

KINETIC AND THERMAL CHARACTERIZATION OF PEROXIDASE FROM PEELS OF *CITRUS RETICULATA* VAR. KINNOW

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ABSTRACT

In this study peroxidase was isolated from peels of *Citrus reticulata* var. Kinnow and partially purified using ammonium sulphate precipitation method. Kinetic and thermal characterization of the partially purified enzyme was explored. The optimum pH and temperature were found to be 6 and 55 °C respectively. The K_m and V_{max} values for guaiacol as substrate were found out to be 0.66 mM and 380 $\mu\text{mol/mL/min}$. Kinetics of irreversible thermal inactivation was studied from 60-80°C. The enthalpy (ΔH°) and free energy (ΔG°) of thermal inactivation of *Citrus reticulata* peroxidase were 92.92 and 110.06 kJ/mol respectively at 80 °C. The effect of urea as inhibitor was investigated. *Citrus reticulata* var. Kinnow peroxidase showed retention of 84.22 % activity after 60 min with 8 M urea. The surfactants decreased the activity of peroxidase with the exception of lemon max which enhanced the activity of enzyme. Metal ions like Mg^{2+} , Mn^{2+} , Cd^{2+} , Cu^{2+} and Al^{3+} enhanced the activity of peroxidase whereas Co^{2+} , Cr^{3+} , Hg^{2+} , Ca^{2+} , Pb^{2+} , Zn^{2+} , Sr^{2+} and Ni^{2+} showed slightly inhibiting behaviour.

Key words: *Citrus reticulata* var. Kinnow peroxidase; Thermal denaturation; Enthalpy of inactivation; Free energy of inactivation; Kinetics.

INTRODUCTION

Peroxidases (E.C. 1.11.1.7), representing a huge family of heme containing enzymes are widely distributed in plants, microbes, and animal tissues (Boeuf *et al.*, 2000). Plant peroxidases are found in tonoplast and plasmalemma, inner and outer side of the cellular wall both in soluble as well as, ionically bound forms (Chen *et al.*, 2002). They oxidize several substrates in the presence of hydrogen peroxide and usually contain a protoporphyrin IX prosthetic group and have various physiological roles in plant cells. It is one of the key enzymes controlling plant growth and development. It takes place in various cellular processes including construction, rigidification and eventual lignification of cell walls (Quiroga *et al.*, 2000)

Peroxidases being ubiquitous proteins have wide applications in different areas such as chemical synthesis, medicine, and in the analysis of food, chemicals, clinical and environmental samples (Agostini *et al.*, 2002). In recent times, peroxidases are being used for several novel applications in different processes including detoxification and removal of variety of organic pollutants, e.g. aromatic amines, phenols, dyes, etc., from contaminated wastewater (Duran and Esposito, 2000; Shaffiqu *et al.*, 2002; Bhunia, 2001; Akhtar *et al.*, 2005). Peroxidases have also enormous applications as catalysts in phenolic resin synthesis, in fuel and chemical production from wood pulp, in the production of dimeric alkaloids, in bio-bleaching processes and in the oxidation/biotransformation of organic compounds (Fatima *et al.*,

2007). This great diversity of applications is due to the wide substrate specificity of peroxidase catalysis.

Horseradish peroxidase (HRP) is the most extensively studied peroxidase. It has vast diagnostic, biosensing and biotechnological applications (Regalado *et al.*, 2004). The availability and cost of commercially available HRP restricts its applications. Peroxidases from other plant sources have also been explored; however these investigations have been unsuccessful in terms of identifying peroxidases able to knock out HRP as the preferred plant peroxidase in biotechnology. The availability of highly stable and active peroxidases from sources other than horseradish would go a long way towards the development of a catalytic enzyme with broad commercial and environmental applications. Microbes are the potential source for the production of peroxidase enzyme and a number of such reports (Mtui and Nakamura, 2008) are available in literature but the production in a stirred tank fermenter is not suitable as these microbes are known to be sensitive to shear stress.

Peroxidase activity has been investigated in a range of fruits such as oranges (Clemente, 2002) and peach (Neves, 2002) pears (Regalado *et al.*, 2004) and apples (Singh *et al.*, 2010), Mohammed *et al.* (2010) reported that the major pool of peroxidase activity is present in the peel of some Egyptian citrus species and cultivars compared to the juice and pulp.

