

Full Length Research Paper

The effect of heavy metals on peroxidase from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers

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Peroxidases (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase, POD) are part of a large group of enzymes. In this study, peroxidase, a primer antioxidant enzyme, was partial purified with 2.49 fold and 29.3% efficiency from Jerusalem artichoke (*Helianthus tuberosus* L.) by ammonium sulphate precipitation and dialysis purification steps. The specific activity of enzyme was calculated as 612.1 EU/mg. The substrate specificity of peroxidase was investigated using 2-methoxyphenol (guaiacol)/hydrogen peroxide (H₂O₂) substrate pattern. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were calculated from Lineweaver-Burk graph for this substrate pattern. The enzyme had K_m values of 0.263 and 1.143 mM for guaiacol and H₂O₂, respectively. The enzyme had V_{max} of 33.3x10⁵ and 0.213x10⁵ EU/mL.min for guaiacol and H₂O₂, respectively. Also, the *in vitro* effect of some heavy metals such as iron (Fe²⁺ and Fe³⁺), cobalt (Co²⁺), strontium (Sr²⁺), zinc (Zn²⁺), mercury (Hg²⁺), nickel (Ni²⁺), aluminium (Al²⁺) and lead (Pb²⁺) on POD from Jerusalem artichoke (*H. tuberosus*) was evaluated. These heavy metals inhibited POD activity. IC₅₀ values, represents the inhibitor concentration required for obtaining 50% of inhibition of peroxidase. The above mentioned metals had IC₅₀ values of 12.58, 9.48, 12.59, 24.51, 13.57, 7.32, 10.57, 18.69 and 6.00 mM for Fe²⁺, Fe³⁺, Co²⁺, Sr²⁺, Zn²⁺, Hg²⁺, Ni²⁺, Al²⁺ and Pb²⁺, respectively.

Key words: Jerusalem artichoke, *Helianthus tuberosus*, peroxidase; enzyme, metal ions, inhibition.

INTRODUCTION

Peroxidase (POD) is a heme protein, which is a member of oxidoreductases [E.C.1.11.1.7] and catalyses the oxidation of a wide variety of organic and inorganic substrates in the presence of hydrogen peroxide (Banci, 1997; Yemenicioğlu et al., 1998; Köksal and Gülçin, 2008). Peroxidases are widely distributed in living organisms including microorganisms, plants and animals. POD and catalase are two major systems for the enzymatic removal of H₂O₂ and peroxidative damage of cell walls is controlled by the potency of antioxidative peroxidase enzyme system (Sreenivasulu et al., 1999; Velikova et al., 2000). This enzyme is one of the key enzymes controlling plant growth and development. POD is involved in various cellular processes including construction, rigidification and eventual lignifications of cell walls, protection of tissue from damage and infection by pathogenic microorganisms (Farrel et al., 1989; Sakharov et al., 2000). This enzyme participates in the formation of lignins in the secondary cell walls during normal growth (Sato et al., 1993; Pedreno et al., 1995) and in the formation of phenolic polymers such as lignins, suberins, etc when

plants are infected or wounded (Dixon and Palva, 1995; Köksal and Gülçin, 2008). POD is also widely used as an important reagent for clinical diagnosis and microanalytical immunoassay. Some applications for POD have been suggested in the medicinal, chemical and food industries (Kwak et al., 1996). Peroxidase has been used for food processing as an indicator of enzyme's stability (Keleş, 1986) and biotransformation of organic molecules (Adam et al., 1999; Gülçin and Yıldırım, 2005). Because of its broader catalytic activity, a wide range of chemicals can be modified using POD. Also, it can be used for other applications such as synthesis of various aromatic compounds, removal of phenolics from waste waters and the removal of peroxides from foodstuffs, beverages and industrial wastes (Torres et al., 1997). POD is also related to quality of plant commodities, particularly the flavour, in both raw and processed foods. POD activity is also correlated to fruit ripening as has been shown in a number of cases and it is also involved in enzymatic browning, either or together with polyphenol oxidase activity. A precise understanding of the implication of

POD in these mechanisms is an essential step towards a more efficient control of these undesirable reactions, particularly in heat-processed products, which frequently contain residual peroxidase activity (Cardinali, et al., 2007; Köksal and Gülçin, 2008).

