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Extraction, Purification and Kinetics of Guaiacol Peroxidase from Leaves and Friuts of Black Blueberry (MORUS NIGRA)

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ABSTRACT

Peroxidase (EC 1.11.1.7) is one of the key enzymes controlling plant growth, differentiation and development. Peroxidase from black mulberry (*Morus Nigara*) leaves and fruits was purified using ammonium sulfate salt precipitation and anion-exchange chromatography on DEAE-Sepharose column. The preparation gave an overall yield of 28.2%, 80-fold purification for leaves and of 12.4%, 97-fold purification for peroxidase of fruits. Specific activity of 15.3 and 31.1 U mg^{-1} protein for peroxidase of leaves and fruits were earned, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the purified enzymes were homogeneous and peroxidase of leaves have two isoenzymes with molecular weight of approximately 56 and 33 kDa, but peroxidase of fruits has a isoenzyme with molecular weight of approximately 55 kDa. Activity of peroxidase in leaves and fruits in the presence of guaiacol and H_2O_2 were optimum after incubation at 40 and 45 °C, respectively. Temperature stability profile showed that the purified Guaiacol peroxidase of leaves had maximum stability at 60 °C and retained 79% activity after incubation for 30 min. PGPF was 100% stable for 1 h at 45 °C and retained 45% of its initial activity after 4 h of preincubation at this temperature. The purified Guaiacol peroxidase of leaves seemed to have considerable thermostability, which can be favorable in industrial operations for traditional brewing and food processing. Kinetic parameters assesment for the purified Guaiacol peroxidase of leaves and purified Guaiacol peroxidase of friuts showed V_{max} of 16.8 and 25.3 U mg^{-1}, and K_m of 6.8 and 5.6 mM, respectively. NaN_3 and kojic acid were potent inhibitors of peroxidase and $ZnSO_4$ showed an activatory effect on peroxidase in leaves and fruits of black mulberry.

Keyword: Guaiacol, Peroxidase, Morus nigra, Temperature

INTRODUCTION

Peroxidase (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase, POD) is involved in various essential physiological processes of plant growth and development throughout their life cycle. The enzyme participates in construction, rigidification and eventual lignification of cell walls, biosynthesis of ethylene from 1-aminocyclopropane-1-carboxylic acid and H_2O_2, regulation of auxin level through auxin catabolism, protection of tissue from damage and infection by pathogenic microorganisms, the oxidation of indoleacetic acid [1,2]. *In vitro*, this enzyme is widely employed in microanalysis [3]. More than 80% of immunoenzymatic kits contain peroxidase as labeling enzyme. Recently, peroxidases have been used for biotransformation of organic molecules [4]. It has been well established that peroxidase as one of the most stable enzymes can contribute to deteriorative changes in quality of the processed products [5]. Enzymatic browning is one of the most important colour reactions that affect fruits and vegetables. It is catalyzed by polyphenoloxidases and peroxidases [6]. It was estimated that over 50 percent losses in fruits and vegetables occur as result of enzymatic browning [7]. It was reported that the rate of enzymatic browning in fruits and vegetables is governed by the active polyphenoloxidase and peroxidase content of the tissues, the phenolic content of the tissues, pH, temperature and oxygen availability with the tissue. Mulberries have recently been received much attention as potential sources of functional foods due to several biological effects. Findings on antioxidant activity of mulberry fruits and leaves could justify some biological uses of this plant. Isolation and identification of phenolic compounds existed mulberry fruits and leaves will be the subject of further research

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projects [8]. The objective of this work was to study the effect of pH and heat on peroxidase activity and to extend the knowledge of vegetables peroxidase in particular.

MATERIALS AND METHODS

Plant Material

Leaves and fruits of Black mulberry were obtained from a farm during the summer in sanandaj, Kurdistan province, Iran. Then, it was washed, peeled, broken up, packed in polyethylene bags, and stored at -83 °C until the extraction procedure was performed separately for leaves and fruits at the Department of Botany of the Payame Noor University of Kurdistan, Iran. Also, all chemicals of analytical grade were obtained from Merck.