Citrus represents one of the most important and widely grown fruits in the world and Pakistan is one of the major citrus producing countries. As the focus of most of today's research is to explore economical sources so use of peels of oranges would be encouraged.

The aim of present study was to isolate, partially purify and thermally characterize the peroxidase from economical source of Kinnow, which can be utilized in the industry as biocatalyst and to remove dyes from waste water.

MATERIALS AND METHODS

Isolation of peroxidase from peels: Peels of *Citrus reticulata* var. Kinnow were first thoroughly washed with distilled water. Then 10 g of peels were cut into small pieces and homogenized in 100 mL of 100 mM phosphate buffer of pH 7.0 using a blender. The homogenate was filtered through Whatman filter paper No. 1 and filtrate was centrifuged at 10,000 x g for 15 min. After the centrifugates were pooled the remaining residue was re-extracted with extraction buffer by centrifuging the residue as above. The centrifugates were pooled and assayed for peroxidase activity and protein contents (Bhatti *et al.*, 2006).

Peroxidase assay: Peroxidase activity was determined colorimetrically using spectrophotometer (Cecil 7200) following the formation of tetraguaiacol ($A_{\max}=470$ nm, $= 26.6 \text{ mM}^{-1}\text{cm}^{-1}$) with slight modification (Bhatti *et al.*, 2006). The reaction mixture contained 1mL of 0.1M acetate buffer (pH 5), 1 mL of 15 mM guaiacol, 1mL of 1.6 mM H_2O_2 and 60 μL of enzyme extract.

One unit of peroxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol in 1min.

Partial purification of peroxidase: Solid ammonium sulphate (561g) was added to 1L crude extract of Kinnow peels in order to get 80 % saturation. It was left over night at 4 °C. After that it was centrifuged at 10,000 x g for 15 min at 4°C, the precipitate was dispensed in 0.1M phosphate buffer of pH 7.0 and dialyzed against distilled water. The dialyzed enzyme was assayed and further used for kinetic and thermal characterization.

Optimum pH and temperature: Optimum pH of *Citrus reticulata* var. Kinnow peel peroxidase was determined by assaying the enzyme using different pH buffers (2-10) at 30 °C following the same procedure as mentioned before. The buffers used were acetate, phosphate, glycine/HCl, glycine/ NaOH and Tris/ HCl. The optimum temperature was determined by incubating the enzyme at different temperatures ranging from 30-80 °C (Bhatti *et al.*, 2007).

Effect of substrate concentration: *Citrus reticulata* var. Kinnow peel peroxidase was assayed in the reaction mixtures containing variable amounts of guaiacol (0.5-30 mM) while keeping H_2O_2 concentration constant whereas in a similar experiment varying H_2O_2 concentration (0.4-40 mM) and keeping guaiacol as constant substrate. The

data were plotted to determine the values of kinetic constants V_{\max} and K_m (Amin *et al.*, 2010).

Kinetics of thermal denaturation: Kinetics and thermodynamics of irreversible thermal denaturation for *Citrus reticulata* var. Kinnow peroxidase by placing the enzyme at elevated temperatures. Aliquots were collected at different time intervals, cooled on ice for 2-3 h (Bhatti *et al.*, 2007) and assayed for enzyme activity at 30°C as mentioned before. This procedure was repeated for 5 different temperatures ranging from 60-80°C. The data was fitted to pseudo-first order plots. For calculation of thermodynamic parameters Eyring's equation derived from transition state theory was taken into account as reported by Bhatti *et al.* (2007.):

$$k_d=(k_bT/h)e^{(-H^\circ/RT)}.e^{(S^\circ/R)} \quad (1)$$

Where h=Plank's Constant= 6.63×10^{-34} Js

K_b =Bltzman's constant (R/N)= $8.314 \text{ JK}^{-1}\text{mol}^{-1}$

N=Avogadro's No.= 6.02×10^{23}

T=absolute temperature

$$H^\circ \text{ (enthalpy of inactivation)} = E_a - RT \quad (2)$$

$$G^\circ \text{ (free energy of activation)} = -RT \ln (k_d h / k_b T) \quad (3)$$

$$S^\circ \text{ (entropy of activation)} = (H^\circ - G^\circ) / T \quad (4)$$

Energy of activation for thermal denaturation was determined from Arrhenius plots.

Effect of urea: *Citrus reticulata* var. Kinnow peel peroxidase was incubated with 8.0 M urea for varying times at 30 °C. Peroxidase activity was determined at the indicated time intervals by assaying the enzyme as mentioned before. The activity of enzyme without incubation with urea was taken as control (100 %).

