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Full Length Research Paper

Biochemical Characterization of Peroxidases from the Fruits of *Mallus pumilus*

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Ionically bound peroxidases were salt extracted and ethanol precipitated from the pulp of four Indian apple varieties *i.e.,* Chocklaty, Kali devi, Kinnaur and Maharaji. All peroxidases showed their temperature optima at 40°C. Various phenolic compounds activated all apple peroxidases by 25-240% but protocatechuic acid inhibited (32%) the Kali devi peroxidases. The activities from all apple varieties were strongly improved in the buffer containing metal ions like Fe^{2+} (169-364%) and Cu^{2+} (3-103%), indicating their role as cofactors. Mg^{2+} inhibited (23%) the peroxidases from Chocklaty, Kinnaur and Kali devi. 25-45% inhibition was observed in all the apple peroxidases by Mn^{2+}. Zn^{2+} established an inhibition (24-38%) to Chocklaty, Kinnaur and Maharaji peroxidases. Methionine, proline, tryptophan and valine stimulated all apple peroxidases. Cysteine stimulated (100%) the Maharaji peroxidases but inhibited (17-57 %) the others.

Keywords: Apple Peroxidases, Amino acids, Phenolic compounds, Metal ions, Kinetics.

INTRODUCTION

Class III peroxidases or the secreted plant peroxidases (EC 1.11.1.7, donor: hydrogen-peroxide oxidoreductase) are found only in plants, where they form large multigenic families (Bakalovic et al., 2006; Cosio and Dunand, 2008). The high number of isoenzymes and their remarkable catalytic versatility allow them to be involved in a broad range of physiological and developmental processes all along the plant life cycle (Almagro et al., 2009). Peroxidases oxidize several substrates in the presence of hydrogen peroxide and usually contain a protoporphyrin IX prosthetic group (Vianello et al., 1997; López-Serrano et al., 2004) and have various physiological roles in plant cells such as plant hormone regulation (Gutiérrez et al., 2009), defense mechanisms towards pathogens (Kuzaniak and Sklodowska, 2005; Almagro et al., 2009), control of cell elongation (De Gara , 2004), polymerization of extension (Ahmed et al., 1995), detoxification of reactive oxygen species (Mika et al., 2004), hydrogen peroxide scavenging (Mika et al., 2004), lignin polymerization (Fagerstedt et al., 2010), suberization processes (Quiroga et al., 2000; Kolattukudy et al., 2001) and various abiotic stresses, including UV (Kim et al., 2007; Krishnamurthy et al., 2009), air pollution (Lee, 2002), heat (Lin et al., 2010) and cold (Tao et al., 1998).. Plant peroxidases are hypothesized to mediate changes in the mechanical properties of plant cell walls by catalyzing cross-linking of cell wall components resulting in the formation of diferuloyl bridges between pectin

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residues and isodityrosine bridges between hydroxyproline-rich extensin molecule (Deepak et al., 2010).

Color, flavor, texture and nutritional value are four attributes considered by consumers in evaluating food quality. Color may be influenced by naturally occurring pigments such as chlorophylls, carotenoids and anthocyanins in food, or by pigments resulting from both enzymatic and non-enzymatic reactions. Enzymatic browning results mostly from polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (Šukalović et al., 2010; Slatnar et al., 2010). Thus, the control of activities of these enzymes is very importance in preservation of foods (Prabha and Patwardhan, 1986; Clement and Robinso, 1995).Besides these peroxidases play important role in the estimation of blood sugar and cholesterol, immunoassays, biosensors (Hamid and Khalil-ur-Rehmana, 2009), treatment of waste water containing phenols and aromatic amines (Husain and Husain, 2008; Hamid and Khalil-ur-Rehmana, 2009), biobleaching processes (Machii et al., 2006), lignin degradation in fuel, production of dimeric alkaloids, oxidations, and biotransformation of organic compounds (Kumar et al., 2008).