Peroxidase Purification

The leaves and fruits of black mulberry were homogenized in cold phosphate buffer (60 mM), pH 6.5 (0.17 g ml^{-1} of leaves and 0.17 ml^{-1} fruits), in a domestic blender for 5 min, separately. The homogenates were rapidly filtered through six layers of cheesecloth and centrifuged at 10,000 x g for 15 min at 4 ºC. The supernatants were taken as the source of peroxidase purification, immediately [12]. All purification steps were carried out at 15 ºC. The enzymatic fractions were obtained by a two-step purification procedure. For preparation of crude extract, the extracts of leaves and fruits of Black mulberry were homogenized with 40% and 75% ammonium sulfate saturation, separately. After standing for 1 h at 4 ºC, the homogenates were centrifuged at 20,000 x g for 15 min at 4 ºC. The supernatants were discarded, and the pellet was dried at a temperature of 10-15 ºC and used for purification. The partially purified peroxidase of leaves and fruits were stored at -18 ºC until use. An adequate amount of partial purified peroxidase of leaves and fruits were dissolved in 50 mM sodium phosphate buffer, pH 6.5, separately. The enzymes were then loaded on a DEAE-Sepharose column (1 ml) and eluted with a 0 to 0.5 M NaCl linear gradient in 50 mM sodium phosphate buffer pH 6.5, at a flow rate of 1.0 ml min^{-1}. The protein concentration of the fractions were routinely estimated by measuring the absorbance at 280 nm and the activity in each eluted fraction was determined in triplicate by measurement of the absorbance at 470 nm of production of tetraguaiacol ($\varepsilon = 6.65 \times 10^3\ M^{-1} cm^{-1}$) [13]. Active fractions from the DEAE-Sepharose column were pooled and dialyzed against deionized water, then concentrated by freeze dryer and stored at -20 ºC to use for biochemical characterization of the enzymes.

Protein Electrophoresis

The purified Guaiacol peroxidase of leaves (PGPL) and fruits (PGPF) were used for PAGE or SDS-PAGE in order to estimate the enzyme activity, MW (molecular weight) and purity. The sample preparation for native PAGE was performed according to Davis [14], after dialysis overnight. Enzyme-substrate staining of the native PAGE gels was carried out using guaiacol-hydrogen peroxide [15]. For SDS-PAGE the samples were boiled in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol and separated in a 12% gel according to Laemmli [16]. The proteins were stained with coomassie brilliant blue R-250. The standard molecular weight used were: phosphorylase b (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100 Da), and a-lactoalbumin (14,400 Da).

Molecular Weight Determination

Gel filtration chromatography of adequate quantities of PGPL and PGPF were performed on a Sephacryl S-200 column (internal diameter of 16 mm, height of the packed gel bed of 60 cm, giving a bed volume of 120 ml), using blue dextran for the void volume determination and soybean trypsin inhibitor (20,100 Da); carbonic anhydrase (30,000 Da); ovalbumin (43,000 Da); bovine serum albumin (67,000 Da); phosphorylase b (94,000 Da) as standards. The molecular weight of the PGPL and PGPF were calculated from a regression curve, where log of the molecular weights of the standards were plotted against the ratio of the elution volumes of the standards and the void volume of the column.

Peroxidase Activity and Total Protein Determinations

PGPL and PGPF were determined in triplicate by measurement of the absorbance at 470 nm for tetraguaiacol production (ε = 26.6 mM^{-1} cm^{-1}) [17]. Briefly, we used 2.4 ml of 1% guaiacol and 0.5 ml of 0.1 M hydrogen

peroxide in 50 mM phosphate buffer pH 6.5 at 35 ºC and the reaction was started by adding 0.1 ml of PGPL and PGPF solution, separately. The peroxidase activity was measured while the reaction was at the initial rate (1.5-3.0 min). One unit of activity was defined as the amount of enzyme that causes an increase of 0.001 absorbance per min. Total protein concentration was determined in triplicate, using bovine serum albumin as standard, according to Lowry *et al*. [18].

