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Purification of phenylalanine ammonia lyase (PAL) and peroxidase (POX) enzymes obtained from lucerne (*Medicago sativa* L. cv. Vertus) following inoculation with *Verticillium albo-atrum*

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ABSTRACT:

Phenylalanine ammonia lyase (EC 4.3.1.24, PAL) and peroxidase (EC 1.11.1.7, POX) enzyme activities were determined in seedlings of lucerne (*Medicago sativa L. cv. Vertus*) after the inoculation of *V. albo-atrum* (V2, a weak pathogenic isolate to lucerne). PAL and POX activities of lucerne inoculated with V2 were found significantly higher than that of the control group after 48 h inoculation. Partial purification of enzymes with 0-50% $(NH_4)_2SO_4$ precipitation in control groups resulted in 2.74 and 2.42 fold increases. On the other hand, 1.78 and 1.74 fold increases in PAL and POX activities, respectively were evident in V2-inoculated plants. This study showed that the purification of the enzymes with little effort enabled us to see the differences between the treatments clearly. This type of work could be suggested where the protein concentration of the sample is low or a time-scale experiment is to be carried out to see the enzyme changes or to characterize the pathogen-related proteins.

Keywords:
Ammonium sulphate precipitation, *Verticillium*, lucerne, PAL, POX.

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INTRODUCTION

V. albo-atrum is a major problem in lucerne, cotton and vegetable grown areas. Various cultivars have different responses to the various isolates of *V. albo-atrum.* For example, various levels of resistance to *V. albo-atrum* were found to vary from one plant to another within the same cultivar (personal communication with Prof. Barbara W. Pennypacker, USA). Defense responses such as peroxidase (EC 1.11.1.7, POX) and phenylalanine ammonia lyase (EC 4.3.1.24, PAL) have been reported to change during infecion stage. These enzymes are a good criteria to measure the responses of the crop plants. However, during infection process, it is difficult to differentiate the control plants from the infected ones by measuring the enzyme activities in early stages through crude extract of plants since the level of enzymes in both control and infected groups show similar patterns due to similar protein profiles or similar molecular weights, therefore, it is difficult to judge about the defence responses at this stage. Under these circumtances, it would be better to purify the proteins and measure their enzymatic activities if very low concentration of proteins exist.

Peroxidase (EC 1.11.1.7, POX) enzymes, in general, are divided into three groups differing in molecular weight and in absorption spectra including ascorbate type, fungal derived and plant derived enzymes (Das *et al.,* 2011). Of them, plants are the richest sources of peroxidases and primarily found in roots and leaves of higher plants, which play important roles in lignifying cell walls after infection, and thus increasing plant resistance (Tang, 2001; Dikilitas, 2003; Boka and Orban, 2007; Renugadevi *et al*., 2011). Plant derived peroxidase enzymes were isolated, purified and characterized from a various plant species including horseradish, soybean, beet, tomato, carrot, wheat, apricot, pears, bananas and tea (Silva and Franco, 2000; Lavery *et al*., 2010). Among them, horseradish peroxidase was the most commonly used enzyme for the standard preparation and characterization of the protein profiles. Isolation of peroxidase enzymes from microorganisms also led to increased research interest in this area. Many fungal agents have been known to produce peroxidase enzymes (Tsujimura *et al.,* 1994; Hamid and Rehman, 2009). The role of peroxidase in metabolism is not clear due to the large number of reactions involved and the considerable number of isoenzymic species (Kim and Lee, 2005) although it is clearly involved in the enzymatic reactions for removal of H_2O_2 and oxidants in cells.