In the present study, we have discussed the biochemical characterization of peroxidases from apple varieties (Chocklaty, Kali devi, Kinnaur and Maharaji. Kali devi) of India.

EXPERIMENTAL

Experimental Plant Materials

Various varieties of apples *i.e.,* Chocklaty, Kali devi, Kinnaur and Maharaji were collected at their ripened stage from different parts of Northern India. The fruits were cleaned with sterile water, peeled and pulp was obtained.

Preparation of Crude Enzyme

Juice was separated after crushing 250 gm of apple pulp with a mortar and pestle and pressed through cheesecloth. Pulp was suspended in 125 mL of salt solution (0.5 M $CaCl_2$, 0.5 M NaCl, and 0.125 M $MgCl_2$) for one hour at room temperature. The suspension was passed through the cheesecloth and the filtrate was centrifuged at 15,000 rpm for 15 min. The supernatant was collected and used as a source of crude enzyme (Kumar et al., 2008).

Solvent Precipitation

The crude enzyme was precipitated with double volume of chilled absolute ethanol and centrifuged at 15,000 rpm for 15 min. Pellets were collected, dissolved in 10 mL of deionised water, and used as a source of ionically bound peroxidases (Kumar et al., 2008).

Enzyme Assay

Peroxidase activity was measured by a change in absorbance at 470 nm, due to oxidation of o-dianisidine (250 µl) in the presence of hydrogen peroxidase (500 nM) in one milliliter reaction mixture (Dubey et al., 2007). One unit of enzyme activity is defined as the amount of enzyme producing a 0.001 absorbance change per minute under the standard assay conditions (Kumar et al., 2008; Dalal et al., 2010).

Determination of Temperature Optima and Thermal Stability

To determine the optimum temperature, various peroxidases were incubated with substrate in the temperature range of 20-80^{o}C and the activity was measured (Kumar et al., 2008; Dalal et al., 2010). Stability of enzyme was determined by incubating peroxidase, substrates and buffer for 30 min, samples were withdrawn at regular intervals of time, rapidly cooled in an ice bath, and residual activity was calculated.

Determination of pH Optimum

Sodium acetate (4.0–6.0) and Tris-HCl (pH 7-9) buffers (100mM) were used under standard assay conditions for determining optimum pH (Dubey et al., 2007 ; Márquez et al., 2008; Motamed et al.,2009).The relative peroxidase activity (%) was calculated after assaying the enzyme at different buffers.

Effect of Effectors (Metal Ions, Phenolic Compounds and Amino Acids)

Metal ions ($FeSO_4$, $CuSO_4$, $MgSO_4$, $MnCl_2$, $ZnSO_4$: 0-4mM), amino acids (cysteine, methionine, proline,

tryptophan, valine: 0-10 mM) and phenolic compounds like hydroxycinemic acid derivatives such as caffeic acid (0–0.45 mM), ferulic acid (0–3.5 mM), p-coumaric acid (0–6.0 mM), and a hydroxybenzoic acid derivative like protocatechuic acid (0–6.0 mM) was incubated with fixed enzyme concentrations and the relative activity (%) was measured (Kumar et al., 2008).

Determination of Kinetic Constants

The apparent K_M and V_{max} were determined from the Lineweaver-Burk plot at optimum pH and temperature conditions (Kumar et al., 2008).

RESULTS AND DISCUSSION

Effect of pH

Figure 1 showed pH optima of 4.5 for all apple peroxidases, however another pH optima was recorded for Chocklaty (6.0), Kali devi and Kinnaur (7.0) peroxidases indicating the possibilities of isozymes (Dubey et al., 2007) obtained pH optima in the range of 5.0-7.0 from other apple varieties *i.e.,* Golden delicious HP, Golden delicious JK, Red delicious and Royal delicious. Similarly a broad pH range from 5.0 to 7.0 was determined by Deepak et al. (2010) for silk peroxidase. Acidic pH optima of the peroxidases were reported from vegetable sources (Vamos-Vigyazo,1981 ; Motamed et al., 2009), *Cassia didymobotrya* (Vitali et al., 1998) and soyabean (Kamal and Behere, 2003 ; Ryan et al., 2006). A maximal reduction of beta strands and beta turns at pH 5.5, causing the haem to be further exposed to the solvent and increasing the overall conformational flexibility of the protein (Kamal and Behere, 2003).

