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Full Length Research Paper

Properties of peroxidase from chewing stick miswak

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Miswak is a chewing stick prepared from the roots, twigs or stems of *Salvadora persica L.* and widely used in Middle Eastern and Estern African cultures. Currently, its chemical components had antimicrobial and antioxidant activities. In the present study, peroxidase, as antioxidant and antibacterial enzyme, was screened in 4 parts of miswak and the level of peroxidase activity was recorded in the order of peel of stem > root without peel > peel of root > stem without peel. Generally, the people used the root without peel. By chromatography of miswak root without peel on DEAE-Spharose 3 peroxidases POI, POII and POIII were separated. Peroxidase POII with highest activity was reached to homogeneity by chromatography on Sephacryl S-200. The molecular weight of POII was found to be 70 kDa. o-Phenylenediamine was found to be the best substrate for the enzyme followed by guaiacol, o-dianisidine, pyrogallol and p-aminoantipyrine. The apparent Km for catalysis of H_2O_2 and guaiacol were 0.9 and 17.33 mM respectively. The enzyme had an optimum pH and temperature at 5.5 and 40°C respectively. POII was stable at 10 to 40°C and unstable above 50°C. Most of the examined metal ions had partially inhibitory effects on POII, while Co^{2+}, Fe^{3+} and Ca^{2+} were able to greatly enhance the activity. The metal chelators caused moderate inhibitory effects. The enzyme was highly inhibited by mercptoethanol, cysteine and ascorbic acid, while thiourea had moderate inhibitory effect. POII exhibited high resistance to protyolysis by trypsin. The highest storage stability at 4°C was detected for POII in powder form compared to liquid form.

Key words: Miswak, *Salvadora persica*, chewing stick, peroxidase.

INTRODUCTION

The use of the chewing stick (miswak) for cleaning teeth is an ancient custom which remains widespread in many parts of the world (Hyson, 2003). The World Health Organization has recommended and encouraged the use of chewing sticks as an effective tool for oral hygiene in areas where such use is customary (WHO, 1987). *Salvadora persica* or arak is the major source of material for chewing sticks in Saudi Arabia and much of the Middle East (Eid et al., 1990). It has been shown that extracts of miswak posses various biological properties including significant antibacterial (Al-Lafi and Ababneh, 1995) and anti-fungal effects (Al-Bagieh et al., 1994). Extracts of S. persica and other related plants may be effective against the bacteria that are important for the development of dental plaque. Therefore, it has been claimed that miswak sticks may have anti-plaque effects and may also affect the pathogenesis of periodontal diseases by reducing the virulence of periodonto phathogenic bacteria (Homer et al., 1992). Almas et al. (2002) reported that miswak and chlorahexidine gluconate had the same effect on healthy human dentin.

The anti-microbial and cleaning effects of miswak have been attributed to various chemicals detectable in its extracts. These effects are believed to be due to its high content of sodium chloride and potassium chloride as well as salvadourea and salvadorine, saponins, tannins, vitamin C, silica and resin (Ezmirly and El-Nasr, 1981), in

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Abbreviations: H_2O_2, Hydrogen peroxide; POI, POII, POIII, Peroxidases.

addition to cyanogenic glycoside and benzylisothiocyanate. Thus, it has been reported that some anionic components naturally occurring in plant species exert anti-microbial activities against various bacteria. Thiocyanate (SCN-) acts as a substrate for salivary lactoperoxidase to generate hypothiocyanite (OSCN-) in the presence of hydrogen peroxide (Nishioka et al., 2003; Hannig et al., 2006; Aizenbud et al., 2008). OSCN- has been demonstrated to react with sulfhydryl groups in bacterial enzymes which in turn lead to bacterial death (Darout et al., 2000). Acid production in human dental plaque in vitro has been reported to be inversely proportional to the concentration of OSCN- in the test system, while supplementing saliva with hydrogen peroxide and SCN- inhibited acid production (Tenovuo et al., 1981). Sofrata et al. (2007) reported that miswak extract raised the plaque pH after an acidic challenge, suggesting that it may play a potential role in caries prevention. Oral bacteria especially Streptococci produce hydrogen peroxide (Ryan and Klleinberg, 1995). Hydrogen peroxide, one of reactive oxygen species, can damage a variety of molecules within cells, leading to oxidative stress.

Two important and interrelated factors are involved in the patho-physiological progression of periodontal diseases, that is, the activation of immune system and the production of oxygen radicals and their related metabolites. Increased production of oxygen radicals may contribute to oxidative stress, which is reported to be involved in many diseases, including periodontal diseases (Wei et al., 2004). Within 5 min of intravenous injection into rats, horseradish peroxidase (HRPO) was detected in the gingival fluid of the molar teeth. Thirty minutes after its injection, HRPO was accumulating in lysosome-like bodies in epithelial cells of the epithelial attachment and disappeared from these structures in 1 or 2 days. This lysosome-like activity of the epithelial attachment may play a role in the maintenance and defense of the underlying periodontal tissues (McDougall, 1970).

