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Comparative Characterization of Diphenolases from Two Mulberry Fruits (*Morus alba* L. and *Morus nigra* L.)

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Fruits of two mulberry cultivars (*Morus alba* L. and *Morus nigra* L.) were investigated for their polyphenol oxidase potentials. Native electrophoresis of the crude extracts prepared from the cultivars stained with *L*-DOPA showed similar patterns with R_f values of 0.15 (one major band), 0.42 and 0.54 (two minor bands), respectively. The crude extracts from each mulberry cultivars (*Morus alba* L. and *Morus nigra* L.) were highly active against 3-(3,4-dihydroxyphenyl)propionic acid at acidic and neutral pH values and possessed temperature optima of 40 and 20ºC, respectively. pH-stability profiles showed that crude enzymes were extremely stable at both their optimum pH and alkaline pH values. The diphenolase activities from the two mulberry cultivars were very sensitive to ascorbic acid and metabisulfite with IC_{50} values lower than 1 mM. Moreover, both inhibitors exhibited complete inhibition of 3-(3,4-dihydroxyphenyl)propionic acid oxidation at 1 mM and 2 mM concentrations, respectively. 5 mM sodium dodecyl sulfate is required for the fully active diphenolase from *Morus nigra* L. It can be concluded from the present study that the crude enzymes prepared from the ripe mulberry fruits of two cultivars possess diphenolase activities sharing similar functional properties.

Key Words: *Morus alba* L., *Morus nigra* L., Mulberry, Polyphenol oxidase, Diphenolase.

INTRODUCTION

Plant polyphenol oxidases are a group of copper-containing enzymes[1] responsible for enzymatic browning reactions occuring during harvesting, handling, processing and storage of plant materials. From industrial point of view, these enzymes have gained much attention since browning reactions may cause undesirable taste, aroma and appearance of fruits and vegatables. Polyphenol oxidases catalyze two different types of reactions by utilizing molecular oxygen. The first, hydroxylation of monophenols to *o*-diphenols (monophenolase, EC 1.14.18.1) and the second, oxidation of *o*-diphenols to *o*-quinones (diphenolase, EC 1.10.3.1) which then polymerize to brown, red or black melanine pigments depending on the natural components present in plant material[2].

Mulberry fruits are a member of Moraceae family and only three types (*Morus alba* L., *Morus nigra* L. and *Morus rubra* L.) are widespread in Anatolia. These plants are also native to China, the far east, central Asia, eastern and central USA and widely cultivated in south Europe and south-west Asia[3]. Although mulberry leaves are the principle food source for silkworms, the fruits and its products such as syrup, jam, juice, pulp, paste, marmalade and jelly are consumed for its nutritional quality and medicinal properties such as hypoglycemic and hypotensive activities, antioxidant capacity, fever treatment, liver protection, eyesight improvement, facilitating discharge of urine and lowering blood pressure[3-7]. Mulberry fruits can be consumed as fresh or dried and are also be used in alcoholic beverage production such as wine[4]. The nutritional value of mulberry fruits comes from their phenolic acid, vitamin, mineral and sugar contents[8]. Although the production of mulberry was approximately 68,000 tons in year 2000 in Turkey[7], the fruits of cultivated plants are not sufficiently known as a potential food source.

In this work, polyphenol oxidase potentials of fruits from two different mulberry cultivars were investigated and compared for their biochemical properties. The two mulberry cultivars (*Morus alba* L. and *Morus nigra* L.) can be easily found in Trabzon, Turkey. The crude enzyme extracts were used and their polyphenol oxidase potentials were studied in terms of substrate specificities, thermal activation, pH optimum and stability and degrees of inhibition by general polyphenol oxidase (PPO) inhibitors, in order to help to predict the behaviour of the enzymes present in the two mulberry cultivars.

EXPERIMENTAL

Mulberry fruits from both cultivars (*Morus alba* L. and *Morus nigra* L.) were harvested directly from local gardens at the same altitude (170 meter above sea level) in Trabzon (Turkey) in June of year 2004. The fruits were carried into the laboratory in an ice bath and stored deep-frozen at -34°C for 1-2 months until used. A half kg of ripe mulberry fruits was randomly collected in triplicate from each cultivar.

Subtrates and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the other reagents were of analytical grade and used as obtained.