Effect of Temperature and Thermostability

All the apple peroxidases *i.e.* Chocklaty, Kinnaur, Kali Devi and Maharaji peroxidase observed optimum temperature and thermostability a 40°C (Figure 2). Dubey et al. (2007) observed temperature optima and thermostability in the range of 50-60°C for all the apple varieties. Reversible conformations in cases of horse radish peroxidases were observed between 20–55°C, while their activity was lost at a temperature of 60°C or above (Artiukhov et al., 2003 ; Kumar et al ., 2008).

Heat lability of apple peroxidase in the present study was supported by Dubey et al. (2007) and Moulding et al. (1987). Similar thermostability was also reported in the peroxidases of peach (Neves and Lourenc, 1990), apple (Dubey et al., 2007), papaya (Silva et al., 1990), orange (Mohamed et al., 2008), ricinus, (Kumar et al., 2008) and turnip (Dalal et al., 2010).

Effect of Effectors (Metal Ions, Phenolic Compounds and Amino Acids)

The effect of metal ions on peroxidase activity is shown in Figure 3. All apple peroxidases were strongly stimulated with Fe^{2+} recording maximum increase of 364% with Kali devi variety, however, Cu^{2+} increased the activity in the range of 3-103%, thereby, indicating their potential role as cofactor. Zaalishvili et. al. (1990) observed that at low Cu^{2+} concentrations, the binding of these ions to the high-affinity sites of the enzyme increases the polymerase activity, whereas at high Cu^{2+} concentrations, the binding may also occur to sites with a lower affinity for the metal, which results in the inhibition of the enzyme activity. Dubey et al. (2007) recorded similar trends of stimulation for Fe^{2+} and Cu^{2+}. Mg^{2+} recorded 23% inhibition (2-3 mM) in Chocklaty, Kinnaur and Kali devi peroxidases but slight activation (4%) was established for Maharaji. Dubey et al. (2007) observed 2.5 fold more inhibition for Mg^{2+}. Mn^{2+} inhibited (25-45% at 1-4 mM) all the apple peroxidases. This study was similar to other apple peroxidases (Dubey et al., 2007) and large lima bean seed peroxidases (Wang et al., 2008). Zn^{2+} established an inhibition (24-38% at 2-4 mM) to Chocklaty, Kinnaur and Maharaji peroxidases but Kali devi peroxidases observed a slight stimulation. Dubey et al. (2007) observed comparatively less inhibition but Wang et al. (2008) recorded a stimulation to large lima bean seed peroxidases in the presence of Zn^{2+}.

Further stimulation of 25-240% was showed for all apple peroxidases with caffeic acid, ferulic acid and p-coumaric acid (Figure 4). These results are similar to other apple peroxidase (Dubey et al., 2007).Similarly types of results were also established for Ricinus peroxidase in the presence of ferulic acid and (0.02-0.08 µM) and caffeic acid (1.0-4.5 µM) (Kumar et al., 2008). Protocatechuic acid showed 32% inhibition for Kali devi peroxidase, marginal stimulation of 3 and 10% to Chocklaty and Kinnaur peroxidases and 100% stimulation to Maharaji peroxidase. Similar results were reported by Dubey et al. 2007. Similarly, the derivatives