Peroxidases are heme-containing enzymes that oxidize a variety of xenobiotics by hydrogen peroxide (Saunders, 1973). Peroxidases reduce hydrogen peroxide and oxidize a wide number of compounds including phenols, aromatic amines, thioanisoles, halide ions, thyocianate ions, fatty acids and also degrade hydrogen peroxide (Vojinovic et al., 2007). Thus, peroxidases are oxidoreductases which use hydrogen peroxide as electron acceptor for catalyzing different oxidative reactions. Plant peroxidases appear to play a role in the metabolism of auxin, a hormone that signals growth: development. They also play a role in extracellular defense against pathogens and stress, biosynthesis and degradation of lignin, intracellular removal of hydrogen peroxide, and oxidation of toxic reductants. Plant peroxidases are induced by stress, for example, pathogen attack, wounding, heat, cold or draught, UV light (O'Brien, 2000). Horseradish peroxidase (HRP) is one of the most extensively studied enzymes. Practical uses of HRP include: removal of phenols from polluted water (Wu et al., 1999), organic syntheses (Wagner and Nicell, 2005). Applications for analytical purposes are also being investigated for cancer gene therapy (Azevedo et al., 2003).

Despite several studies had been focused on the miswak chemical components, which had antimicrobial and antioxidant activities, its bioactive compounds especially oxidoreductase enzymes such as peroxidase has not yet been established, where peroxidase act as antioxidant and antimicrobial enzyme. Screening of the peroxidase level in different parts of miswak and biochemical characterization of peroxidase was studied.

MATERIALS AND METHODS

Plant material

Miswak *Salvadora persica* L. (Salvadoraceae) was purchased from local market of Jeddah, Kingdom of Saudi Arabia.

Peroxidase assay

Peroxidase activity is carried out according to Miranda et al. (1995). The reaction mixture containing in one ml: 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and least amount of enzyme preparation. The change of absorbance at 470 nm due to guaiacol oxidation was followed at 30 second intervals. One unit of peroxidase activity is defined as the amount of enzyme which increases the O.D. 1.0 per min under standard assay conditions.

Protein determination

Protein was quantified by the method of Bradford (1976) with bovine serum albumin as standard.

Preparation of crude extract

Peroxidase crude extracts were prepared by homogenization the miswak in 20 mM Tris–HCl buffer, pH 7.2 using a mortar. The homogenates were centrifuged at 10,000 g and the supernatant was designated as crude extract.

Purification of peroxidase from root without peel

Unless otherwise stated all steps were performed at 4 to 7°C. The crude extract from root without peel was filtered though ultrafiltration membrane Cut-Off 10 kDa. The ultra-filtration fraction was applied directly to a DEAE–Sepharose column (4 × 1.6 cm i.d.) which was previously equilibrated with 20 mM Tris–HCl buffer, pH 7.2. The adsorbed material was eluted with a stepwise gradient ranging from 0 to 0.4 M NaCl prepared in the same buffer at a flow rate of 60 ml/h and 3 ml fractions were collected. Protein fractions exhibiting peroxidase activity were eluted with 0.0, 0.05 and 0.1 M NaCl, respectively and designated peroxidases POI, POII and POIII according to elution order. POII with the highest peroxidase activity was applied to a Sephacryl S-200 column (90 × 0.6 cm i.d.) which was previously equilibrated with 20 mM Tris–HCl buffer, pH 7.2. The enzyme was eluted with the same buffer.

Table 1. Screening of peroxidase activity in different parts of miswak.

Tissues	Units/g tissues	mg protein/g tissues	Specific activity (Units/mg protein)
Peel of stem	1527	2.5	610
Stem without peel	325	1.83	177
Peel of root	683	4.79	142
Root without peel	967	1.6	604

Molecular weight determination

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90 × 1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for the calibration curve.

Characterization of peroxidase

Optimum pH and temperature

The optimum pH for the peroxidase activity is determined by assaying the activity at different pH values, using the following buffers: 50 mM sodium acetate buffer (pH 3.6 to 6.5) and 50 mM Tris-HCl buffer (pH 7.0 to 9.0). The optimum temperature for peroxidase activity was determined by assaying the enzyme at temperatures from 20 to 70°C at pH 5.5.

Thermal stability

Heat stability is measured by incubating the enzyme at 20 to 70ºC for 15 min in 0.05 mM sodium acetate buffer, pH 5.5. After heat treatment, the enzyme solution is cooled and the residual activity assayed under standard assay conditions.

Kinetic constant

Kinetic parameters of the peroxidase for hydrogen peroxide and guaiacol as substrates are determined at pH 5.5. The values of Michael's constants (Km) were determined from Lineweaver Burk plot.

Substrate specificity

To determine the substrate specificity of the enzyme, a number of potential natural electron donors are used under the standard assay conditions.

Metal ion effect

The effects of various metal ions on enzyme activity were determined by pre-incubating the enzyme with 1 and 5 mM metal ions for 15 min and then assaying the enzyme activity. The activity in absence of metal ions is taken as 100%.