Crude polyphenol oxidase preparation: Crude enzyme fractions were prepared as reported previously[9-11]. Mulberry fruits (*ca.* 50 g) were placed in a dewar flask under liquid nitrogen for 10 min in order to decompose cell membranes. The cold fruits were homogenized by using a blender in 50 mL of 50 mM cold potassium phosphate buffer (pH 6.0), containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF)

and 6% (w/v) Triton X-114, for 2 min at 4°C. Before homogenization, 0.1 g poly(vinylpolypyrrolidone) was added into the blender mixture for 1 g of mulberry fruit. The homogenate was filtered and kept at 4°C for 1 h before being centrifuged at 17,000 rpm for 0.5 h at 4°C. The supernatant was used as crude enzyme which retained PPO activity for at least 15 d at 4°C.

Protein determination: Protein contents in the enzyme extracts was determined according to the Lowry method with bovine serum albumin as a standard[12]. The values were obtained by graphic interpolation on a calibration curve at 650 nm and it was found that the crude extracts from *Morus alba* L. and *Morus nigra* L. had an average of 5.9 ± 1.1 mg protein/mL and 7.2 ± 1.0 mg protein/mL, respectively.

Assay of polyphenol oxidase activity: Polyphenol oxidase activity was assayed by measuring the rate of increase in absorbance at a given wavelength using a double beam model ATI Unicam UV2-100 spectrophotometer (ATI Unicam, Cambridge, UK) as described previously[9,11]. The activity was determined using different phenolic substrates by measuring the increase in absorbance at 494 nm for 4-methyl catechol and 500 nm for all other substrates[13]. The enzymatic assay was carried out in air saturated solutions. 3-(3,4-dihydroxyphenyl) propionic acid (DHPPA), catechol and 4-methylcatechol were assayed as diphenolic substrates and *L*-tyrosine and (*p*-hydroxyphenyl)propionic acid (PHPPA) as monophenolic substrates with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in 50 mM potassium phosphate buffer at pH 7.0.

The reaction mixture contained substrates (stock 100 mM), an equal volume of MBTH (stock 10 mM) and 20 μL dimethylformamide (DMF) and the solution was diluted to 950 μL with buffer and 50 μL enzyme extract was added. The reference cuvette included all the reactants except the crude enzyme. Under the assay conditions, the oxidation of phenolic compounds in the reference mixture was negligible during the measurement time. One unit of diphenolase activity was defined as 1 μM of product formed per min. Specific activity was defined as unit of enzyme activity per mg of protein[14,15].

Properties of mulberry diphenolases-pH-optimum and stability: Diphenolase activity as a function of pH was determined in a pH range of 3.0 in 50 mM glycine-HCl buffer, 4.0-5.0 in 50 mM acetate buffer, 6.0-7.0 in 50 mM phosphate buffer and 8.0-9.0 in 50 mM *Tris*-HCl buffer. The pH stability was determined by incubation of crude enzyme solution in the buffer solutions indicated above in a ratio of 1:1 (v/v) for 24 h at 4°C. Residual diphenolase activity was determined in the form of per cent residual diphenolase activity at pH 7.0 by mixing 0.1 mL of 100 mM DHPPA as a substrate, 0.1 mL of 10 mM MBTH and 0.02 mL DMF with the incubated enzyme solution[9,10].

Thermal activity: Temperature optimum for each mulberry diphenolase was determined by measuring the enzyme activity at various temperatures over the range of 20-70°C with 10ºC increments using a circulation water bath. The reaction mixture at pH 7.0 containing all the reagents except crude enzyme was incubated for 5 min at different temperatures indicated above. After the enzyme was added, the relative activity was determined spectrophotometrically at 500 nm as rapidly as possible from the each cultivar using DHPPA as a substrate[11].

Enzyme kinetics: Enzyme kinetics for the diphenolases from each mulberry cultivar was studied by using DHPPA as a substrate and the rate of the diphenolase reaction was measured at various DHPPA concentrations (0.2-20 mM) in the standard reaction mixture in terms of the increase absorbance at the wavelength of maximum absorption for the corresponding chromophore[16].