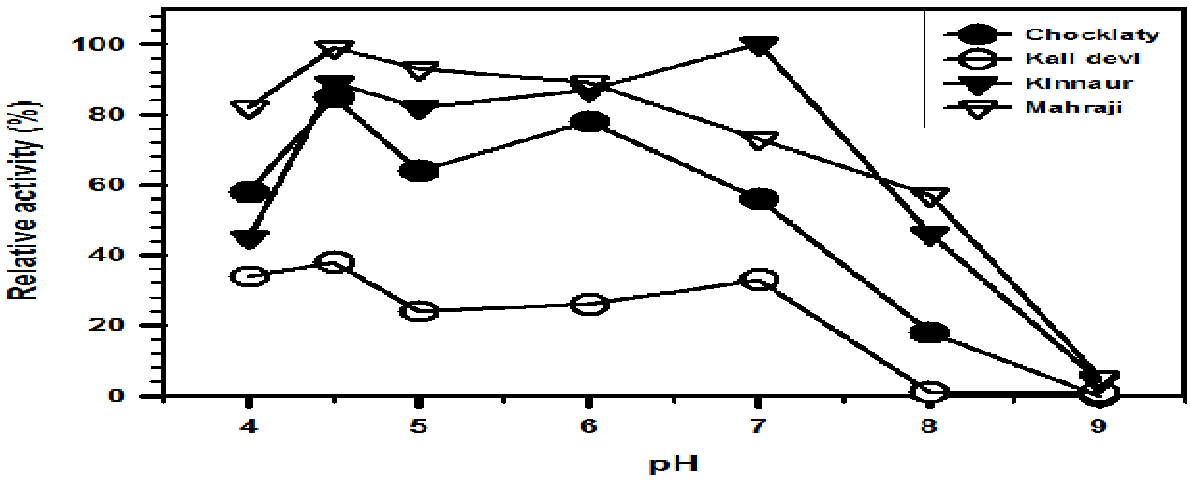

Figure 1. Effect of pH on the activity of apple peroxidases.

of hydroxybenzoic acid (protoctatechuic acid) act as potent inhibitor for *Ricinus* peroxidases (Kumar et al., 2008).

Methionine, proline, and valine stimulated (38-260% at 2.5-10 mM) all apple peroxidases (Figure 5).These results are similar to other apple peroxidases (Dubey et al., 2007). L-cysteine inhibited peroxidases from Kali devi (57% at 5.0 mM), Chocklaty (21% at 2.5 mM) and Kinnaur (17% at 5.0 mM) but stimulated the Maharaji (100% at 2.5 mM) peroxidases. Dubey et al. (2007) reported similar results in the presence of Methionine, proline and valine but L-cysteine observed 12% more inhibition for peroxidases from Golden delicious JK and stimulated the rest of the varieties (4-55%). Similarly DL-methionine and DL-valine worked as inhibitors for *Ricinus* peroxidase at 4.0 mM concentration while L-cyteine worked as a potent inhibitor at a very low concentration (0.4 mM). However, D-alanine and L-proline activated the of *Ricinus* peroxidase activity (Kumar et al., 2008).

The apparent K_M (substrate concentration at 1/2 the maximum velocity) and V_{max} were determined from Lineweaver-Burk plots (1/V vs 1/[S]) by following the standard assay conditions at optimum temperature and pH conditions (Kumar et al., 2008). Higher value of V_{max}/K_m of Chocklaty, Kali devi, Kinnaur and Maharaji indicated a preferential action of the enzyme for hydrogen peroxide as compared to o-dianisidine (Table 1). Similar findings were also observed by Dubey et al. (2007) on other apple peroxidases. Contrary results were reported from peroxidases of papaya (Silva et al., 1990), ricinus (Kumar et al., 2008) and kiwifruit (Soda et al., 1991).