Effect of chemical compounds

The effect of metal chelators (EDTA, sodium citrate and sodium oxalate), mercptoethanol, cysteine, thiourea and ascorbic acid on the enzyme activity was determined by assaying the enzyme in presence of these compounds. The activity in absence of these compounds is taken as 100%.

Storage stability

Storage stability was detected by measuring of peroxidase activity in liquid and powder (lyophilization) forms of enzyme during 8 weeks at 4°C.

RESULTS AND DISCUSSION

Screening of peroxidase activity in different parts of miswak

Peroxidase activity was screened in four parts of miswak for the first time, peel of stem, stem without peel, peel of root and root without peel. Table 1 showed that the level of peroxidase activity was recorded in the order of peel of stem > root without peel > peel of root > stem without peel (Table 1). The specific activity (units/mg protein) had the same order except for peel of root where it had high level of protein. Generally, the people used the root of miswak without peel (chewing stick) which had high level of peroxidase (967 units/g). This study showed that the higher levels of peroxidase exist in the peel of stem (1527 units/g) and we recommended the people used this part of miswak. Mohamed et al. (2008a) demonstrated that the major pool of peroxidase activity was present in peel of some Egyptian citrus species and cultivars compared to the juice and pulp.

Purification of peroxidase from miswak root without peel

The purification of peroxidase from miswak root without peel, which is generally used as chewing stick, was summarized in Table 2. The crude extract with 2100 units and specific activity of 677 units/ mg protein was subjected to ultrafiltration using ultrafiltration membrane cut-off 10 kDa, where the ultrafiltration fraction had 2000 units and specific activity of 714 units/mg proteins. The ultrafiltration fraction was applied onto DEAE-Sepharose

Table 2. Purification scheme of peroxidases from miswak root without peel.

Sample	Total units*	Total protein (mg)	Specific activity (units/mg protein)	Fold purification	Recovery (%)
Crude extract	2100	3.1	677	1	100
Ultrafiltration fraction	2000	2.8	714	1.05	95
DEAE- Sepharose					
0.0 M NaCl (POI)	516	0.27	1876	2.77	24
0.05 M NaCl (POII)	840	0.3	2800	4.13	40
0.1 M NaCl (POIII)	70	0.36	194	0.2	3
Sephacryl S-200 POII	450	0.1	4500	6.6	21

*One unit of peroxidase activity is defined as the amount of enzyme which increases the O.D. 1.0 per min under standard assay conditions.