Phenylalanine ammonia lyase (EC 4.3.1.24, PAL), synthesized by a multistep phenylpropanoid biosynthesis pathway, is the entry-point enzyme catalysing the deamination of phenylalanine to transcinnamic acid. The induction of PAL is associated with the accumulation of secondary pruducts such as flavonoids and lignins involved in defence responses providing specific means to protect the plant from stress. PAL is also involved in phenolics synthesis, including phytoalexins and suberin (Tang, 2001; Agrios, 2002). Studies with several different species of plants showed that PAL activity increases with the biotic and abiotic stresses (Hammerschmidt, 1999). The resistance of plants to the pathogen may depend on the speed and the extent of synthesis of the enzymes. Therefore, PAL shows the level of resistance of the organism against stress. For example, Dunn *et al.,* (1998) reported that after 30 days of high salinity (0.1 M NaCl), citrus plant grew more slowly and produced lower PAL activity and as a result of that they became more susceptible to nematode attack (*Tylenchulus semipenetrans*). Similarly, Dikilitas (2003) stated that increase in PAL activity after the inoculation of elicitor derived from *Verticillium albo-atrum* on cell cultures of lucerne *(Medicago sativa cv. Maris Kabul)* decreased when the culture was grown in Murashige and Skoog (M&S) medium containing 50 mM NaCl. As a result, the resistant cultivar became more susceptible to the effect

of pathogen when exposed to salinity.

Partial prufication of enzymes was reported by many research people (Altunkaya and Gökmen, 2011; Hu *et al*., 2012) to characterize and further investigate their properties. In this study, partial purification of the enzymes obtained from the lucerne plants after the pathogen attack was achieved by $(NH_4)_2SO_4$ precipitation method to differentiate the enzyme levels at similar end-points with little effort. Via this method, we would then be able to see the differences between the severity of stress factors such as salinity and the pathogens in crop plants by demonstrating their combined and seperate effects of each stress factor. Data of this study would also enable us to establish other purification systems for further purifications.

MATERIALS AND METHODS

Inoculation of plants

V. albo-atrum, isolate V2 were isolated from tomato plants and maintained in PDA and Dox agar media. The fungal culture was originally obtained from the culture collection of Dr Chris J. Smith (University of Wales, Swansea-UK). The isolate was characterized as a weak pathogen according to studies carried out by Dikilitas (1997 and 2003). Lucerne (*Medicago sativa cv. Vertus*) plants were inoculated with the root dipping method with the spores of *V. albo-atrum,* V2 ($1x10^7$ conidia ml^{-1}). For this, plants were removed from pots and the soil was removed by shaking the plants gently, followed by washing in tap water. The root of the plants were then placed in the spore suspension for 10 min. The inoculated plants were replanted using the same soil. Distilled water was used for the control plants.

Preparation and purification of tissue homogenate for POX

Ten grams of fresh lucerne leaves were homogenized at 4°C with 100 ml of 50 mM Na phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant obtained at this stage was designated as crude extract.

The crude extract was then partially purified by adding solid ammonium sulphate (calculated through the web site http://www.encorbio.com) to give 0-50% saturation and the precipitate was collected by centrifugation at 10,000 g for 90 min at 4°C. The precipitate was then suspended in about 10 ml of the same buffer which was used for homogenization and dialyzed overnight at 4°C against 40 mM Na phosphate buffer (pH 7.0) to remove ammonium sulphate and other minerals. The supernatant at this stage was designated as purified enzyme.

The activity of POX in the supernatant was determined before and after partial purification.

Determination of POX activity

POX activity was assayed with guaiacol according to the modified method of Meriga *et al.,* (2004). A 3 ml reaction mixture containing 13 mmol l^{-1} guaiacol, 5 mmol l^{-1} H_2O_2, 50 mmol l^{-1} Na phosphate buffer (pH 6.5) and 500 ml of enzyme solution was prepared. Oxidation of guaiacol was followed by the increase of absorbance at 470 nm, after initiating the reaction with H_2O_2. One unit of POX activity was defined as 0.01 ΔA_{470} per min.