CONCLUSION

This paper established some unique finding in comparison to previous study on apple peroxidases. Ionically bound peroxidases extracted from four Indian apple varieties *i.e.,* Chocklaty, Kali devi, Kinnaur and Maharaji showed their temperature optima and thermostability at 40°C. Mild inhibition was observed for all the apple peroxidases in the presence of Mn^{2+}. Zn^{2+} stimulated the Kali devi peroxidase and inhibited the others. Cysteine stimulated the Mahraji peroxidase and inhibited all other peroxdases. Protocatechuic acid inhibited Kali devi peroxidases and stimulated the others.

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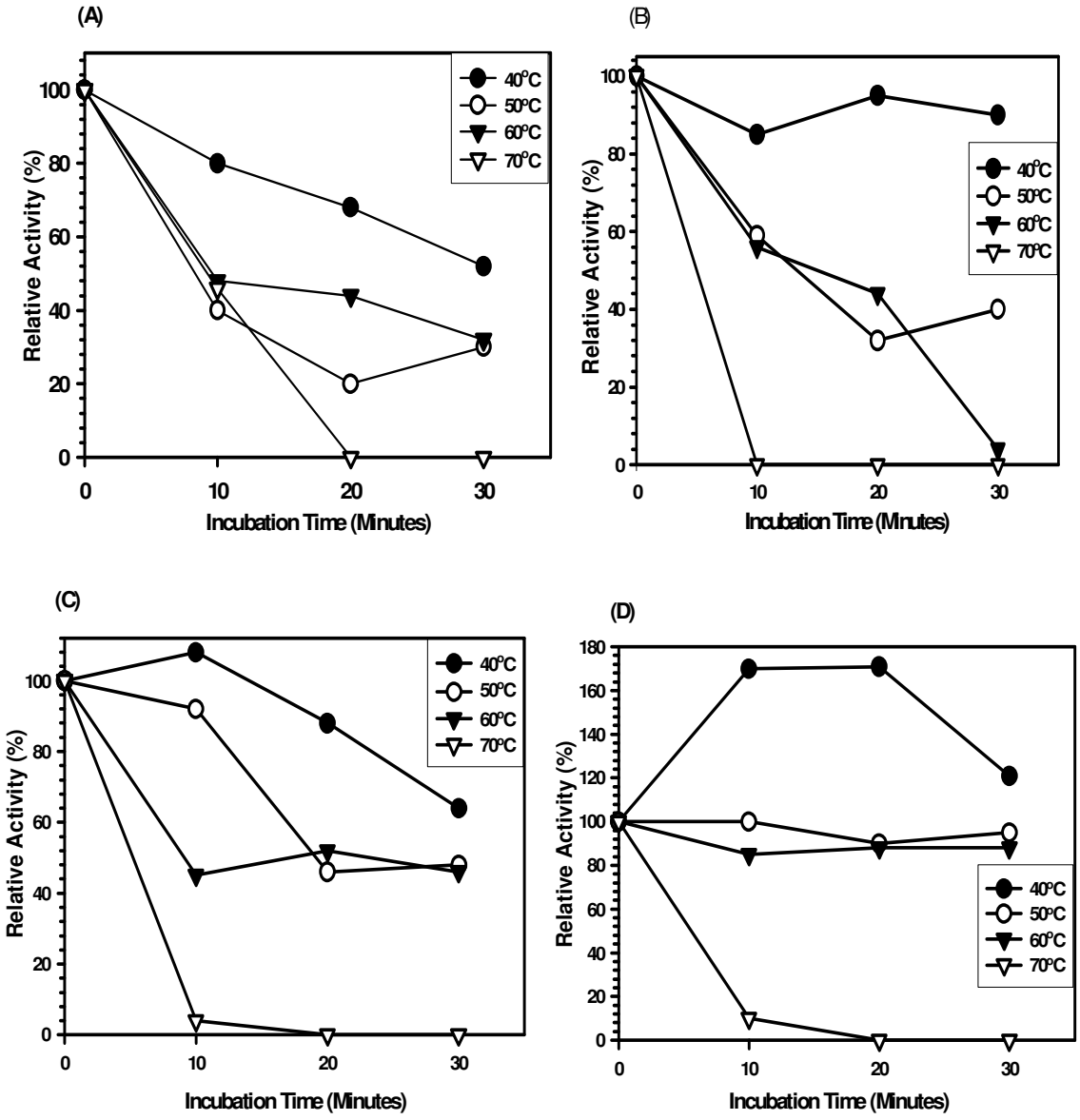