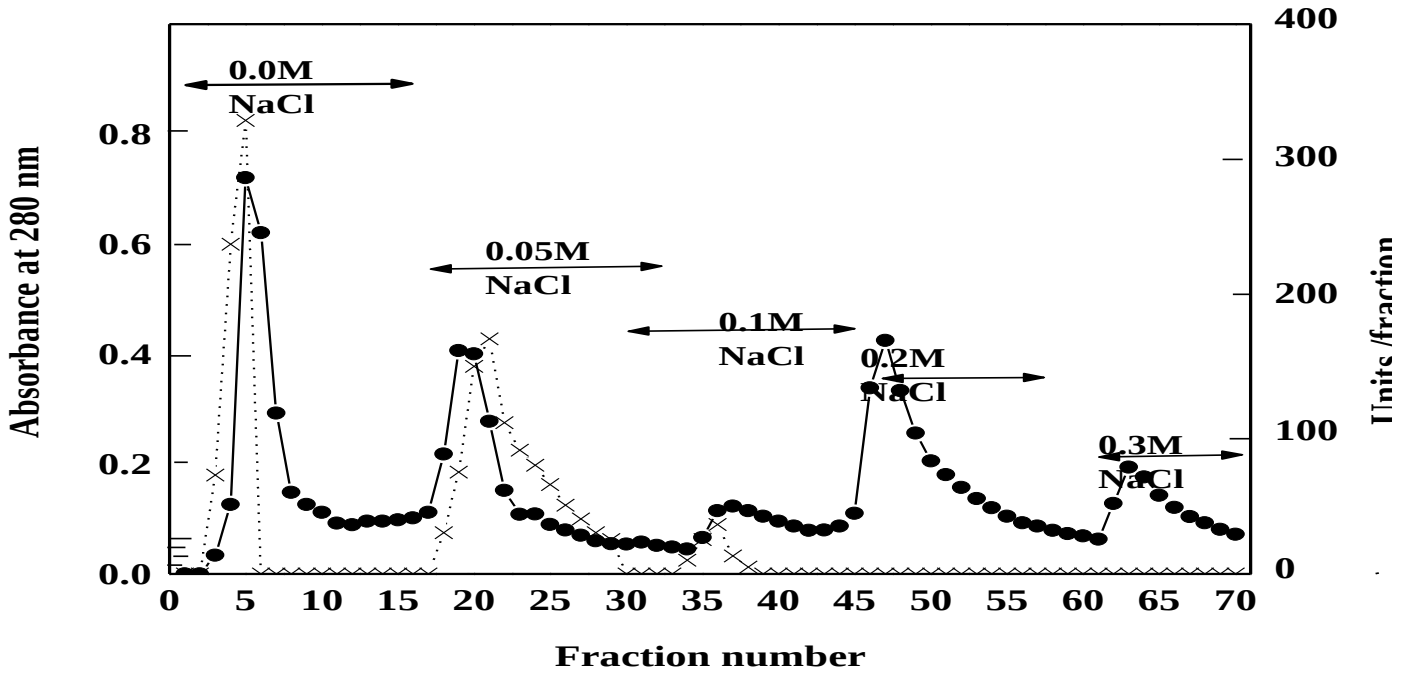

Figure 1. A typical elution profile for the chromatography of miswak root without peel peroxidase on DEAE-Spharose column (4 x 1.6 cm i.d.) equilibrated with 20 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions. Absorbance at 280 nm (•—•), peroxidase activity (x---x).

column (4 × 1.6 cm i.d.). From the elution profile of the chromatography on DEAE-Sepharose column, three forms of peroxidases were separated POI, POII and POIII (Figure 1) with specific activity of 1876, 2800 and 194 units/mg protein and 24.5, 40.0 and 3.0% recovery, respectively. A number of cationic and anionic isoperoxidases from C. sinenis (Clemente, 1998) and apple (Valderrama and Clemente, 2004) were detected. Four anionic and one cationic peroxidase isoenzymes from Citrus Jambhiri peel were detected using DEAE-cellulose and CM-cellulose columns (Mohamed et al., 2008a). It has been suggested that the number of peroxidase isoenzymes from the same kind of fruit may differs depending on ecological and environmental differences, as well as differences in variety, and also differences in stage of maturity and detection techniques (Gaspar et al., 1982). The presence of more than one peroxidase enzyme in miswak supports the hypothesis that a gene family gives rise to multiple isoenzymes belonging to class III peroxidase as in several other plant species (Boucoiran et al., 2000). The complete purification was restricted on miswak POII with highest peroxidase activity. A Sephacryl S-200 column was used to obtain POII with the highest possible specific activities of 4500 units/mg protein which represented 6.6fold purification over the crude extract with 21% recovery

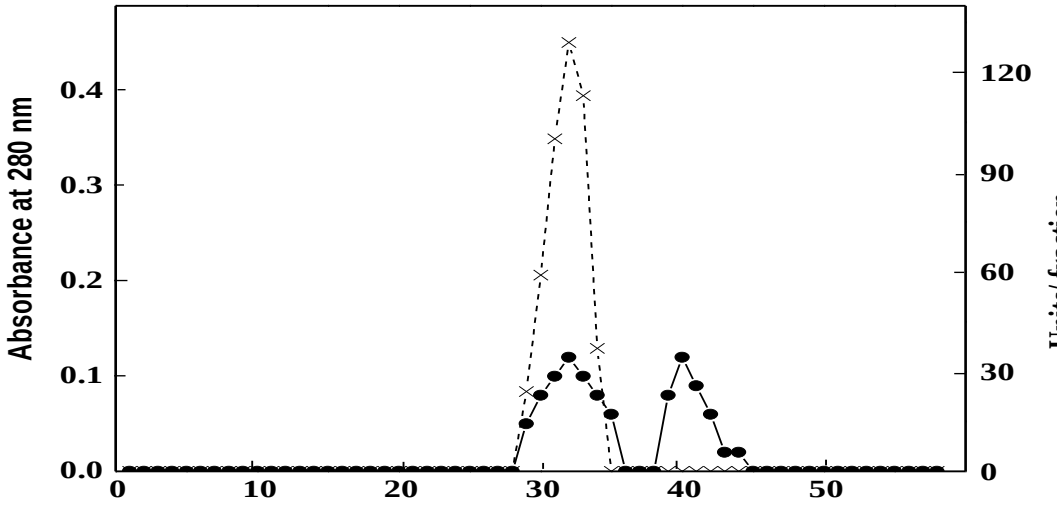

Figure 2. A typical elution profile for the chromatography of miswak DEAE-Sepharose fraction POII on Sephacryl S-200 column (90 × 1.6 cm i.d.) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions, absorbance at 280 nm (•—•), peroxidase activity (x ----x).

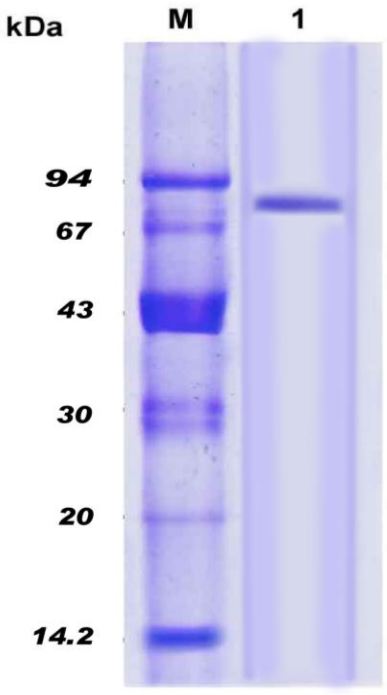

Figure 3. SDS-PAGE for homogeneity and molecular weight determination of Miswak POII. M- Protein markers; 1- Sephacryl S-200 POII.