Preparation and purification of tissue homogenate for PAL

To assess the early defence responses of lucerne leaves, PAL activity was measured. Ten grams of fresh leaves were homogenized at 4°C with 100 ml of 50 mM Tris-HCl, pH 8.4, containing 4 mM Na_2EDTA, 10 mM mercaptoethanol, 2 mM ascorbic acid and 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 g acid-washed sand with a cold pestle and mortar. The homogenate was filtered through two layers of wet Mira Cloth (Calbiochem), and the resulting filtrate was centrifuged (20,000 g, for 20 min at 4°C).

The supernatant was then dialyzed overnight

against two litres of dialysis buffer (50 mM Tris-HCl, pH 8.4 containing 10 mM mercaptoethanol, 4 mM Na_2EDTA and 0.5 mM PMSF).

The crude extract was then partially purified as in that of POX except that the buffer was changed into Tris-HCl (pH 8.8).

Determination of PAL activity

PAL activity was assayed using a method described by Bolwell *et al.,* (1985) with slight modifications (Dikilitas, 2003). The reaction mixture contained the following components: 1.5 ml of 50 mM Tris-HCl (pH 8.4) containing 4 mM Na_2 EDTA, 10 mM mercaptoethanol, 5 mM ascorbic acid and 1µM PMSF and 1 ml of 10 mM L-phenylalanine (final concentration). The reaction was then started by the addition of enzyme extract (0.5 ml) containing approximately 500 mg protein to the mixture, which was incubated at 40°C for two hours. Assays were performed in duplicate and the control incubation was prepared using D-phenylalanine (10 mM final concentration) in place of the L-isomer. Changes in absorbance was recorded at 30 min intervals at 290 nm using UV-1700 Shimadzu Spectrophotometer.

Activities have been calculated from the molar absorption coefficient of trans-cinnamic acid at 290 nm, which was determined to be 10, 900 litre mol^{-1} cm^{-1} under the conditions of assay. PAL activity was expressed as: nmol cinnamic acid mg^{-1} protein h^{-1}. The change in absorbance was converted to nmol of trans-cinnamic acid using the following equation;

$$A = Ecl$$

A : Absorbance at 290 nm.

c : Concentration of cinnamic acid in Moles/L.

l : Path length of light (1 cm).

E: The molar absorption coefficient of cinnamic acid at 290 nm, which was determined to be 10,900 litres mol^{-1} cm^{-1}

The rate of formation of trans-cinnamic acid was taken as a measure of enzyme activity. One unit of PAL was defined as 0.01 A at A_{290} per hour, which was calculated as 2.74 nmol of trans-cinnamic acid.

Protein determination

Protein in the samples was determined in a Coomassie blue dye-binding assay by the method of Bradford (1976) with standard curves prepared using bovine serum albumin (BSA, Sigma).

Chemicals

All chemicals were from Sigma and Fisher Co. except where specified.

RESULTS AND DISCUSSION

POX and PAL enzymes from lucerne plants inoculated with the weak pathogen *V. albo-atrum*, isolate V2 showed higher activities than that of control group. The activities from control and inoculated plants in respect to both enzymes were found much higher following purification with ammonium sulphate precipitation, 0-50% (Table 1). Ammonium sulphate precipitation was done by using the finely ground ammonium sulphate. The rich form of proteins and enzymes after precipitation were subjected to dialysis to remove extra salts and minerals. Civello *et al.,* (1995) reported an increase in POX activity in strawberry fruit following purification of ammonium sulphate. Similarly, Zia *et al*., (2011) stated that 1.16 and 4.14 fold purification was achieved in apple and orange seeds, respectively following ammonium sulphate precipitation. Again, Mohamed *et al*., (2008) reported the peroxidase activity of orange and apple seed extracts were increased as compared to crude enzyme extract up to 30.64 and 8.34 fold with their specific activity of 18.16 and 9.20 U/mg, respectively following purification.