Heavy metal pollution occurs in many industrial wastewaters such as those produced by metal plating facilities, mining operations, battery manufacturing processes, the production of paints and pigments, ammunition, ceramic and glass industries. These heavy metals are not biodegradable and their presence in streams and lakes leads to bioaccumulation in living organisms, causing health problems in animals, plants, and human beings (Oliver, 1997; Argun and Dursun, 2008).

Lead is ubiquitous and can be found naturally occurring in many different foods as metallic lead, inorganic ions and salts. Lead has no essential function in man, but has a number of adverse effects and young children and the developing foetus which are considered to be at most risk from its toxic effects (WHO, 1995). Mercury has a wide variety of effects, and range in value from therapeutic agents to lethal chemicals. Exposure to mercury vapor over periods of months or years can result in chronic poisoning (Harrison, 2002).

Aluminum and its compounds are used in the paper, glass and textile industries as well as in food additives. Raised concentrations of aluminium in water used for renal dialysis can lead to toxic effects (Harrison, 2002). Zinc is an essential element for all plants and animals. It is necessary for the correct function of various enzyme systems (Festa et al., 1985). Cobalt is used in the manufacture of alloys and in nuclear technology (Delves et al., 1996). Cobalt compounds are also included in trace element supplement preparations for ruminants. The cobalt concentrations in vegetables and other foods have been found to be between < 0.01 and 0.83 mg/kg (Ministry of Agriculture, 1985; Delves et al., 1996). Nickel salts are widely used in industry for plating and as pigments. Alloys of nickel are used in storage batteries, coins, cooking utensils and other products. Fats and oils are hydrogenated using a nickel catalyst. Nickel has not been shown to be an essential nutrient for humans, but is considered to be a normal constituent of the diet (Ministry of Agriculture, 1985; Harrison, 2002). The human body absorbs strontium as if it were calcium. Due to the elements being sufficiently similar chemically, the stable forms of strontium do not pose a significant health threat, but the radioactive strontium can lead to various bone disorders and diseases, including bone cancer (Reginster et al., 2005).

The ferrous state of iron (Fe^{2+}) accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction. Fe^{2+} ion is the most powerful prooxidant among the various species of metal ions (Gülçin, 2006a). However, ferric (Fe^{3+}) ions also produce radicals from peroxides although the rate is

tenfold less than that of ferrous (Fe^{2+}) ions (Gülçin, 2006b).

Living organisms require trace amounts of some heavy metals, including iron, cobalt, copper, manganese, molybdenum, vanadium, strontium, and zinc, but excessive levels can be detrimental to the organism. Other heavy metals such as mercury, lead and cadmium with the exception for the latter are toxic metals. They have no known vital or beneficial effect on organisms, and their accumulation over time in the bodies of mammals can cause serious illness (Duffus, 2002).

Jerusalem artichoke (*Helianthus tuberosus* L.) is native to North America and is presently cultivated in Europe, Asia and Australia. In Turkey, Jerusalem artichoke (*H. tuberosus*) is cultivated in all the regions. The tubers accumulate high levels of polysaccharides during their growth. On a dry weight basis, the tubers contain 68 - 83% fructans, 15 - 16% proteins, 13% insoluble fibre and 5% ash (Fleming and GrootWassink, 1979). Remarkably, its tubers do not contain starch. It has traditionally been used as food and animal feed and, more recently, as a raw material for the industrial production of fructose and fructans (Kosaric et al., 1984). In this study the *in vitro* effect of some heavy metals such as Fe^{2+} , Fe^{3+} , Co^{2+} , Sr^{2+} , Zn^{2+} , Hg^{2+} , Ni^{2+} , Al^{2+} and Pb^{2+} on POD from Jerusalem artichoke (*H. tuberosus*) tubers was investigated.