(Figure 2). The homogeneity of the purified POII was detected by SDS-PAGE (Figure 3).

Molecular mass

Peroxidases have a molecular weight ranging from 30 kDa to 150 kDa (Hamid and Khalil-ur-Rehman, 2009). The purified enzyme was chromatography on Sephacryl S-200, which was calibrated with standard proteins. The molecular weight of the enzyme was calculated from the plot of Ve/Vo versus log of molecular weight and was found to be 70 kDa. The purified enzyme showed a single protein band on SDS-PAGE (Figure 3) and the molecular weight was found to be 70 kDa. Similar molecular weight was detected for peroxidase from marular fruit (71 kDa) (Mdluli, 2005). However, it was different from the molecular weight of turnip peroxidase and horseradish peroxidases, which were reported, have molecular weights of 49 kDa and 40-46 kDa, respectively (Duarte-Vazquez et al., 2003).

Substrate specificity

Different compounds were tested as hydrogen donor substrates for POII. o-Phenylenediamine was found to be the best substrate for the enzyme followed by guaiacol, o-dianisidine, pyrogallol and p-aminoantipyrine (Table 3). Similar results were reported for peroxidase from *Cirus jambhiri* cv. Adalia, where the enzyme had highest affinity toward o-phenylenediamine compared with the other tested substrates (Mohamed et al., 2008a). As the enzyme showed wide substrate specificity POII may belong to class III of the plant peroxidase superfamily with EC 1.11.1.7 (donor : hydrogen peroxide

Table 3. Relative activities of miswak POII toward different substrates.

Substrate	Relative activity (%)
Guaiacol	100
o-Phenylenediamine	198
o-Dianisidine	77
Pyrogallol	14
p-Aminoantipyrine	7

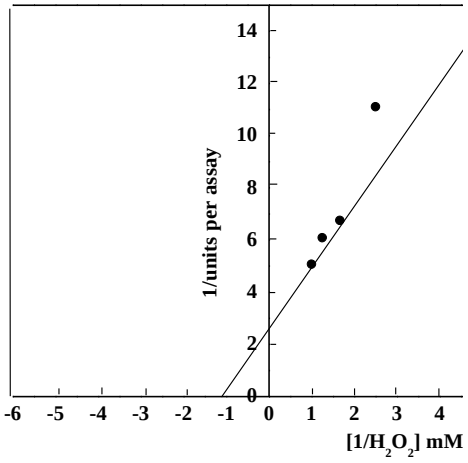

Figure 4. Lineweaver-Burk plot relating miswak POII reaction velocity to H_2O_2 concentrations. Each point represents the average of two experiments.

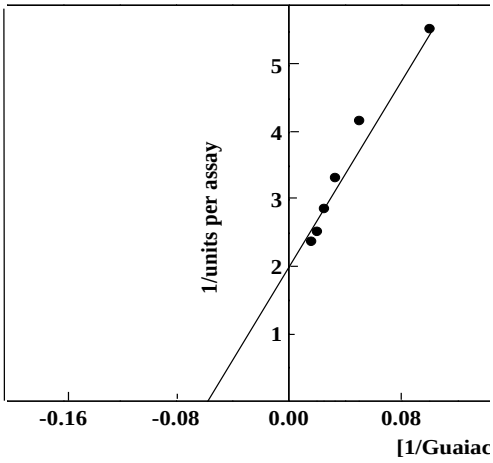

Figure 5. Lineweaver-Burk plot relating miswak POII reaction velocity to guaiacol concentrations. Each point represents the average of two experiments.

oxidoreductase), similar to horseradish peroxidase or seed coat soy protein (Veitch, 2004).

Km value

Using guaiacol as a reducing substrate, the activity of POII showed a Michaelis-Menten relationship at varying H_2O_2 concentrations (Figure 4). The Km value of H_2O_2 calculated from the Linewever-Burk plot is 0.9 mM for POII. The significance of low Km value for H2O2 reflects a high number of H_2 or hydrophobic interactions between the substrate and the heme group at the enzyme active site (Richard-Forget and Gauillard, 1997). This affinity of interaction with H_2O_2 was higher than that reported for some peroxidases from marula fruit (1.77 mM, Mdluli, 2005), Brussels sprouts (11.4 and 6.2 mM Regalado et al., 1999), and pear (1.5 mM, Richard-Forget and Gauillard, 1997). However, Duarte-Vazquez et al. (2000) reported higher affinity for H_2O_2 interaction with turnip peroxidase isoenzyme C1 at 0.04 mM, while the isoenzyme C2 and C3 showed higher Km values of 0.25 and 0.85 mM. Guaiacol showed lower affinity toward POII, where its Km value is 17.33 mM (Figure 5). Various Km values, using guaiacol, were reported for peroxidases from buckwheat seeds (0.202-0.288 mM, Suzuki et al., 2006), *Pleargonium graveolense* (7.3 mM, Seok et al., 2001), Brassica napus (3.7 mM, Duarte-Vazquez et al., 2001) and *Cirus jambhiri* cv. adalia (5 mM, Mohamed et al., 2008a).

