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Extraction, purification and characterization of peroxidase from *Vitis vinifera* wastes

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ABSTRACT

The proper consumption of plant wastes could not only provide a possible source of natural products, but it also is an environmental friendly research. The aim of this study was to use grape wastes as a source of peroxidase. In practice, one isoenzyme of peroxidase (POD_1) was partially purified from *Vitis vinifera* wastes, the plant which is widely harvested in Iran. The activity of this novel peroxidase was determined using guaiacol as its substrate. The new peroxidase was partially purified and its kinetic parameters determined. The values of K_m and V_{max} of peroxidase for guaiacol were 83.2 mM and 0.35 M/min respectively. Optimum pH and temperature were determined for guaiacol to be 6.2 and 60°C respectively. According to SDS-PAGE results, the molecular weight of isozymes was 38-40 KD. The results indicate that agricultural leftovers from *Vitis vinifera* are a considerable source for a peroxidase with reliable kinetic behaviors.

Key words: *Vitis vinifera*, plant wastes, peroxidase, kinetic parameter, guaiacol.

INTRODUCTION

Due to the difficulty in synthesis and high prices of many important industrial enzymes, they are often extracted from various natural sources (Badan-Ara Marzdashti *et al*. 2018) with high purity rates. Peroxidases (PODs) are groups of enzymes belonging to the oxidoreductases family which catalyze oxidation reactions using hydrogen peroxide as an electron acceptor (Conesa *et al*. 2002; Cardinali *et al*. 2011). They can be found in almost all living organisms and have important biological roles in different organisms (Saboora *et al*. 2010, 2012). Despite the crucial role of oxygen for all aerobic organisms, generation of reactive oxygen species (ROS), as by-products of normal metabolic processes, could be quite harmful to cells (Hancock *et al*. 2001). In a healthy person, ROS are needed for normal functions of cells such as gene expression, defense against pathogens and cell growth (Guo *et al*. 2012). However, overproduction of ROS could cause oxidative damages (Apel *et al*. 2004). This can lead to modification of macromolecules such as proteins and induction of apoptotic pathway (Wall *et al*. 2012).

Peroxidases may contain or lack heme group in their structures (Battistuzzi *et al*. 2010). Most of heme peroxidases are divided into two superfamilies, the plant, fungal and bacterial peroxidases with the second family being mammalian enzymes. The first superfamily includes three classes I, II and III based on structural similarities and amino acid sequence. All of the three classes contain Fe (III) protoporphyrin-IX as their prosthetic group (Mathé *et al*. 2010; Zámocký *et al*. 2015). Secretory plant peroxidases belong to class III and have important role in cellular processes including control of ROS production (Welinder *et al*. 1993). They are involved in many processes such as biosynthesis of lignin (Barceló *et al*. 2004), suberin (Bernards *et al*. 2004), cross link between cell wall proteins (Welinder *et al*. 1993), plant growth and development (Saboora *et al*. 2012), wound healing (Cosio *et al*. 2009), protection against pathogens (Almagro *et al*. 2008) and programmed cell death (Circu *et al*. 2010). They are also used for biotransformation of organic molecules (Adam *et al*. 1999; Liu *et al*. 2007) and removal of phenolic compounds and peroxides from industrial wastes (Bansal *et al*. 2012). A more common use

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of peroxidases is in diagnostic kits for determination of some biochemicals such as glucose, uric acid and cholesterol (Agostini *et al*. 2002). In ELISA test, peroxidase is most frequently used as enzyme-labeled antibody (Acharya *et al*. 2013). The widespread use of peroxidase in biotechnology, medicine and industry has encouraged scientists and industries to find novel enzymes from this family. For many years horseradish peroxidase (HRP), has been the only commercial source of peroxidase (Bansal *et al*. 2012). However, many researchers are investigating new sources (Murakami *et al*. 2007; Şişecioğlu *et al*. 2010; Cardinali *et al*. 2011; Saboorsi *et al*. 2012; Gui *et al*. 2012). Plant wastes including stems, leaves, barks, dead flowers and leftovers from fruit processing industries are natural sources of many important biochemical compounds. If used in a proper way, they could be valuable sources of low price natural compounds such as some useful enzymes. This could also provide a logical strategy to make best of them and control environmental pollution from their deterioration. Depending on the plant type, the utilized part and the extraction procedure, the wastes may contain inorganic and organic secondary metabolites (Afsharnezhad *et al*. 2017). For example, the content of iron can help to relieve anemia and have the power to counteract calcium loss leading to bone strengthening. Research on the environmental pollutants that can be recycled has improved during the last decade (Alizadeh *et al*. 2018).