Figure 2. Temperature stability of apple peroxidases.
A): Temperature stability of Chocklaty peroxidase.
B): Temperature stability of Kali devi peroxidase.
C): Temperature stability of Kinnaur peroxidase.
D): Temperature stability of Maharaji peroxidase.

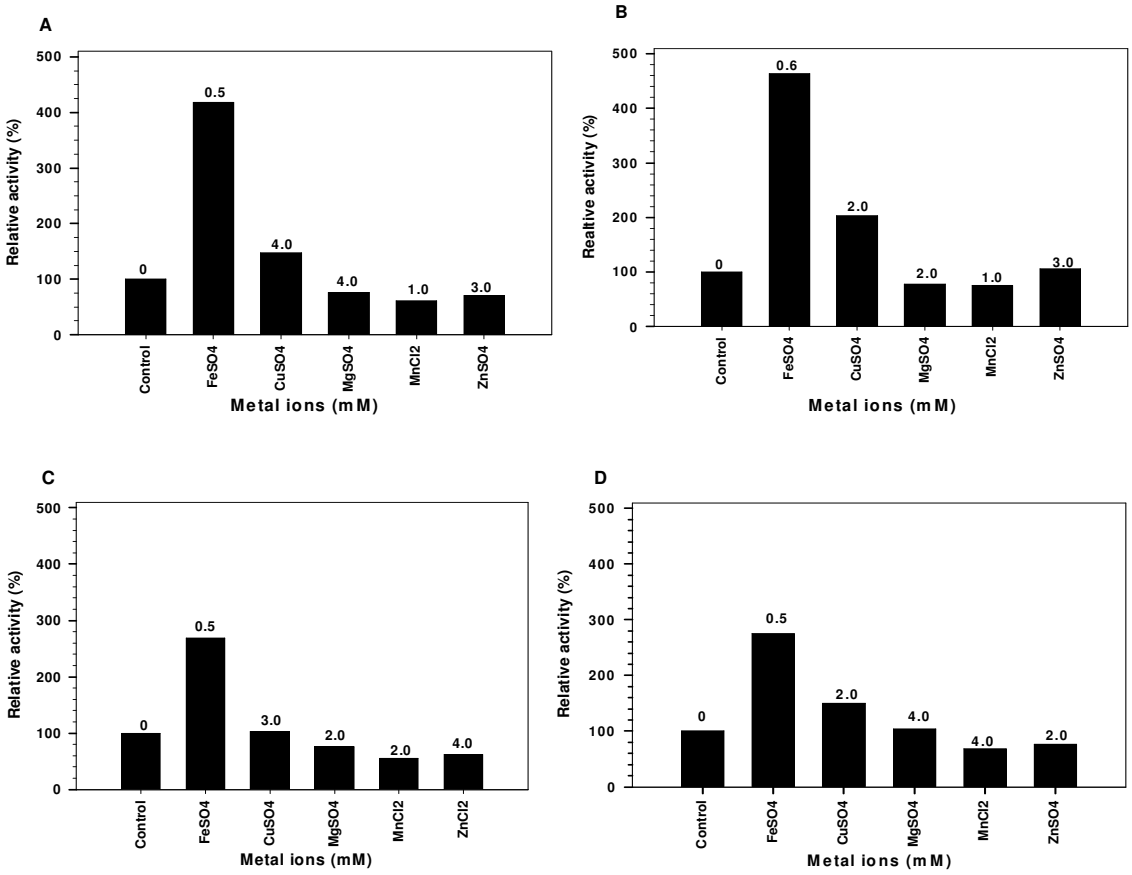
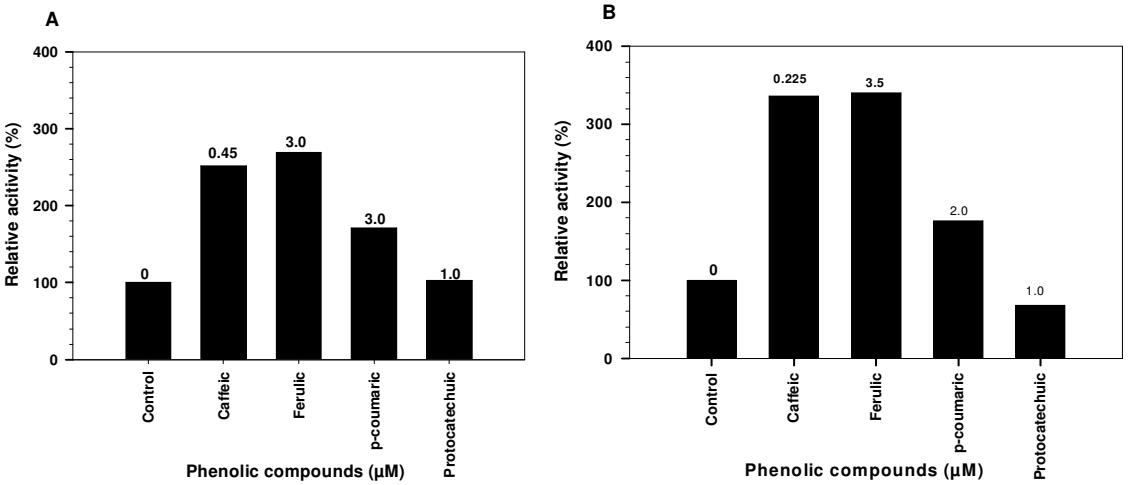