pH optimum

The effect of pH on the activity of miswak POII was shown in Figure 6. The enzyme had a pH optimum of 5.5, in sodium acetate buffer, with guaiacol as substrate. Around 60 to 70% of the enzyme activity was retained at pH's ranged from 4.5 to 7.5. The same pH optimum was detected for peroxidase from *Cirus jambhiri* cv. Adalia (Mohamed et al., 2008a). *Withania sommifera* peroxidases were optimally active at pH 5.0 in sodium acetate buffer (Johri et al., 2005). Both *Cucumis sativus* peroxidases showed a bell-shaped pH dependence of the catalytic activity, which reaches a maximum between 5.5 and 7.5 and sharply decreases outside interval (Battistuzzi et al., 2001). The POD isoenzymes from broccoli were reported to have an optimum pH of 6.0 for neutral POD and 4.0 for acidic POD (Thongsook and Barrett, 2005).

Effect of temperature

Miswak POII showed its maximum activity at 40°C and retained more than 50% of activity at temperature 20 to 50°C (Figure 7). This optimal temperature was higher than that reported for peroxidases from Vanilla (16°C, Marquez et al., 2008) and buckwheat seed (10 to 30°C, Suzuki et al., 2006). POII was stable at 10 to 40°C and unstable above 50°C (Figure 8). However, the low thermal stabilities of POX I and II from buckwheat seeds

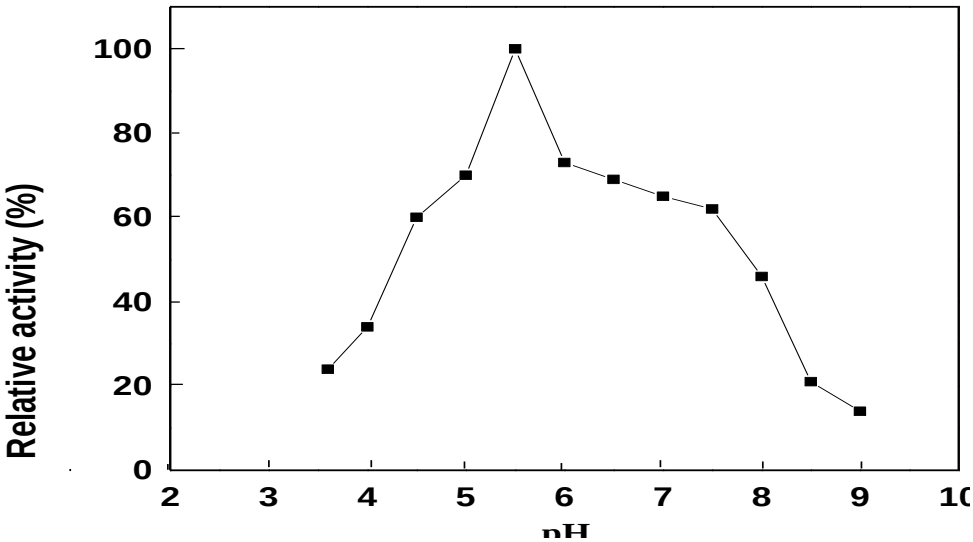

Figure 6. pH optimum of miswak POII. Each point represents the average of two experiments.

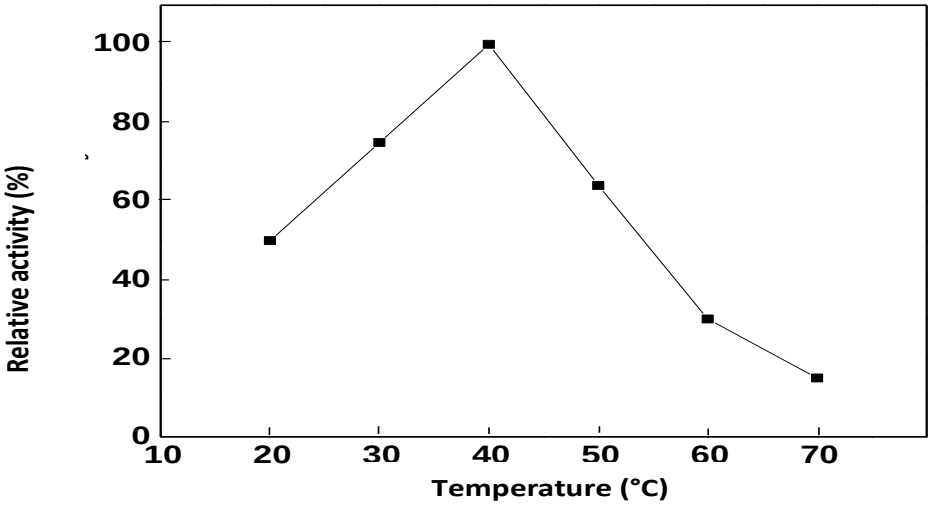

Figure 7. Temperature optimum of miswak POII. The enzyme activity was measured at various temperatures using the standard assay method as previously described. Each point represents the average of two experiments.