A number of bacteria are sensitive to environment pollutants such as wastes from various sources (Mazaheri & Fergusen 2018). They can act as sensors to show the presence of harmful wastes. *Vitis vinifera* (common grape vine) is a member of *Vitis* that is grown in east and north parts of Iran. The plant could grow to about 32 m in length and its barks are flaky with berry like fruits having a wide range of color and size. Traditionally, the grape is used as a fresh fruit, processed to make wine or juice, or dried to produce raisins (Heuzé *et al*. 2017).

Considering the high volume of wastes produced in autumn from various plants and agricultural processes together with their low price, the aim of this study was to extract peroxidase from residual leaves, stems and barks of grape plant after its fruiting stage.

MATERIALS AND METHODS

Plant wastes and chemicals

All types of grape wastes including barks, leaves, damaged fruits and roots from various grape plants were collected, with permission of owners, from houses and gardens in Rasht. After removing other contaminants, they were washed, dried at 37°C and kept in sealed plastic bags until experiments.

Guaiacol, hydrogen peroxide (30% v/v), triton X-100, coommassie brilliant blue G250 were purchased from Sigma™. Molecular weight marker was obtained from Fermentase™ chemical company.

Preparation of mixed waste extracts

Using liquid nitrogen, 30g of grape wastes were powdered and mixed with 60 ml of extraction buffer (50 mM potassium phosphate buffer, pH 7.0 containing 0.5 mM EDTA and 1% triton X-100). The mixture was centrifuged for 20 min at 12000 × g at 4°C. The pellet was then discarded and supernatant collected for determination of POD activity and protein concentration (Bradford 1976).

Enzyme assay

POD activity was determined by spectrophotometer using guaiacol as substrate in 470 nm (A470; $\Delta\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$) at 25°C. A typical reaction mixture contained 495µl guaiacol (50 mM), 495 µl H_2O_2 (180 mM) and 10 µl diluted enzyme extract. One unit of peroxidase activity is defined as the amount of enzyme being able to oxidase one µmole of guaiacol to tetraguaiacol in one minute (Saboora *et al*. 2012).

Quantitative protein determination

Protein concentration was determined using a slight modification of Bradford's method with bovine serum albumin (BSA) as standard (Bradford 1976). In practice, the shift in absorbance of Coomassie Brilliant Blue G-250 was followed at 540 nm.

Native Polyacrylamide Gel Electrophoresis (Native- PAGE)

Peroxidase isoenzymes were detected using native polyacrylamide gel electrophoresis (PAGE) according to Laemmli's protocol (He 2011).

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In practice, electrophoresis was performed at 4°C in the cold room of laboratory using 5% stacking gel and 12% separating gel. 20µl of extracted enzyme was loaded onto stacking gel and electrophoresis was conducted at 100V for three hours. Then POD isoenzymes of the plant were partially purified and characterized.

Peroxidase purification

Purification was performed by electroelution method using native page. The electrophoresis gel was divided into two unequal parts and the strip fragments were placed in a container to react with substrates until isoenzyme bands were appeared.

This strip gel was used as reference. After alignment of the reference strip with the other portion (not exposed to substrates), desired band was cut out of the gel. Elution buffer was then added to excised gel pieces followed by crush of the gel pieces and centrifuged at 12000 × g for 10 min at 4°C. The supernatant contained partially purified enzyme (Mujeeb *et al*. 2018; Mafulul *et al*. 2018).