The increase in enzyme activities were reported in many plant & abiotic and plant & microorganism interactions (Chutia *et al.,* 2012). An increase in enzyme activities are regarded as a general defense response of the organism to the effect of stress (Dikilitas, 2003). In our study, the effect of *V. albo-atrum,* isolate V2 led to

Table 1. Purification and activities of POX and PAL of lucerne inoculated with *V. albo-atrum*, isolate V2.

Enzymes-Treatments	Purification Steps	Activity (EU ml^{-1})	Total Volume (ml)	Protein Content (mg ml^{-1})	Total protein (mg)	Total activity (EU)	Specific Activity (EU mg^{-1})	Yield (%)	Fold purification
POX									
Control	Crude	16	90	0.50	45.0	1440	32	100	1
	$(NH_4)_2SO_4$	48	11	0.62	6.82	528	77.42	37	2.42
V2	Crude	136	92	0.49	45.08	12512	277.55	100	1
	$(NH_4)_2SO_4$	265	11	0.55	6.05	2915	481.81	23	1.74
PAL									
Control	Crude	22	90	0.45	40.5	1980	48.88	100	1
	$(NH_4)_2SO_4$	87	11	0.65	7.15	957	133.84	48	2.74
V2	Crude	150	94	0.47	44.18	14100	319.14	100	1
	$(NH_4)_2SO_4$	329	12	0.58	6.96	3948	567.24	28	1.78

increase in activities of enzymes such as POX and PAL after 48 h of inoculation. The increased activities of enzymes and their mechanisms which led to various defense responses were discussed in detail in the works of (Tang, 2001; Eldeen *et al.,* 2010). The main issue here is to evaluate the purification of enzymes by determining their activities. In this study, the purification procedure allowed us POX and PAL to be purified 2.42- and 2.74-fold with 37 and 48% recovery, respectively as compared to those of the crude extracts of controls. Similarly, POX and PAL enzymes were purified 1.74- and 1.78-fold with 23 and 28% recovery, respectively in V2-incoulated plants as compared to their crude extracts. In POX enzyme, the ratio between control and V2 was 0.11 (32/277) in crude extracts while this ratio increased up to 0.16 (77/481) in ammonium precipitated extracts. Similar case was also observed in PAL in which the ratio between control and V2 was 0.15 (48/319) in crude extracts while the ratio between control and V2 was 0.23 (133/567) in precipitated extracts. Although the ratios between control and V2 showed similar trends in both enzymes in respect to their activities before and after purification, however, purification enabled us to see the differences more clearly through the higher absorbance values. This type of work could well be suggested in similar absorbance values obtained in which the control and treatment groups do not differ from each other.

CONCLUSIONS

In this work, POX and PAL enzymes from lucerne leaves were extracted and partially purified with ammonium sulphate precipitation. According to the results of analysis, the purified enzymes gave higher absorbance values and clear cut-offs between treatments were evident when compared to those of crude extracts. Purification could be helpful to find out the differences between control and treatment groups as well as in determining the primary and secondary infections in which the induced enzyme activities might not differ in crude extracts.

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REFERENCES

Agrios GN. 2002. Plant Pathology. Academic Press. 4th ed.

Altunkaya A, Gökmen V. 2011. Purification and characterization of polyphenol oxidase, peroxidase and

lipoxygenase from freshly cut lettuce *(L. sativa).* Food Technol. Biotechnol., 49(2):249-256.

Boka K, Orban N. 2007. New aspect of H2O2 signaling. Plant Signal Behav., 2:498-500.

Bolwell GP, Bell J, Cramer J. Schuch W, Lamb CJ, and Dixon RA. 1985. L-phenylalanine ammonia-lyase from *Phaseolus vulgaris. European J. of Biochem.*, 149:411-419.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 136:175-179.

Chutia J, Borah SP, Tanti B. 2012. Effect of drought stress on protein and proline metabolism in seven traditional rice (*Oryza sativa Linn*.) genotypes of Assam, India. Journal of Research in Biology, 2(3):206-214.