Effect of surfactants: Surfactant effect was determined by incubating the enzyme with different surfactants (1 %) for 60 min and then performing enzyme assay under the same conditions as mentioned in the text. The activity of Kinnow peel peroxidase in assay buffer without any surfactant was taken as control (100%) for the calculation of percent activity.

Effect of metal ions: Metal ion effect was determined by incubating the enzyme with 2 mM solution of each metal for 1h and then assaying the enzyme by the same procedure as mentioned before.

Statistical analysis of data: All the experiments were conducted in triplicate and the results are reported as mean \pm S.D.

RESULTS AND DISCUSSION

Partial purification of peroxidase: Peroxidase from the peels of Kinnow was isolated using 0.1 M phosphate buffer and then partially purified through ammonium sulphate (80 %) fractionation. After dialysis the partially purified enzyme was then characterized in terms of kinetic and thermodynamic parameters.

Optimum pH and temperature: The optimum pH of the enzyme was determined by incubating the enzyme using different pH buffers ranging from 2-10. The results regarding the effect of pH on the enzyme activity are reported in Fig. 1. The optimum pH was found to be 6. The peroxidase activity was low above and below the optimum pH. The pH at which an enzyme catalyses a reaction at the maximum rate is called the optimum pH. Change in pH above and below the optimum level affects the charges on the amino acids within the active site such that the enzyme-substrate complex formation is disturbed. Our findings are in close agreement with the reported results in which peroxidase isolated from, Turkish black radish and *Euphorbia cotinifolia* (Sisecioglu *et al.* 2010; Kumar *et al.* 2011) showed the same pH optima.

The effect of temperature on activity of peroxidase was determined by incubating the enzyme at different temperatures ranging from 30-80 °C for 3 min and the results are represented in Fig. 2. The results indicated that the optimum temperature was 55 °C although there was not much decrease in activity up till 80 °C. These results reveal that peroxidases are stable in broad range of temperatures. Similar trend was obtained in case of peroxidase isolated from *L. leucocephala* (Pandey, 2011). An optimum temperature in the range of 40 to 55°C has been reported for turnip peroxidases (Duarte-Vazquez *et al.*, 2000).

Effect of substrate concentration: In order to determine substrate specificity, K_m and V_{max} values for guaiacol (Fig. 3) and H_2O_2 (Fig. 4) as substrate were determined by Lineweaver-Burk plot. Effect of guaiacol on the peroxidase activity was determined by varying the concentration of guaiacol while keeping a fixed and saturated concentration of second substrate H_2O_2 and vice versa. The enzyme had K_m values of 0.66 and 1.428 mM for guaiacol and H_2O_2 substrates, respectively. Thus, *Citrus reticulata* var. Kinnow peroxidase showed lower K_m value for guaiacol suggesting higher affinity of enzyme for guaiacol. On the other hand, the enzyme had V_{max} value of 380 and 625 $\mu\text{mol}/\text{mL}/\text{min}$ for guaiacol and H_2O_2 substrates, respectively. Peroxidases have different K_m and V_{max} depending upon sources e.g. for the same pair of substrates, Sisecioglu *et al.* (2010) had reported 0.036 and 0.0084 mM for guaiacol and H_2O_2 substrates

where as V_{max} values of 38728 and 35122 EU/ mL/ min for above substrates in Turkish Black radish. The higher value of V_{max} and K_m ratio for guaiacol represents guaiacol as preferential substrate than H_2O_2 .

Kinetics of thermal denaturation: Thermostability is the ability of enzymes to resist against thermal unfolding at elevated temperatures in the absence of substrate. The results of thermal inactivation of *Citrus reticulata* var. Kinnow peroxidase at different temperatures are shown in Fig. 5. The Arrhenius plot to calculate energy of activation for thermal denaturation (E_a) is shown in Fig. 6. It is obvious from the results that the half life of the *Citrus reticulata* var. Kinnow peroxidase is 223 min at 60°C and decreases with increase in temperature until it remains 30.37 min at 80°C (Table 1). The free energy (G°) of thermal unfolding was 109.18 kJ/mol at 60°C. With increasing temperature slight decrease in the value of free energy was observed which again started increasing at 75°C and reached at 110.06 kJ/mol, indicating resistance of *Citrus reticulata* var. Kinnow peroxidase against higher temperatures. This value is comparable to cauliflower peroxidase (109.73 kJ/mol), reported by Kalsoom *et al.* (2010). Thermodynamically *Citrus reticulata* var. Kinnow peroxidase can be considered as a stable enzyme, having high G° . The entropy of inactivation entropy (S°) exhibited almost constant value (-0.048 J/mol/K) at all temperatures which represented the ordered state of the active site of the orange peroxidase. Plant peroxidases from different sources exhibit differential thermostability. A peroxidase extracted from wheat bran exhibited a sharp decrease in activity even heating at 40°C for 5 min whereas a turnip anionic peroxidase retained good activity (21 %) even after 25 min heating at 80°C (Manu and Rao, 2009; Duarte -Vazquez *et al.*, 2003).