SDS – PAGE electrophoresis

SDS–PAGE electrophoresis was performed under denaturing conditions as described by He (2011) for determination of enzyme purity and molecular mass. In practice, 30µl of samples were loaded onto stacking gel. Electrophoresis was performed at 150 V for 45 min. Protein band was then detected using Coomassie brilliant blue staining.

Determination of K_m and V_{max}

Substrate specificity of enzyme was calculated for guaiacol using varying concentrations (0-200 mM) of guaiacol at a constant saturation of hydrogen peroxide. The apparent K_m and V_{max} were then determined using the Lineweaver–Burk double reciprocal plot.

Optimum pH profile

Optimum pH was determined by assaying enzyme in a range of buffers to provide the desired pH, i.e. 50 mM acetate buffer (for pH of 3-5), 50 mM phosphate buffer (for pH of 6-8) and 50 mM Tris-HCl buffer (to provide pH 9.0).

The effect of temperature

To measure the heat stability and optimum temperature, the enzyme was heated in optimum pH at 30, 40, 50, 60 and 70°C for 15 to 40 min and cooled in ice bath. The residual enzyme activity was obtained using the unheated enzyme for comparison.

RESULTS AND DISCUSSION

Purification

The changes in enzyme activity and specific activity due to purification are summarized in Table 1. The native PAGE was used as a simple and one stage procedure to isolate and partially purify the possible isozymes for peroxidase. As seen, while the activity is decreased after purification, the specific activity is highly improved.

Table1. Purification of peroxidase isoenzyme from *Vitis vinifera* wastes.

Extract	Activity (Units)	Protein (mg)	Specific activity (U mg^{-1})	Purification fold	K_m (mM)	V_{max} ($Mmin^{-1}$)
Crude extract	41225.5	9.2	5101.5	1	-	-
Pure enzyme	14980.5	0.24	70112	15.05	83.2	0.35

Molecular mass

The electrophoresis pattern of crude extract on native-PAGE is shown in Fig. 1. As seen, two isozymes were identified on the gel. In this research, the POD_1 isoenzyme was cut from the gel as mentioned above. This was followed by denaturing electrophoresis to obtain its molecular weight. Purified peroxidase from previous step was migrated in SDS–PAGE as a single band corresponding to a molecular weight of 38 kDa as compared to standard

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protein molecular weight marker (Fig. 2). Most of plant peroxidases have molecular weight around 32-55 kDa (Dąbrowska *et al*. 2007). For instance 34.5 kDa for peroxidase isoenzyme from tea leaves (Kvaratskhelia *et al*. 1997), 55 kDa for peroxidase from royal palm tree (Watanabe *et al.* 2007) and 44 kDa for peroxidase from cauliflower (Köksal & Gülçin 2008) have been reported. However, enzymes with different mass range have also been reported, e.g. 90 kDa for brussels sprouts peroxidase (Regalado *et al.* 1999), 66 kDa for *Raphanus sativus* L. (Şişecioğlu *et al*. 2010) and 22 and 27 kDa for two mango isoperoxidases (Khan & Robinson 1993).

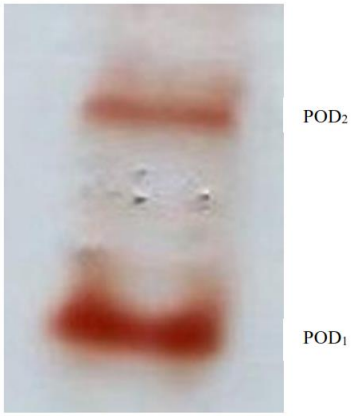

Fig. 1. The result of native polyacrylamide gel showing both isozymes of *Vitis vinifera* wastes peroxidase.