The kinetic data were plotted as reciprocals of activities *vs.* DHPPA concentrations. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined as the reciprocal absolute values of the intercepts on the *x*- and *y*-axis, respectively, of the linear regression curve[17]. Substrate specificity (V_{max}/K_m) was calculated by using the data obtained on a Lineweaver-Burk plot.

Effect of general PPO inhibitors and sodium dodecyl sulfate: Sodium metabisulfite (0.2-2 mM), ascorbic acid (0.1-1 mM) and *L*-cysteine (0.05-20) as PPO inhibitors and sodium dodecyl sulfate (SDS, 0.2-20 mM) as a detergent were used for evaluating their effect on the oxidation of DHPPA for both mulberry diphenolases. An aliquot of each compound at various final concentrations was added to the standard reaction mixture before the addition of enzyme extract. Relative enzymatic activity was calculated as a percentage of the activity in the absence of each specific compound. The concentration of inhibitor giving 50 % inhibition (IC_{50}) was determined from plot of residual activity against inhibitor concentration[10,15].

Native polyacrylamide gel electrophoresis: Native polyacrylamide gel electrophoresis was performed on Hoeffer SE 600 Series Electrophoresis dual slab cell unit (California, USA) using preparative 10 % polyacrylamide gels[18] under native conditions as reported previously[10]. After electrophoresis, the gels were stained for diphenolase activity in 24 mM *L*-DOPA in 50 mM potassium phosphate buffer (pH 7.0) at room temperature for 2 h then in 1 mM ascorbic acid solution until appearance of isoenzyme bands.

Statistical analysis of data: Analysis of variance of the data was performed with SPSS 10.0 for Windows (USA). The LSD multiple range test was employed to determine the statistical analysis. In all figures and tables, data points represent mean of three determinations ($p \leq 0.05$).

RESULTS AND DISCUSSION

Native electrophoresis resulted isoforms of mulberry PPO having R_f values of 0.15 (major band), 0.42 and 0.54 (minor bands), respectively, indicating the presence of at least three isoforms of PPO in the ripe mulberry fruits from each cultivar (Fig. 1). The presence of two-four PPO isoenzymes has also been reported for fruits from Rosaceae and Ebenaceae families[9-11,19]. The extracts from both mulberry cultivars, *Morus alba* L. and *Morus nigra* L., contained three polyphenol oxidase isoenzymes having different mobilities on native-PAGE, therefore their activities were characterized on crude enzyme preparations extracted from ripe mulberry fruits to confirm high polyphenol oxidase activity.

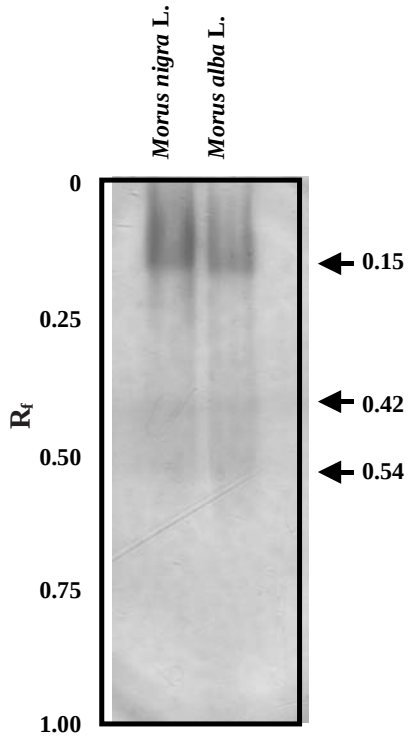


Fig. 1. Native polyacrylamide gel electrophoresis of both crude mulberry extracts from *Morus nigra* L. and *Morus alba* L. by *L*-DOPA staining

Catechol, 4-methylcatechol and 3-(3,4-dihydroxyphenyl)propionic acid (DHPPA) as diphenolic substrates were both oxidized by the crude enzymes from the two cultivars. A high level of activities was observed in the presence of DHPPA for both cultivars (Table-1). It seems from the assays that the enzymes from mulberry fruits were almost unable to oxidize monophenolic compounds either *L*-tyrosine or (*p*-hydroxyphenyl)propionic acid (PHPPA). These data indicate that crude enzymes from both mulberry

cultivars contain a diphenolase responsible for the oxidation of catechol, 4-methylcatechol or DHPPA which is consistent with previous reports on the PPOs from other plant sources[9-11,21,22]. Catalytic efficiencies using V_{max}/K_m ratio (Table-2) indicated that both diphenolases from mulberry fruits possessed similar activities against DHPPA as a diphenolic substrate. Crude diphenolases showing specificity against DHPPA from both medlar[9] and persimmon fruits[10] were also reported to have very similar catalytic efficiency values (Table-2).