Effect of pH

pH profile of guaiacol peroxidase activities PGPL and PGPF were determined spectrophotometrically at 25 °C. The activity of PGPL and PGPF were determined in the pH range of 3-10 by using a citrate-phosphate-borate mix buffer, 0.1 M. The optimum pH for PGPL and PGPF activity were obtained in the presence of 20 mM guaiacol and 10 mM of H_2O_2 as substrates. Enzymatic activity was determined by measuring the increase in absorbance at 470 nm (spectrophotometer, 6305 JENWAY). Assays were carried out by addition of 100 µl of purified enzyme to the sample cuvette, and changes in absorbance were recorded. The reference cuvette contained just 3 ml of substrate solution. PGPL and PGPF activity were calculated from the linear portion of the curve. Assays were carried out at room temperature and results are the averages of at least three assays and the mean and standard deviations were plotted.

Effect of Temperature on Peroxidase Activity of Black Mulberry

pH profile of *Morus nigra* peroxidase of leaves (PGPL) led to two peaks at 5.5 and 6.5 that probably belong to atleast two isoenzymes and pH profile of PGPF showed a peak at pH = 6.5. For determining the optimum temperature values, PGPL and PGPF activity were measured, at constant temperatures (27, 30, 40, 45, 50, 60 and 70 °C separately) using guaiacol (20 mM) and H_2O_2 (10 mM). Effect of temperature on the activity of PGPL and PGPF were tested by heating the purified extract of peroxidase to the appropriate temperatures in different times. The desired temperatures were provided by using a Memmert model ST/70 temperature controller attached. After different times (0-60 min) at a same temperature, purified enzymes cooled in ice and were added and the reaction were followed spectrophotometrically at given time intervals as described above, separately.

Heat Inactivation of Peroxidase

The thermal denaturation of the PGPL and PGPF were studied at different temperature (30-70 °C) and constant concentration of guaiacol (20 mM) and H2O2 (10 mM). 100 µl of the purified solution in a test tube was incubated at the required temperature for fixed time intervals. At the end of the required time interval, the test tube was cooled in an ice bath. The activity of the peroxidase was then determined for PGPL and PGPF, separately.

Statistical Analysis

All the experiments were repeated three times and expressed as means ± SD. The statistical significances of different factors were evaluated by analysis of variance (ANOVA), using Microsoft excel. P-value less than 0.05 ($p < 0.05$) was considered to be statistically significant.

RESULTS AND DISCUSSION

Enzyme Purification

From 80 g of fresh leaves and fruits of black mulberry, we extracted 70 ml and 80 ml of protein extract, respectively. The results for each purification step are summarized in Table 1. At higher concentrations of ammonium sulfate, protein solubility usually decreases and leading to salting-out. This principle is used to precipitate the peroxidase. Crude extracts of leaves and fruits of black mulberry, were precipitated by ammonium sulfate in range of 30-75% saturation. For leave's and fruit's extract, the best results were obtained by 75% ammonium sulfate saturation. Purification fold for PGPL was 7.5 and yield 71% and purification fold for PGPF was 1.5 and yield 71% (Table 1). After chromatography with DEAE-Sepharose, we concentrate and dialyze active fractions against distilled water for 24 h. We reach a reasonable yield 36 for PGPL and 27 for PGPF, So, leave's peroxidase (PGPL) was purified 11 time and fruit's peroxidase (PGPF) was purified 6 time.

Molecular Weight

The molecular weight and purity were determined by

Table 1. Summary of each Purification Step of Guaiacol Peroxidase from *Black Mulberry* Leaves and Fruits

	Source	Volume (ml)	Total protein (mg)	Enzyme activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude extract	Leaves	115	350	125.2	0.36	1	100
	Friut	129	395	135.3	0.34	1	100
Amunium precipitation	Leaves	88	57	68.9	1.2	3.3	56
30%	Friut	111	98	85.1	0.87	2.6	63
Amunium precipitation	Leaves	58	28	75.2	2.7	7.5	60
75%	Friut	98	45	68.7	1.5	4.4	51
DEAE-Sepharose	Leaves	1.1	12	45.3	3.78	11	36
	Friut	0.6	17	36.8	2.1	6	27