Effect of urea: Incubation of *Citrus reticulata* var. Kinnow peroxidase with 8.0 M urea for 60 min resulted in the retention of 84.22% activity (Fig. 7) which proved orange peroxidase to be quite resistant against chaotropic agent, urea. Fatima *et al.* (2007) reported that BGP retained 88 % its activity after 120 min. It is obvious that citrus peroxidase could be used in organic environment for potential application

Table-1. Kinetics and thermodynamics of irreversible thermal inactivation of *Citrus reticulata* var. Kinnow peroxidase

Temp. (K)	K_d (min ⁻¹)	$t_{1/2}$ (min)	H° (kJ mol ⁻¹)	G° (kJ mol ⁻¹)	S° (Jmol ⁻¹ K ⁻¹)
333	5.178×10^{-5}	223.04	93.085	109.18	-0.0483
338	9.762×10^{-5}	118.32	93.044	109.08	-0.0474
343	1.798×10^{-4}	64.25	93.002	108.99	-0.0466
348	2.464×10^{-4}	46.87	92.961	109.71	-0.0481
353	3.804×10^{-4}	30.37	92.919	110.06	-0.0485

$E_a = 95.86$ kJ/mol calculated from Fig. 6

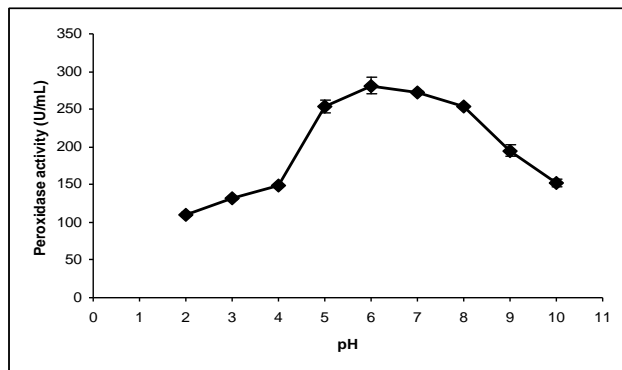


Fig. 1. Effect of pH on the activity of *Citrus reticulata* var. Kinnow peroxidase

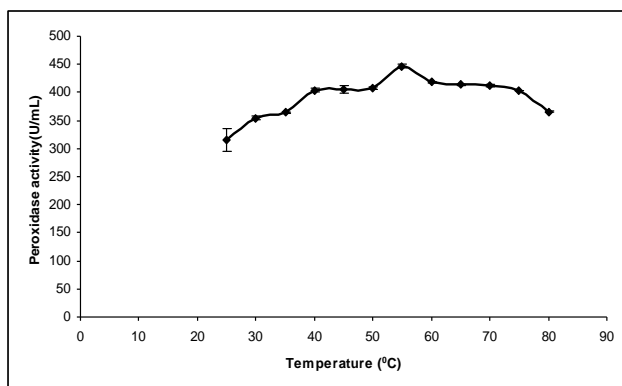


Fig. 2. Effect of temperature on the activity of *Citrus reticulata* var. Kinnow peroxidase

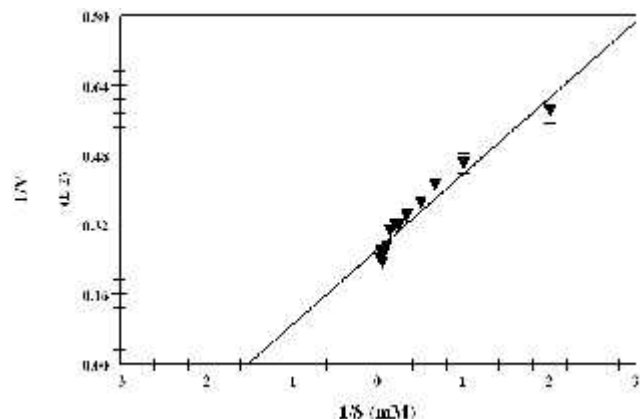


Fig. 3. Effect of guaiacol concentration on the activity of *Citrus reticulata* var. Kinnow peroxidase