MATERIALS AND METHODS

Plant materials

Jerusalem artichoke (*H. tuberosus*) was obtained from a local market in Erzurum, Turkey. It was washed, drained, packed in polyethylene bags and stored at -83°C until required.

Preparation of Jerusalem artichoke (*H. tuberosus*) homogenate

The homogenate preparation procedures for POD were adapted from the methods described by Sakharov et al. (2002). For this purpose, 50 g Jerusalem artichoke (*H. tuberosus*) tubers were taken from frozen storage (-83°C) and ground in a mortar in the presence of liquid nitrogen. This powder then mixed with 150 mL of phosphate buffer (pH 7.0, 0.1 M) and subsequently the collard slurry was centrifuged at $13,000 \times g$ for 30 min at 4°C (Gülçin, 2002; Gülçin et al., 2005). The pellet was discarded (Gülçin et al., 2005).

Ammonium sulphate precipitation and dialysis

The crude extract was subjected to ammonium sulphate fractionation and the precipitate in the 20 - 70% saturation range was collected by centrifugation (30 min at $13,000 \times g$). The precipitate was suspended in about 2 mL phosphate buffers (pH 7.0, 0.1 M) and dialyzed for 12 h at 4°C against 1 L of the buffer (Köksal and Gülçin, 2008).

Peroxidase activity assay

The POD activity in the Jerusalem artichoke (*H. tuberosus*) sample

Table 1. Levels of purification of POD from Jerusalem artichoke (*H. tuberosus*) peroxidase obtained after the application of different purification steps leading to the improvement in the activity of enzyme.

| Purification step | Total volume (mL) | Enzyme activity (EU/mL.min) | Total enzyme activity (EU/mL.min) | Protein (mg/mL) | Total Protein (mg) | Specific activity (EU/mg) | Yield (%) | Purification fold |
|---|-------------------|-----------------------------|-----------------------------------|-----------------|--------------------|---------------------------|-----------|-------------------|
| Homogenate | 20 | 26840 | 536800 | 0.109 | 2.180 | 246.2 | 100 | - |
| (NH ₄) ₂ SO ₄ precipitation | 8 | 37480 | 299840 | 0.095 | 0.760 | 394.5 | 34.9 | 1.60 |
| Dialysis | 11 | 35500 | 390500 | 0.058 | 0.638 | 612.1 | 29.3 | 2.49 |

was measured using guaiacol substrate. Initial rates of free radical formation for substrate were monitored at maximum wavelength of 470 nm. The changes in absorbance were read for 3 min using a double beam UV-VIS spectrophotometer (CHEBIOS s.r.l.). Enzyme activity assay method was as described previously (Yoo and Kim, 1988; Gülçin et al., 2005). Briefly, an aliquot of enzyme sample (25 μ L) was added to a mixture of 1 mL 22.5 mM H₂O₂, 1 mL 45 mM guaiacol, and final volume of this mixture was adjusted to 3 mL by addition of phosphate buffer (pH 7.0, 0.1 M). The change in the absorbance at above wavelength monitored for 3 min at 20°C. One unit of peroxidase activity was defined as 0.01 ΔA_{470} per min (Halpin et al., 1989; Gülçin et al., 2005).