SDS-PAGE, after Coomassie Blue staining. The molecular weights are calculated from a plot of logRf versus protein standard. First column in Fig. 1 shows proteins standards. Column 2 shows purified peroxidase from leaves of black mulberry (PGPL). Column 3 shows purified peroxidase from fruits of black blueberry (PGPF). Two bands at column 2 belong to two isoenzymes of PGPL with the molecular weight around 56 and 33 kDa. Column 3 shows one bands belongs to peroxidase band of fruit (PGPF) with molecular weight of 62.34 kDa. SDS-PAGE analysis shows that the peroxidase in the leaves is different than the peroxidase in the fruits. Molecular weights of most plants peroxidases varies from 30 to 60 kDa [19]. In comparison with Horseradish peroxidase's (40-46 kDa), PGPL and PGPF have an isoenzyme greater and PGPF has an isoenzyme smaller [20].

pH Profile of Peroxidase Activity of Leaves and Friuts in Morus Nigra

pH is a factor for determining of enzymatic activity; it alters the ionization states of amino acid side chains or substrate. For PGPL, two pH optimum were observed at 5.5 and 6.5. No activity was detectable at pHs 3 and 10, regardless of the condition. Figure 2 shows the pH activity profile obtained for PGPL and PGPF. pH optimum of PGPF was observed at pH = 6.5.

Kinetic Assay of Peroxidase

Activity of black mulberry of peroxidase (PGPL and PGPF) increased in presence of various concentrations of guaiacol and constant concentration of H_2O_2 (10 mM). So with increase of guaiacol, activity of PGPL and PGPF increased until reached to maximum rate at 16.8 and 25.3 U mg^{-1} protein, respectively (Table 2). More increase in concentrations of guaiacol accompanied with decrease of activity and showed substrate inhibition of peroxidase. Km of PGPL calculated 6.8 mM and catalytic efficiency is 2.47 U mg^{-1} protein per mM and also, Km of PGPF calculated 25.3 mM and catalytic efficiency is 4.51 U mg^{-1} protein per mM. When different concentrations of H_2O_2 were added to PGPL and PGPF in presence of constant concentration of guaiacol (20 mM) solution at pH 6.5, it was found that the guaiacol peroxidase activity gradually increased, So its apparent Vmax and Km were 18.5 U mg^{-1} protein and 1.8 mM, respectively for PGPL. In these condition, apparent Vmax and Km were 28.2 U mg^{-1} protein and 1.1 mM, respectively. With increase of guaiacol, activity of PGPF increased until reached to maximum of rate at 28.2 U mg^{-1} protein (Table 2). More increase in concentrations of guaiacol accompanied with decrease of activity and showed substrate inhibition of peroxidase (PGPL and PGPF). Km of PGPF calculated 1.1 mM and catalytic efficiency is 25.6 U mg^{-1} protein per mM. When different concentrations of H_2O_2 were added to PGPL in presence of constant concentration of guaiacol (20 mM) solution at pH 6.5, it was found that PGPL activity gradually increased. So its apparent V_{max} and K_m were 18.5 U mg^{-1} protein and 1.8 mM, respectively.

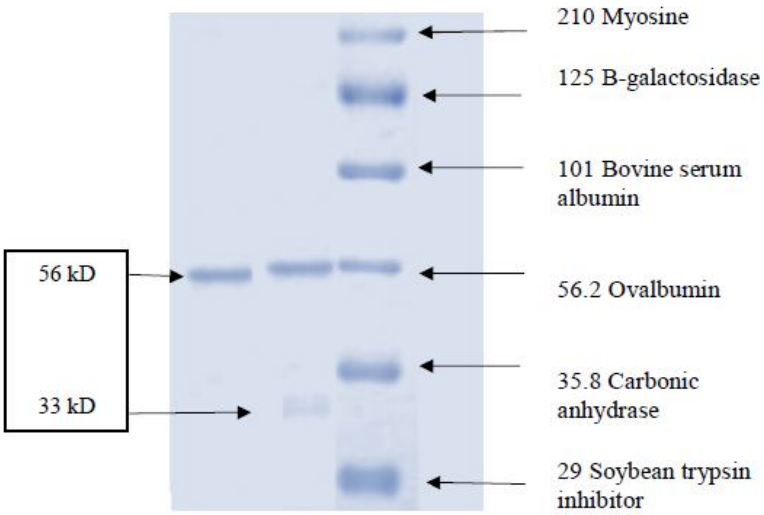

Fig. 1. SDS-PAGE of peroxidases enzymes PGPL and PGPF with Coomassie Blue staining. Lane1: protein molecular weight marker; Lane 2: Purified PGPL; Lane 3: Purified PGPF.