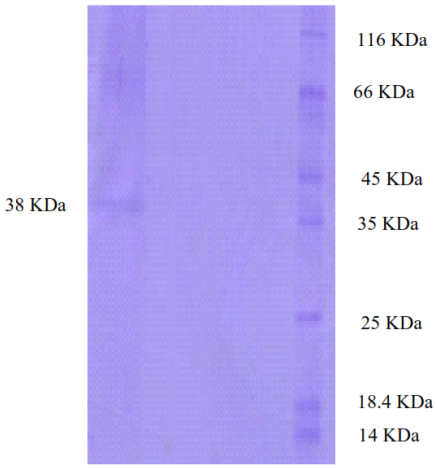

Fig. 2. Electrophoregram of *Vitis vinifera* wastes peroxidase (left line) compared with molecular weight marker (right line) representing the purified isozyme (MW KD).

Substrate specificity

Enzyme activity was assayed in various concentration of guaiacol (0-200 mM) in the presence of constant concentration of H_2O_2. Apparent K_m and V_{max} values were calculated using Lineweaver-Burk plot (Fig. 3). The obtained K_m value was 83.2 mM which is close to *Beta vulgaris* (98.61 mM, BRENDA) and the calculated V_{max} value was found to be 0.35 M min^{-1}.

Optimum pH

To determine the optimum pH, the assay was performed in different buffers as mentioned earlier. It was found that our novel peroxidase could reach its highest activity in pH 6.2 (Fig. 5).

Numerous plant peroxidases have been found that have optimum pH in the range of 6-7. For example, pH 6.0 for *Raphanus sativus* L. (Şişecioğlu *et al*. 2010) and about 6.0 for rye isoperoxidases (Murakami *et al*. 2007) have been reported in literature. Although most of plant peroxidases show a wide range of pH profile, a remarkable point about our enzyme was its considerably high activity in pH 3.6-7 (up to 80%).

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As shown in Fig. 4, the remaining activities were 75 and 80% at pH 4.0 and 7.5 respectively.
The enzyme remained 60% active in the range of 3-7.5 and its optimum pH was 6.0-6.2. This characteristics of our new enzyme is comparable with peroxidase extracted from royal palm tree, *Roystonea regia* (Şişecioğlu *et al.* 2010).

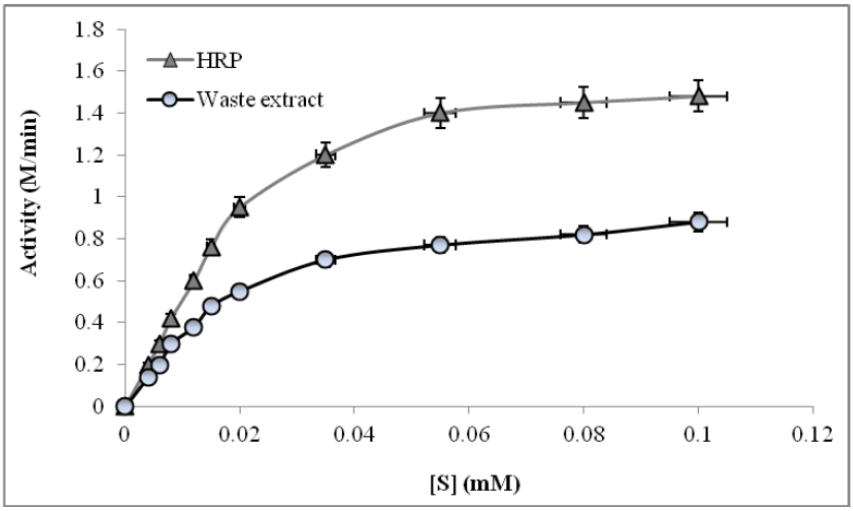

Fig. 3. Activity of waste peroxidase compared to HRP (the Michaelis-Menton plot).