Quantitative protein determination

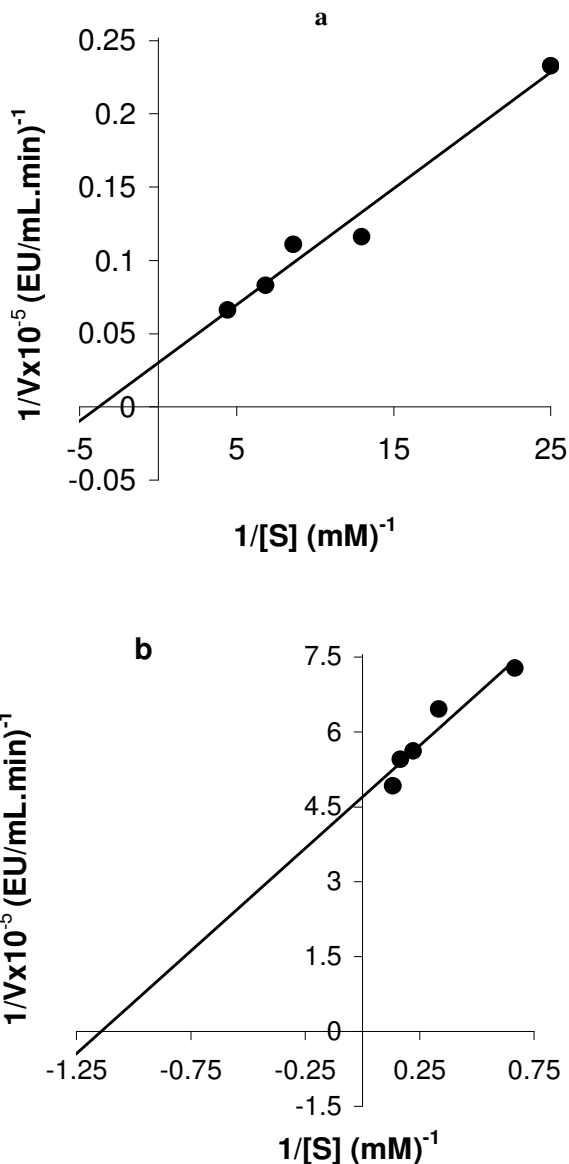
Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford's method (1976), with bovine serum albumin as standard (Gülçin et al., 2004).

RESULTS AND DISCUSSION

Extraction of POD was carried out in 0.1 M phosphate buffer (pH 7.0). Then the enzyme was precipitated with (NH₄)₂SO₄. Several precipitations with solid (NH₄)₂SO₄ between 0 - 10%, 10 - 20%, 20 - 30%, 30 - 40%, 40 - 50%, 50 - 60%, 60 - 70% and 70 - 80% were conducted to find the optimum saturation point. POD activity of the precipitate from 20 - 70% (NH₄)₂SO₄ was found to have the highest activity and this saturation point was used in all the extraction processes. Following ammonium sulfate precipitation, the enzyme extract was dialyzed. The degree of purification of POD was 2.49-fold after dialysis (Table 1).

POD activity was assayed using guaiacol/H₂O₂ substrate pattern. The rate of the reaction was measured in terms of the increase in absorbance. To determine the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), POD activities were measured with variable substrate concentrations in the standard reaction mixture. The K_m and V_{max} of POD for each substrate was calculated from a plot of $1/V$ and $1/[S]$ by the method of Lineweaver and Burk (1934). As can be seen in Figure 1, the enzyme had K_m values of 0.263 and 1.143 mM for guaiacol and H₂O₂, respectively. Also, the enzyme had V_{max} of 33.3×10^5 and 0.213×10^5 EU/mL.min for guaiacol and H₂O₂, respectively.

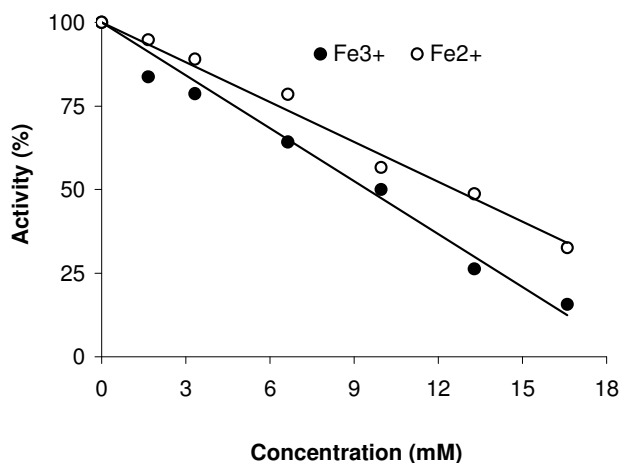
The K_m value for guaiacol is lower than that of H₂O₂,

**Figure 1.** The Lineweaver-Burk plots for 2-methoxyphenol (guaiacol) (a) and hydrogen peroxide (H₂O₂) (b) substrates.

suggesting the higher affinity of POD for guaiacol when compared with H₂O₂. K_m value was found as 141.64 mM for cauliflower peroxidase by using guaiacol substrate

