h, respectively (Figure 4a). These results showed that the ThPOD1 gene was likely to be a regulation factor in ABA and PEG signaling pathways.

DISCUSSION

Peroxidase plays a key role in the detoxification of photoproduced hydrogen peroxide. At present, research on peroxidases mainly manifests in herbaceous plants, such as *Arabidopsis* (Valerio et al., 2004), rice (Li et al., 2000), soybean (Caldwell et al., 1998), and grape (Lin et al., 2006), but few people study the woody plants. However, woody plants are different from herbaceous plants in many respects, including growth, development, physiology, morphology and so on. Moreover, tolerance mechanisms of woody and herbaceous plants are different. Therefore, a gene encoding peroxidase (named *ThPOD1*) was isolated from a NaCl-stress root cDNA library of *T. hispida*, which has a strong resistance to abiotic stresses including drought, salinity and high temperature, making it an ideal species to reverse environmental degradation. Consequently, it is a valuable species for stress-related genes cloning and research on the mechanism of stress resistance (Wang et al., 2006).

In the present study, we have successfully obtained the novel gene (named *ThPOD1*) related to peroxidase, then made it expressed in *E. coli* Rosetta-gami (DE3) and analyzed its peculiarity of expression and biological function. After injecting adult rabbit with recombinant ThPOD1 protein, polyclonal antibody was produced. Western blot with this antibody were used to detect the expression level of ThPOD1 protein in *T. hispida* plants. To further confirm the function of the *ThPOD1*, we will transform this gene into *Populus* (*Populus davidiana* × *Populus bolleana*) cultivar as the gene of interest resistant to the oxidative stress for next study.

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