Civello PM, Arting GA, Chaves AR, Anan MC. 1995. Peroxidase from strawberry fruite by partial purification and determination of some properties. J. Agri. Food Chem., 43(10):2596-2601.

Das MK, Sharma RS, Mishra V. 2011. A novel cationic peroxidase (VanPrx) from a hemi-parasitic plant (*Viscum angulatum*) of western ghats (India): Purification, characterization and kinetic properties. J. Mol. Catal. B: Enzyme, 71:63-70.

Dikilitas M. 2003. Effect of salinity & its interactions with *Verticillium albo-atrum* on the disease development in tomato (*Lycopersicon esculentum* Mill.) and lucerne (*Medicago sativa* & *M. media*) Plants. Ph.D. Thesis, University Of Wales, Swansea, UK.

Dikilitas, M. 1997. A study of interactions between the phytopathogenic fungus *Verticillium albo-atrum* (Reinke & Berth) and tomato (*Lycopersicon esculentum*) and various cultivars of lucerne (*Medicago sativa L.* & *M. media*). MPhil thesis, University of Wales, Swansea, UK.

Dunn DC, Duncan LW, Romeo JT. 1998. Changes in arginine, PAL activity, and nematode behaviour in salinity-stressed citrus. Phytochemistry 49(2), 413-417.

Eldeen D, Radwan M, Fayez KA, Mahmoud SY, Lu G. 2010. Modifications of antioxidant activity and protein composition of bean leaf due to Bean yellow mosaic virus infection and salicylic acid treatments. Acta Physiol Plant., 32:891-904.

Hamid M, Rehman K. 2009. Potential applications of peroxidases. Food Chem., 115:1177-1186.

Hammerschmidt R. 1999. Induced disease resistance: how do induced plants stop pathogens? Physiol. Mol. Plant Pathol., 55:77-84.

Hu Y, Wu J, Luo P, Mo Y. 2012. Purification and partial characterization of peroxidase from lettuce stems. Afr. J. Biotechnol., 11(11):2752-2756.

Kim SS, Lee DJ. 2005. Purification and characterization of a cationic peroxidase Cs in *Raphanus sativus.* J. Plant. Physiol., 162:609-617.

Lavery CB, Macinnis MC, Macdonald MJ, Williams JB, Spencer CA, Burke AA, Irwin DJ, D'Cunha GB. 2010. Purification of peroxidase from Horseradish *(Armoracia rusticana)* roots. J. Agric. Food Chem., 58: 8471-8476.

Meriga B, Reddy BK, Rao KR, Reddy LA, Kavi Kishor PB. 2004. Aluminium-induced production of oxygen radicals, lipid peroxidation and DNA damage in seedlings of rice (*Oryza sativa*). J. Plant Physiol., 161:63 -68.

Mohamed SA, El-Badry MO, Drees EA, Fahmy AS. 2008. Properties of a Cationic Peroxidase from citrus jambhiri cv. Adalia. Appl. Biochem. Biotechnol., 150: 127-137.

Renugadevi R, Ayyappadas MP, Preethy PH, Savetha S. 2011. Isolation, screening and induction of mutation in strain for extra cellular lignin peroxidase producing bacteria from soil and its partial purification. Journal of Research in Biology, 4:312-318.

Silva ME, Franco TT. 2000. Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. J. Chromatogr. B 743: 287-294.

Tang M. 2001. Elicitor-Induced Defence Responses and Mechanisms of Signal Transduction in *Medicago sativa L.* Ph.D. Thesis, University of Wales, Swansea.

Tsujimura H, Takaya M, Katano K, Matsumoto N, Park YS, Okabe M. 1994. Scaleup of peroxidase production by *rthromyces ramosus* based on analysis of fluid velocity distribution. J. Ferment. Bioeng., 77:650-654.

Zia MA, Kousar M, Ahmed I, Iqbal HMN, Abbas RZ. 2011. Comparative study of peroxidase purification from apple and orange seeds. Afr. J. Biotechnol., 10(33): 6300-6303.