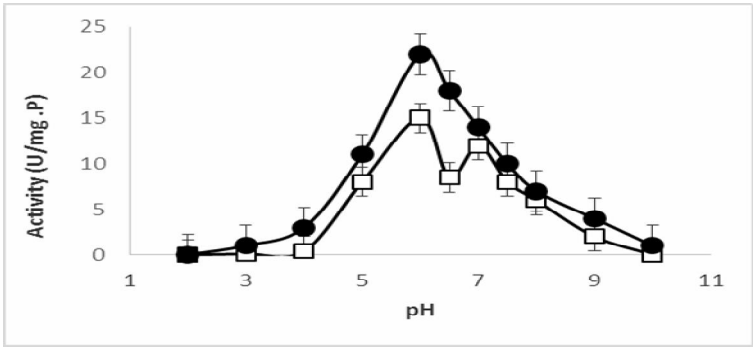

Fig. 2. The pH dependence of peroxidase activity of Morus nigra. Activity was determined in 0.1 M sodium-acetate buffer and phosphate buffer system in the presence of 20 mM guaiacol and 10 mM H_2O_2. (●), PGPF and (□), PGPL.

Optimum Temperature and Thermal Inactivation

Peroxidase in presence of guaiacol and H_2O_2 showed fluctuations in activity with increasing temperature even as high as 60 °C. The plot for guaiacol demonstrated that the PGPL was thermostable between 25 and 60 °C. Our results showed optimum temperature is 40 °C for PGPF activity and 45 °C for PGPL activity. Like most chemical reactions, with increase of temperature from 27 °C, gradually, activity of PGPF and PGPL increased so, reached to maximum of activity at 40 °C (200%) for PGPF and 150% for PGPL (Fig. 3). With more increase in temperature from 40 °C to 70 °C, activity of PGPF decreased gradually and reached to 0% at 70 °C and activity of PGPL reached to 65%. The drop in percentage residual activity at high temperatures can actually be due to the unfolding of the tertiary structure of the enzyme and it is likely to form the secondary structure.

Activity of peroxidase at 70 °C was not observed and is zero related to control (25 °C). These results showed that,

Table 2. Kinetic Parameters of PGPL and PGPF in Constant Concentration of H_2O_2 and Different Concentrations of Guaiacol(●) and Constant Concentration of Guaiacol and Different Concentrations of H_2O_2 (O)

Source		K_m (mM)	V_{max} (U mg^{-1} protein)	V_{max}/K_m (U mg^{-1} mM^{-1})
	PGPL	6.8	168.7	24.8
●	PGPF	5.6	184.3	32.9
	PGPL	1.8	18.5	10.27
O	PGPF	1.1	28.2	25.6

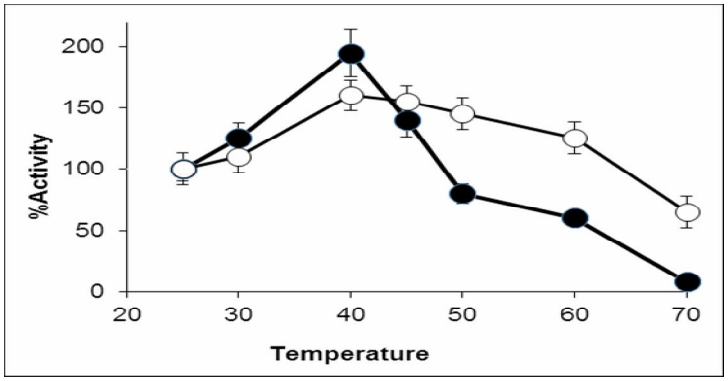

Fig. 3. Effect of temperature on activity of peroxidase in extract of black mulberry (*Morus nigra*) in the presence of guaiacol 20 mM and H_2O_2 10 mM. PGPL (○) and PGPF (●).

peroxidases show activity at high temperatures. A ten degree Centigrade rise from 30 to 40 °C in temperature after 5 min incubation will increase the activity of PGPF from 125 to 200%. Variations in reaction temperature as small as 10 degrees from 30 to 40 °C introduce increase of 56% in the activity of PGPL.