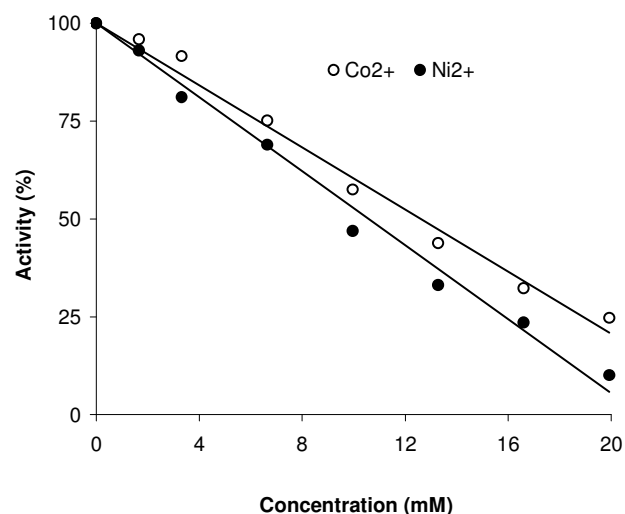
Table 2. IC₅₀ values of different heavy metals on peroxidase from Jerusalem artichoke (*H. tuberosus*)

| Heavy metals | EC ₅₀ values (mM) |
|------------------|------------------------------|
| Fe ²⁺ | 12.58 |
| Fe ³⁺ | 9.48 |
| Co ²⁺ | 12.59 |
| Sr ²⁺ | 24.51 |
| Pb ²⁺ | 6.00 |
| Hg ²⁺ | 7.32 |
| Ni ²⁺ | 10.57 |
| Al ²⁺ | 18.69 |
| Zn ²⁺ | 13.57 |

**Figure 2.** The effect of ferrous (Fe²⁺) and ferric ions (Fe³⁺) on POD from Jerusalem artichoke (*H. tuberosus*) tubers.

(Köksal and Gülçin, 2008). In another study, this value was found as 5.5 mM for *Brassica oleracea* L. var. *Acephala* peroxidase by using guaiacol substrate (Gülçin and Yıldırım, 2005). They were higher than that of Jerusalem artichoke (*H. tuberosus* L.) tubers K_m. The lower K_m value indicates higher substrate affinity. The results obtained from present study showed that Jerusalem artichoke tuber POD has greater substrate affinity for guaiacol than above mentioned plants. Likewise, Jerusalem artichoke (*H. tuberosus* L.) tubers POD had higher V_{max} values than the enzyme from both plants. When the guaiacol used as substrate, *B. oleracea* L. var. *Acephala* and cauliflower demonstrated V_{max} values as 5000 and 7500 EU/mL.min, respectively. However, Jerusalem artichoke (*H. tuberosus* L.) tubers POD had V_{max} value as 33.3x10⁵ EU/mL.min using the same substrate.

Inhibition effects of many substances such as medical drugs, various metals, anions and pesticides have been reported in the literature (Casini et al., 2002; Beydemir and Gülçin, 2004; Ekinci et al., 2007). Many chemicals

**Figure 3.** The effect of cobalt (Co²⁺) and nickel ions (Ni²⁺) on POD from Jerusalem artichoke (*H. tuberosus*) tubers.

affect metabolism by changing normal enzyme activity, particularly inhibition of a specific enzyme and the effects can be dramatic and systemic (Christensen et al., 1982). Hence, heavy metals have various toxicological effects on living organisms. For example, it has been reported that heavy metals, such as mercury and cadmium, exert a toxic action in a synergistic fashion with salinity (Christensen et al., 1982; Vitale et al., 1999). Also, it has been expressed that the binding of heavy metals with membrane transport ligands can alter their catalytic function (Rainbow and Dallinger, 1993; Vitale et al., 1999).