Effect of surfactants: In this study the effect of different surfactants including Triton-X-100, Tween 80, SDS and other detergents commonly used in every household and laundry was checked on activity of *Citrus reticulata* var. Kinnow peroxidase. Fig. 8 shows that all the detergents decreased the peroxidase activity with the exception of Lemon Max which enhanced the peroxidase activity. Kalsoom *et al.* (2010) has also reported a similar trend in case of peroxidase isolated from cauliflower.

Effect of metal ions: A wide variety of proteins and enzymes incorporate metal ions or metal complexes into their overall structure and trigger enhancement of their activity. Peroxidase activity was assayed in the normal manner in the presence of 2 mM of various metal ions and slight increase in activity was observed in case of Mg²⁺, Mn²⁺, Cd²⁺, Cu²⁺ and Al³⁺ however Co²⁺, Cr³⁺, Hg²⁺, Ca²⁺, Pb²⁺, Zn²⁺, Sr²⁺ and Ni²⁺ showed inhibitory effect on peroxidase activity (Fig. 9). The decrease in peroxidase activity with Co²⁺ and Zn²⁺ ions was also observed for isozymes of *Metroxylon sagu* (Onsa *et al.*, 2004) and low inhibitory effect of Ca²⁺ was reported by Ma'quez *et al.* (2008). In a previous study Hg²⁺, Zn²⁺, Co²⁺, Al³⁺ and Mn²⁺ slightly inhibited the chick pea peroxidase activity at a concentration of 1mM (Bhatti *et al.*, 2006) whereas Kumar *et al.* (2011) reported Mg²⁺, Mn²⁺ as stimulator of Santalum peroxidase at a concentration of 2 μM.

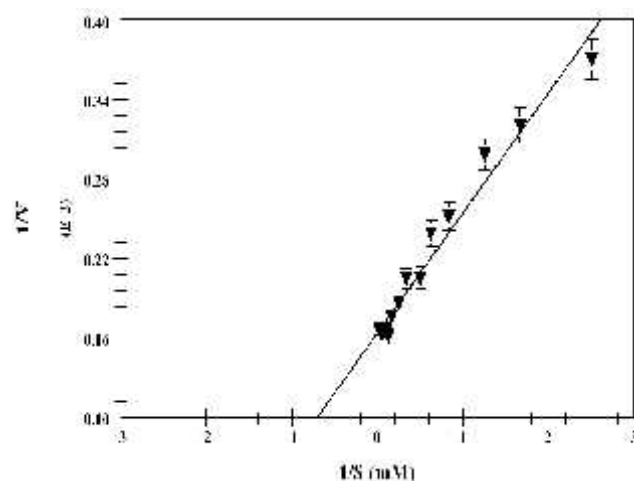


Fig. 4. Effect of H₂O₂ concentration on the activity of *Citrus reticulata* var. Kinnow peroxidase

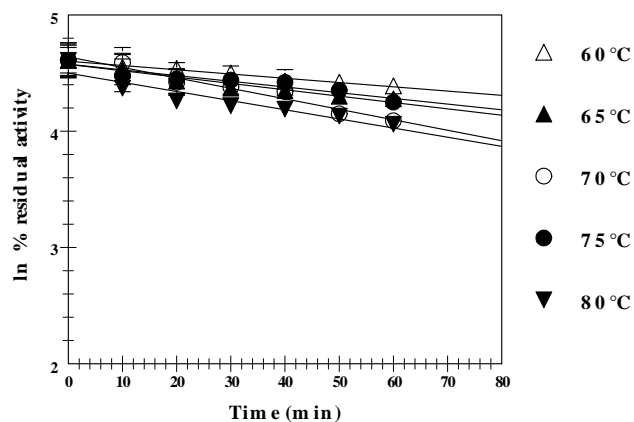


Fig. 5. Pseudo-first order plots of irreversible thermal inactivation of *Citrus reticulata* var. Kinnow peroxidase