Effect of Various Compounds and Metals Ions

The enzymatic activities of PGPL and PGPF were investigated in presence of different compounds such as metal ions, amino acids and inhibitors. Figures 4 and 5 show the results that we obtained from our research. We tested four concentrations of each compounds on the peroxidase activities. PGPL and PGPF were activated by $ZnSO_4$. We investigated effects of NaCl and $FeCl_3$ on PGPL and PGPF. With increasing concentrations of NaCl from 0.05 until 0.5 g l^{-1}, activity of PGPL showed a decrease of 16%. In these condition, activity of PGPF showed a decrease of 11%. Compounds such as $FeCl_3$, Kojic acid, Glycine and Cysteine showed inhibitory effect on PGPF and PGPL. PGPF showed a decrease of 51% in presence of kojic acid and PGPL showed a decrease of 39% in presence of kojic acid. NaN_3 was a potent inhibitor of PGPF and PGPL. So, activity of PGPL and PGPF in presence of 0.15 g l^{-1} of NaN_3 reached to zero and peroxidase became completely inactive. As a results, for PGPL and PGPF, more of metal ions strongly inhibited the peroxidase activity. So, we lost 32% of the activity with 0.5 g l^{-1} of $FeCl_3$, for PGPL, while 42% inhibition was caused for PGPF. On the other side, the enzymatic activities were increased by $ZnSO_4$. The largest increase was observed in PGPL and PGPF by $ZnSO_4$ at the four used concentrations

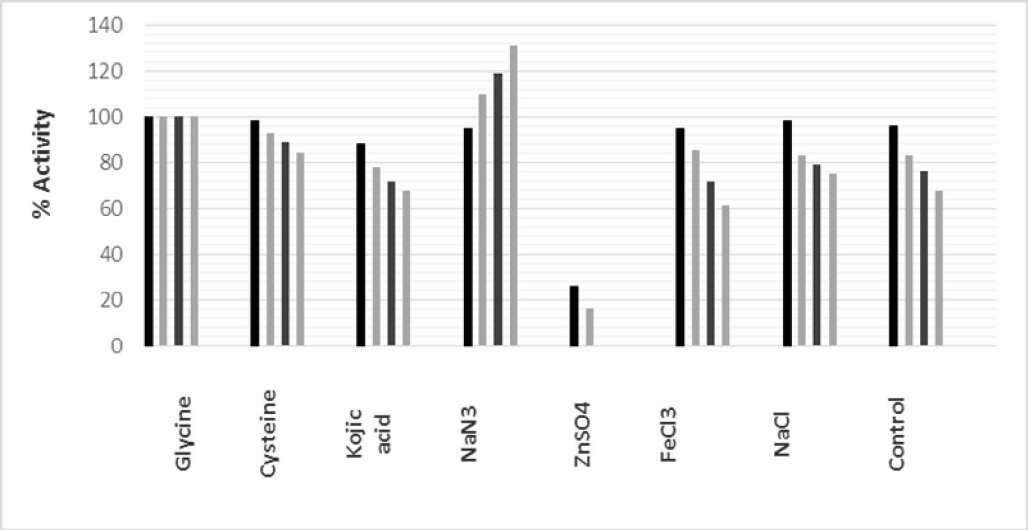

Fig. 4. Effect of metal on PGPL. The enzymes were first incubated with 0.05, 0.1, 0.15 and 0.5 g l^{-1} of various metal ions and inhibitors for 2h, and then the enzymatic activity was measured. Activity of the enzyme without metal ions incubation (control) was set as 100%. Results were earned as mean ± standard deviation of three replicas.