Figure 3. Effect of metal ions on the apple peroxidases.
A): Effect of metal ions on the peroxidase of Chocklaty.
B): Effect of metal ions on the peroxidase of Kali devi.
C): Effect of metal ions on the peroxidase of Kinnaur.
D): Effect of metal ions on the peroxidase of Mahraji.



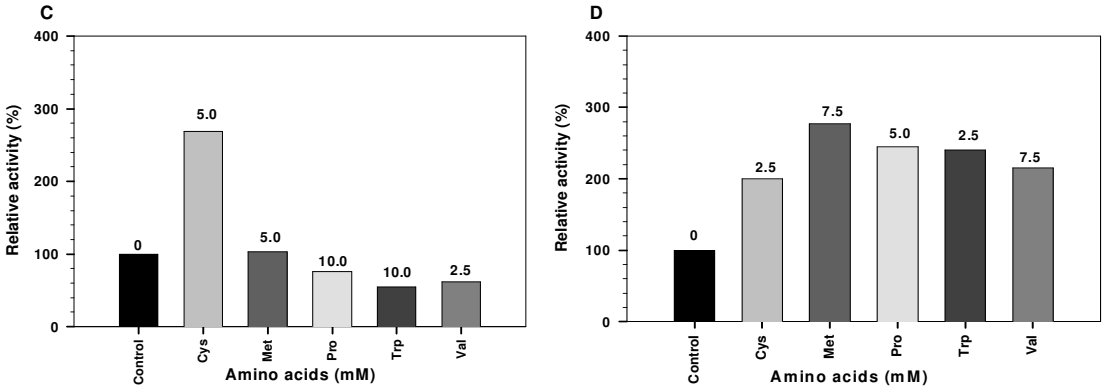


Figure 4. Effect of phenolic compounds on the apple peroxidases.
A): Effect of phenolic compounds on the peroxidase of Chocklaty.
B): Effect of phenolic compounds on the peroxidase of Kali devi.
C): Effect of phenolic compounds on the peroxidase of Kinnaur.
D): Effect of phenolic compounds on the peroxidase of Maharaji.