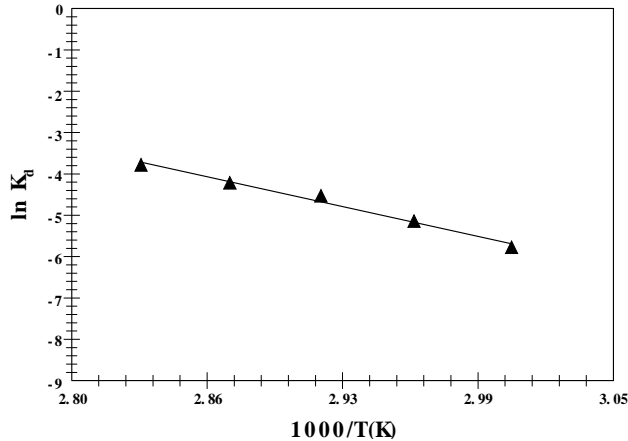


Fig. 6. Arrhenius plot for the determination of energy of activation (E_a) for irreversible thermal inactivation of *Citrus reticulata* var. Kinnow peroxidase

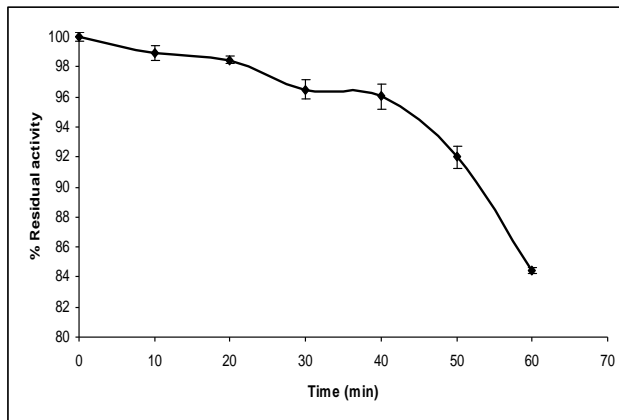


Fig. 7. Effect of urea on the activity of *Citrus reticulata* var. Kinnow peroxidase

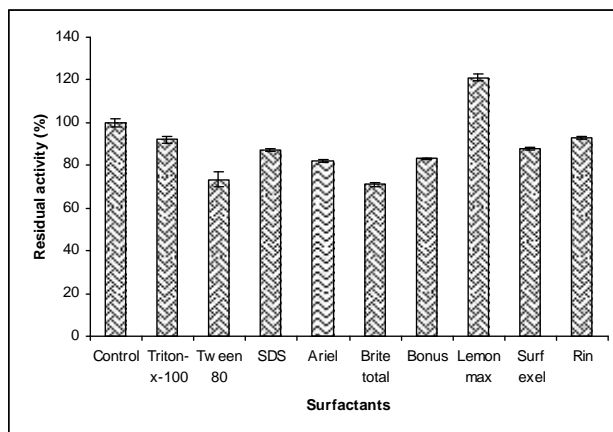


Fig. 8. Effect of surfactants on the activity of *Citrus reticulata* var. Kinnow peroxidase

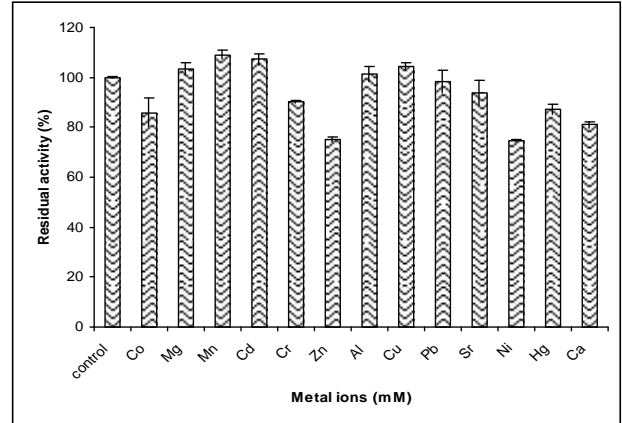


Fig. 9. Effect of metal ions on the activity of *Citrus reticulata* var. Kinnow peroxidase

Conclusions: The present findings revealed that peroxidase extracted from *Citrus reticulata* var Kinnow is very much stable at higher temperatures as well as against chaotropic agent urea. The enzyme could be used for commercial applications as peels of Kinnow are very much economical source for peroxidase extraction and Pakistan is one of the major producers of Kinnow in the world.

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