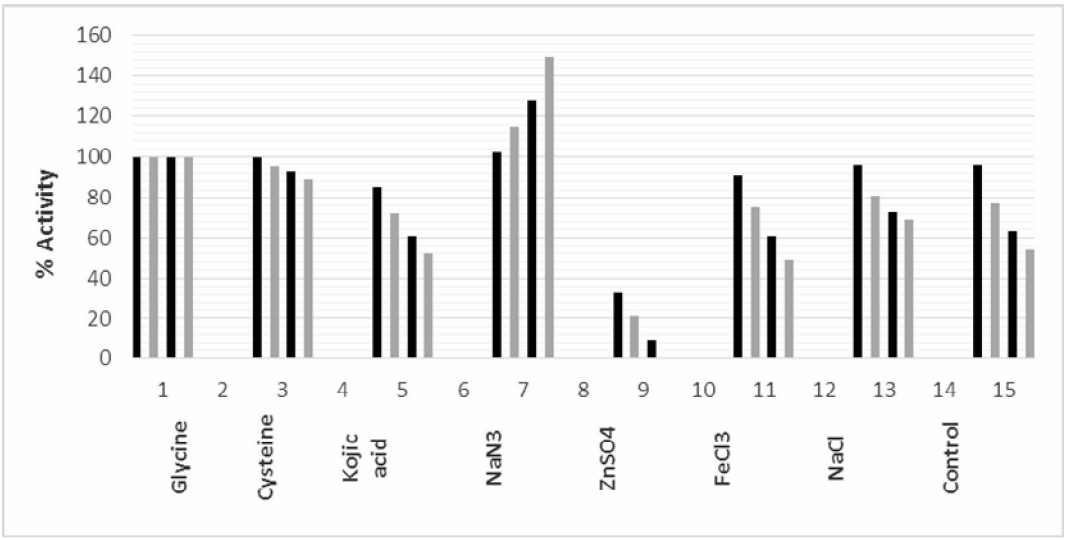

Fig. 5. Effect of metals on PGPF. The enzymes were first incubated with 0.05, 0.1, 0.15 and 0.5 g l^{-1} of various metal ions and inhibitors for 2 h, and then the enzymatic activity was measured. Activity of the enzyme without metal ions incubation (control) was set as 100%. Results were earned as mean ± standard deviation of three replicate.

(more 49% activity by 0.5 g l^{-1}). These results were approved on peroxidase purified from *Jatropha curca* [21]. The activity of PGPL and PGPF decreases with cysteine and glycine. Both PGPL and PGPF were inhibited by NaN_3.

CONCLUSIONS

For peroxidase activity of leaves and fruits in black mulberry, activity of peroxidase is adversely affected by high temperatures. The reaction rate increases with temperature to a maximum level, in the following declines with further increase of temperature. PGPL become denatured at higher temperatures of 40 ºC, and is more resistant at high temperature such 45-70 ºC. These results showed that peroxidase has a potential for activation. It has been noted that heat stability of the enzyme may be related to ripeness of the fruit and molecular forms of the enzyme, and in some cases it is also dependent on pH [22]. Rapid decrease in activity in high temperature after optimum temperature might be due to involvement of disulfide bond in the active site or in three dimensional conformation of the enzyme. There are several other reports that describe high temperature stability of peroxidase from other sources to the same temperature range. The drop in percentage residual activity at high temperatures can actually be due to the unfolding of the tertiary structure of the enzyme to form the secondary structure [23]. Results proved that peroxidase of black blueberry at leaves has at least two isoenzyme. Compound such as NaN_3 is a strong inhibitor of peroxidase, although kojic acid is a potent inhibitor for peroxidase. $ZnSO_4$ showed an activatory effect on peroxidase. PGPL and PGPF was increased with some ions, such as $ZnSO_4$, So the activity has increased about 50%, which make it as a good candidate for biotechnological tools. Guaiacol demonstrated that the PGPL was thermostable between 25 and 60 °C. Our results showed optimum temperature is 40 °C for PGPF activity and 45 °C for PGPL activity. Kinetics of peroxidase showed that, Km of PGPL was 6.8 mM and catalytic efficiency was 2.47 U mg^{-1} protein per mM and also, Km of PGPF calculated 25.3 mM and catalytic efficiency was 4.51 U mg^{-1} protein per mM. The enzymes extracted and purified have different isoenzymes and different molecular weight which means that two different peroxidases are exist in leaves and one isoenzyme exist in fruits of black mulberry.

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