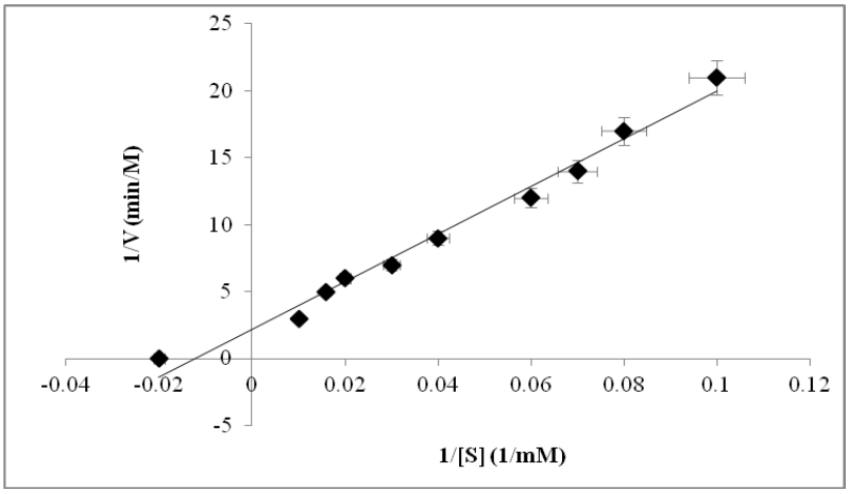

Fig. 4. Effect of substrate (guaiacol) concentration on activity of POD_1 isoenzyme of *Vitis vinifera* wastes.

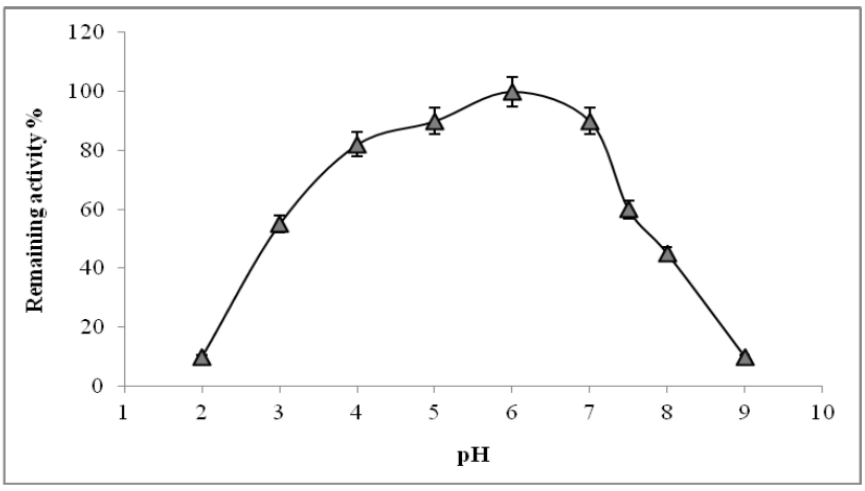

Fig. 5. The activity of *Vitis vinifera* wastes peroxidase at various pH.

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Heat stability

The effect of temperature on POD_1 isoenzyme was studied by incubating the enzyme in 30-70°C. Results showed that heating at 60°C for 20 and 40 min decreased enzyme activity to 19% and 4% respectively and no remarkable activity was detected at 70°C (Fig. 6).

According to these results, the new enzyme has moderate heat stability in comparison with other examined peroxidases. For instance, it has been reported that peroxidase extracted from royal palm tree has remained about 60% active after one hour incubation at 70°C (Şişecioğlu *et al*. 2010), and olive peroxidase preserved its activity until 40°C (Saraiva *et al*. 2007). In the present research, a study was also performed in order to obtain the actual thermal resistance of our novel peroxidase. The enzyme was stored at room temperature (25°C) for 26 days and interestingly, the enzyme retained 20% of its activity. This remarkable feature of the new enzyme is our aim for further investigations and would be explained with more detail in our future publications.