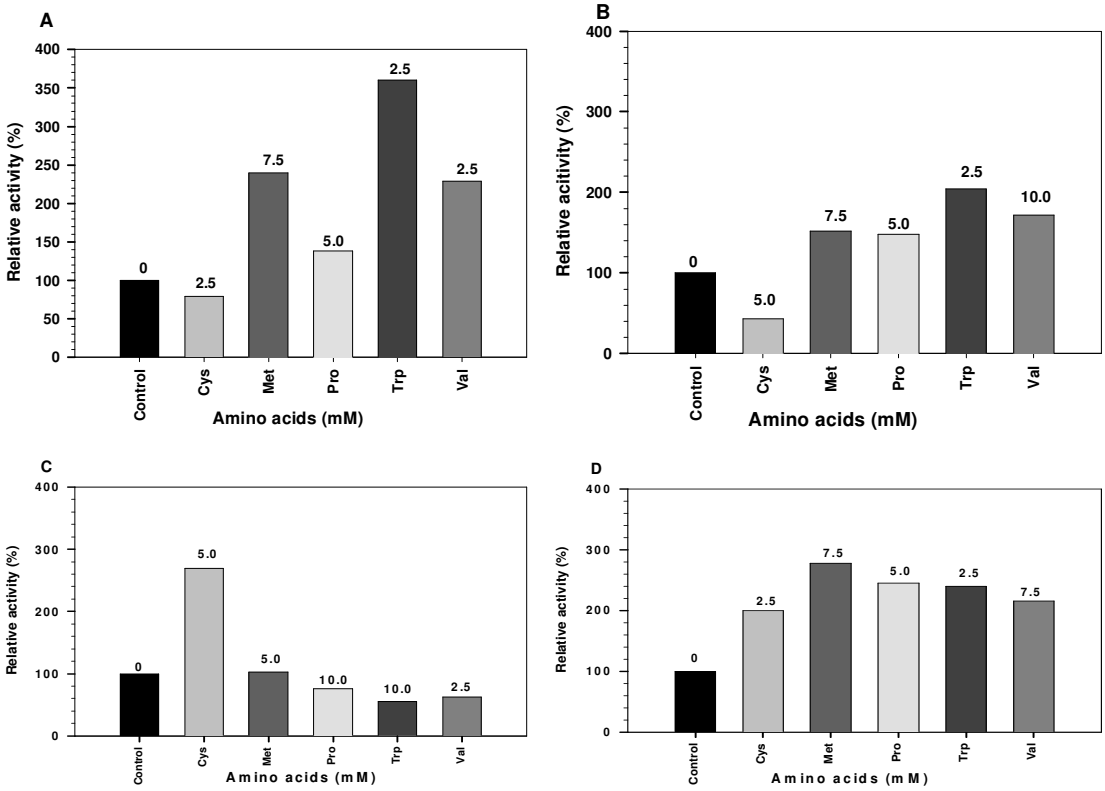


Figure 5. Effect of amino acids on the apple peroxidases.
A): Effect of amino acids on the peroxidase of Chocklaty.
B): Effect of amino acids on the peroxidase of Kali devi.
C): Effect of amino acids on the peroxidase of Kinnaur.
D): Effect of amino acids on the peroxidase of Mahraji.

Table 1. Kinetics of ionically bound peroxidases from different apple varieties of India.

Apple Varieties	o-dianisidine V_{max}/ K_M (Units/min/mL)	H_2O_2 V_{max}/ K_M (Units/min/mL)
Chocklaty	117	30,769
Kali devi	50	29,386
Kinnaur	40	51,760
Maharaji	357	66,138

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