In the present study, Fe (II) and (III), Co (II), Sr (II), Zn (II), Hg (II), Ni (II), Al (II) and Pb (II) were chosen for investigation of their inhibitory effects on POD from Jerusalem artichoke (*H. tuberosus*). IC₅₀ parameters of these metals for peroxidase from Jerusalem artichoke (*H. tuberosus*) were determined. It was found that the heavy metals were potent inhibitors of peroxidase from Jerusalem artichoke (*H. tuberosus*). As can be seen in Table 2 and Figures 2 - 5, IC₅₀ values of 12.58 and 9.48 mM for Fe²⁺ and Fe³⁺ (Figure 2), 12.59 and 10.57 mM for Co²⁺ and Ni²⁺ (Figure 3), 18.69, 24.51 and 13.57 mM for Al²⁺, Sr²⁺ and Zn²⁺ (Figure 4), 7.32 and 6.00 mM for Hg²⁺ and Pb²⁺ (Figure 5). Pb²⁺ was found to be an effective inhibitor for POD from Jerusalem artichoke (*H. tuberosus*) in used heavy metal examples. Also, Sr²⁺ exhibited the lowest inhibitory effect on POD from Jerusalem artichoke (*H. tuberosus*).

In conclusion, living organisms require trace amounts of some heavy metals, including iron, cobalt, copper, manganese, molybdenum, vanadium, strontium, and zinc, but excessive levels can be detrimental to the organism, as they can inhibit a large number of enzymes.

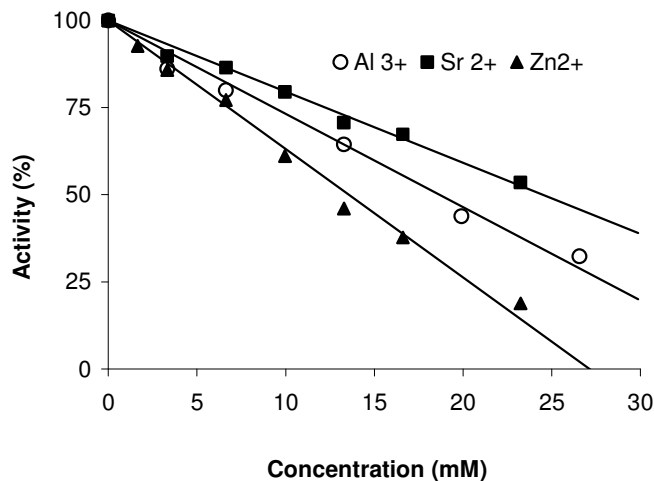


Figure 4. The effect of aluminium (Al^{3+}), strontium ions (Sr^{2+}) and zinc (Zn^{2+}) on POD from Jerusalem artichoke (*H. tuberosus*) tubers.

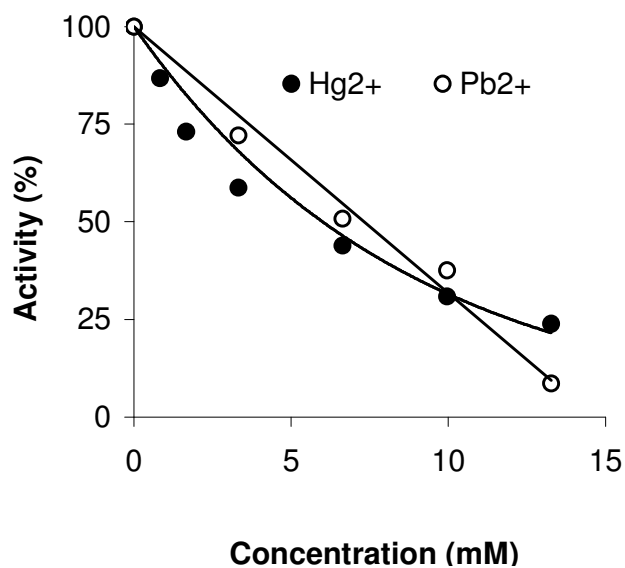


Figure 5. The effect of mercury (Hg^{2+}) and lead (Pb^{2+}) on POD from Jerusalem artichoke (*H. tuberosus*) tubers.

These heavy metals inhibited POD from Jerusalem artichoke (*H. tuberosus*).

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