were detected. POX I was stable at 0 to 30ºC and unstable above 40ºC, whereas POX II was stable at 20ºC and unstable above 30ºC. POX I and II were inactivated at 60 and 50ºC respectively (Suzuki et al., 2006). In addition, the purified isoperoxidases from orange juice lost about from 15 to 80% of their original activity when exposed to 70ºC for 50 s compared with a loss of about 85% when present as a mixture in the soluble crude extract (Clemente, 1998). However, the resistant of enzyme against temperature depends on the source of the enzyme as well as on the assay conditions, especially pH and the nature of the substrate employed. The variability in the heat stability of peroxidase can be attributed largely to the particular enzyme structure. Non-covalent, electrostatic and hydrophobic interactions of individual isoenzymes determine enzyme folding and stability, as well as extra ion pairs, hydrogen bonds and the degree of glycosylation, which has also been found to play a role in enzyme stability (Adams, 1991). It has also been shown that the thermal stability of peroxidase is mainly due to the presence of a large number of cysteine residues in the polypeptide chain (Deepa and

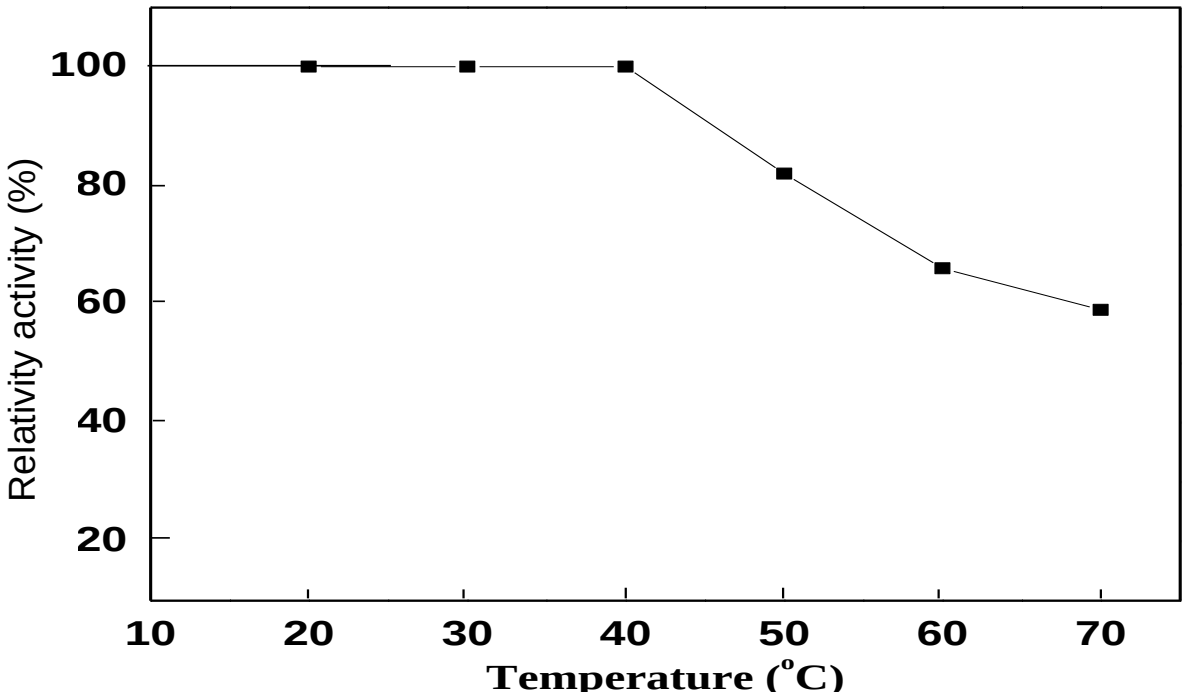

Figure 8. Effect of temperature on the thermal stability of miswak POII. The reaction mixture contained in 1.0 ml: 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and suitable amount of enzyme. The reaction mixture was preincubated at various temperatures for 15 min prior to substrate addition, followed by cooling in ice bath. Activity at zero time was taken as 100 % activity. Each point represents the average of two experiments.

Table 4. Effect of metal ions on miswak POII.

Metal ions	% Relative activity	
	1 mM	5 mM
Control	100	100
Fe^{3+}	100	152
Co^{2+}	98	142
Ca^{2}	97	115
Cu^{2+}	86	80
Ni^{2+}	85	93
Pb^{2+}	88	87
Zn^{2+}	76	75
Hg^{2+}	43	20

Enzyme was pre-incubated for 15 min with metal ions prior to substrate addition. Each value represents the average of two experiments.

Arumughan, 2002).