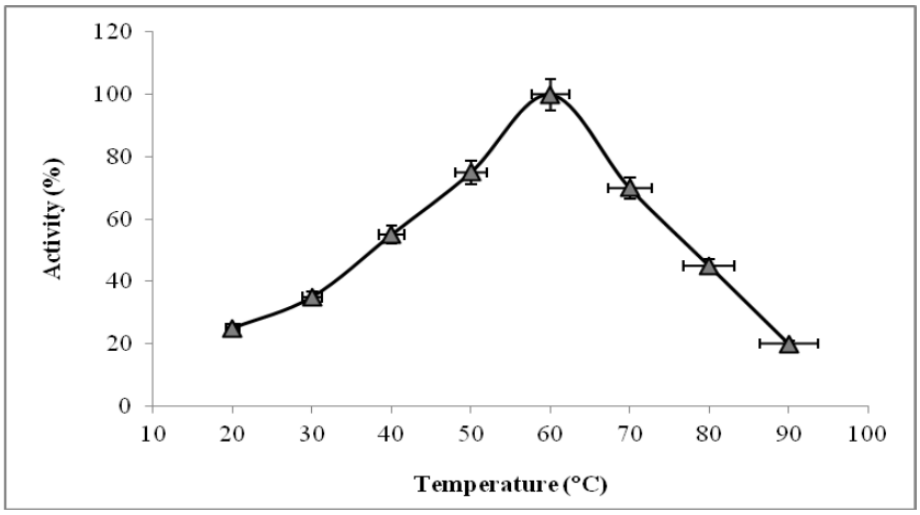

Fig. 6. The biological activity of *Vitis vinifera* wastes peroxidase at various temperatures.

CONCLUSION

One of the two isoenzymes extracted from *Vitis vinifera* wastes was specified. The obtained results provide a starting point on studying the presence and specification of peroxidase in agricultural wastes including *Vitis vinifera*. We have already designed some more research in prospects of our future goal to find and characterize other isozymes. Direct enzyme extraction from N-PAGE, was performed at lower costs and time than prevalent purification methods. Our results showed that polyacrylamide gel electrophoresis and electroelution was an effective method for isolation and purification of our peroxidase. Some of the examined enzymatic properties demonstrated that our peroxidase had high peroxidative activity within a wide range of pH and appropriate heat stability which both make it a suitable candidate for many industrial applications.

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Authors' contribution

Kobra Alijanipoor performed experimental part and wrote the manuscript draft. Reyhaneh Sariri purposed the research idea, guided research team scientifically, altered the various parts of the draft and prepared the final version of the paper. Reza Haji Hosseini designed the study protocol and supervised the whole project. Atusa Vaziri was the advisor and helped the writing up.

Declarations

It is confirmed that work has not been published, not under consideration for publication elsewhere, approved by all authors and, if accepted, it will not be published elsewhere in the same form, in English or in any other language.

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استخراج، تخلیص و تعیین مشخصات پراکسیداز ضایعات انگور (*Vitis vinifera*)

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چکیده

استفاده صحیح از ضایعات گیاهان نه تنها می‌تواند یک منبع ارزان برای ترکیبات طبیعی باشد، بلکه تحقیقی در راستای دوستی با محیط زیست نیز هست. هدف از این مطالعه استفاده از ضایعات انگور به عنوان منبعی برای آنزیم پراکسیداز است. در عمل یک ایزوزیم پراکسیداز (POD_1) از ضایعات انگور (*Vitis vinifera*)، گیاهی که در ایران به فراوانی کشت می‌شود، جداسازی و تخلیص شد. فعالیت این آنزیم جدید با استفاده از سوبسترای گایاکول اندازه‌گیری شد. آنزیم به دست آمده تخلیص شد و پارامترهای سینیتیکی آن تعیین شدند. مقادیر (K_m) و (V_{max}) برای گایاکول به ترتیب ۸۳/۲ میلی مولار و ۰/۳۵ مول/دقیقه به دست آمدند. دما و pH بهینه نیز برای سوبسترای گایاکول به ترتیب ۶۰ درجه سانتی‌گراد و ۶/۲ بودند. نتایج حاصل بیان می‌دارند که ضایعات گیاه انگور منبع قابل توجهی از پراکسیداز با رفتار سینیتیکی قابل قبول می‌باشند.

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