Effect of metal ions

The activity of miswak POII was affected by the presence of metal ions. Cu^{2+}, Ni^{2+}, Pb3+, Ca^{2+} and Zn^{2+} had partially inhibitory effects on POII (Table 4), while Hg^{2+} at 5 mM had strong inhibitory effect. At concentration 5 mM, Co^{2+}, Fe^{3+} and Ca^{2+} were able to greatly enhance the activity of POII by 142, 152 and 115% respectively. Very little articles had been studied the effect of metals on the activity of peroxidase. Fe^{3+} is considered essential for the activity of most plant peroxidase enzymes as it is involved in binding of H_2O_2 and formation of compound I (Whitaker, 1994; Wong, 1995). Ajila and Rao (2009) reported that at 5 mM concentration, Fe^{3+} enhanced the activity of black gram peroxidase to 145%. Ca^{2+} is a cofactor that serves to maintain the conformational integrity of the enzyme's active site (Adams et al., 1996). Activation by Ca^{2+} was reported for peroxidase of avocado (Billaud et al., 1999).

Effect of chemical compounds

In this study, metal chelators (EDTA, sodium citrate and sodium oxalate) had moderate inhibitory effects on miswak POII, except EDTA which caused loss of 50% of activity (Table 5). Metal chelators were unable to fully combine with Fe^{3+} atom found in the POII active center. EDTA had moderate inhibitory effect on peroxidase from vanila (Marquez et al., 2008), while Fujita et al. (1995) reported unsuccessful inhibition of peroxidase by EDTA. Miswak POII was strongly inhibited in the presence of 1.0

Table 5. Effect of chemical compounds on miswak POII.

Chemical compound	Concentration (mM)	Relative activity (%)
Control	--	100
EDTA	1.0	82
	5.0	53
Sodium citrate	1.0	84
	5.0	82
Sodium oxalate	1.0	84
	5.0	79
Mercaptoethanol	0.1	65
	1.0	5
Cysteine	0.1	62
	1.0	9
Thiourea	0.1	64
	1.0	54
Ascorbic acid	0.1	25
	1.0	0.0

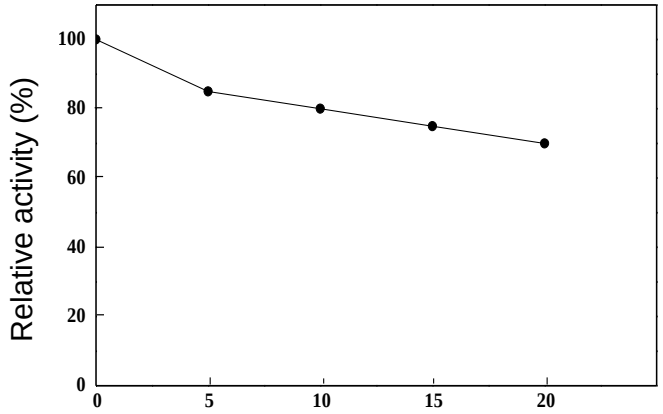

Figure 9. Effect of trypsin concentration on the activity of miswak POII. Enzyme was incubated with increasing concentration of trypsin (5-20 mg) in a total volume of 1.0 ml of 50 mM sodium acetate buffer, pH 5.5 for 1 h. Enzyme activity was assayed under standard assay conditions. Each point represents the average of two experiments.

mM mercptoethanol and cysteine (95 and 91% inhibition), while thiourea at 0.1 and 1.0 mM had moderate inhibitory effect (36 and 46% inhibition), suggesting that peroxidase had disulfide bond in the structure of peroxidase. Similar results were reported for peroxidase from vanilla (Marquez et al., 2008). Thiourea exhibited 92% inhibition of black gram peroxidase activity (Ajila and Rao, 2009). Complete inhibition of miswak POII was achieved by using 1.0 mM ascorbic acid as antioxidant.

Table 6. Storage stability at 4ºC of peroxidase activity in liquid and powder forms of miswak POII.

Week	Relative activity (%)	
	Liquid form	Powder form
1	100	100
2	80	100
3	80	100
4	75	100
6	70	95
8	70	95

Protease inhibition

The stability of miswak POII was detected in presence of trypsin (Figure 9). The activity was slightly decreased with increasing the concentration of trypsin, where the enzyme retained 70% of its activity at 20 mg of trypsin. Mohamed et al. (2008b) reported that the soluble and immobilized horseradish peroxidase retained 44 and 52% of their activities in presence of 2.5 mg trypsin.

Storage stability

The liquid and powder (after lyophilization) forms of miswak POII was stored at 4°C and measured its activity at intervals of week. As shown in Table 6 the liquid form lost 30% of its activity after 8 weeks of storage, while the powder form retained 95% of its original activity at the same time.

In conclusion, we demonstrate that the peel of miswak stem had highest peroxidase activity, while the people used the root without peel (chewing stick) with high peroxidase activity. Therefore, we recommended the people used the peel of miswak stem. In addition, the study reported that miswak POII was more stable to the denaturation induced by pH, heat, metal ions, metal chelators and proteolytic activity. The enzyme oxidized some of phenolic compounds. In the future, we will study the other antioxidant and digestive enzymes in miskwak for using of these enzymes in construction of a potent toothpast.

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