TABLE-1
SUBSTRATE SPECIFICITIES OF CRUDE POLYPHENOL OXIDASES FROM MULBERRY FRUITS (*Morus alba* L. AND *Morus nigra* L.)

Substrate	Wavelength (nm)[13]	Specific Activity (U/mg protein)	
		Morus alba L.	*Morus nigra* L.
Catechol	500	4.8 ± 0.1	28.1 ± 0.2
4-Methylcatechol	494	37.3 ± 0.3	1.6 ± 0.1
DHPPA	500	29.3 ± 0.3	32.8 ± 0.3
PHPPA	500	6.6 ± 0.1	No activity
L-Tyrosine	500	6.2 ± 0.1	4.0 ± 0.1

TABLE-2
COMPARISON OF SOME PARAMETERS OBTAINED FOR DIPHENOLASE-CATALYZED OXIDATION OF DHPPA FROM DIFFERENT PLANT SOURCES

Parameter	Diphenolase source			
	Morus alba L.[a]	*Morus nigra* L.[a]	*Mespilus germanica* L.[9]	*Diospyros kaki* L.[10]
pH optimum	4.0	3.0	5.5	5.5
Temperature optimum (°C)	40	20	35	60
V_{max} (μM/min)	22.6	10.0	7.2	17.2
K_m (mM)	7.6	2.5	1.9	12.8
V_{max}/K_m (min^{-1})	0.0029	0.0040	0.0038	0.0013

[a]This work

pH-optimum and stability: The pH-activity profiles for each diphenolase from mulberry fruits in the presence of DHPPA as a substrate gave two pH maxima one of which was below pH 3.0 and the other at around pH 7.5 for *Morus nigra* L. and one maximum at pH 4.0 and a shoulder above pH 7.0 for *Morus alba* L. (Fig. 2). The second peak of enzyme activities at pH 7.0 or above for diphenolases from both mulberry fruits might indicate enzymatically active different diphenolase isoforms present in the crude enzyme preparations[23]. The relative diphenolase activity of *Morus alba* L. was over 60 % above pH 7.0. However, *Morus nigra* L. diphenolase activity dramatically decreased over pH 8.0. A similar result

was also observed for crude diphenolases from persimmon fruits[10]. It is clear from previous reports that the pH-optimum for PPOs is highly dependent on the enzyme source and the nature of substrate used[9-11,24].

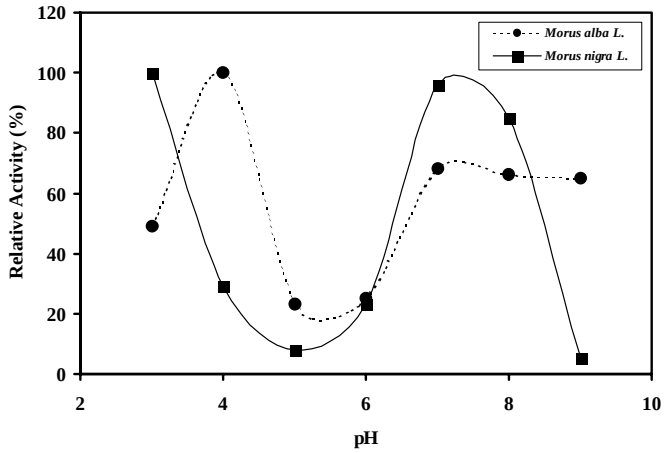


Fig. 2. pH-activity profiles for mulberry diphenolases in 50 mM glycine-HCl buffer (pH 3.0), 50 mM acetate buffer (pH 4.0-5.0), 50 mM phosphate buffer (pH 6.0-7.0) and in 50 mM *Tris*-HCl buffer (pH 8.0-9.0)

pH-stability profiles have shown that both enzymes were highly stable at their optimum pH values (Fig. 3). In addition, the diphenolases prepared from both mulberry cultivars had at least 70 % relative activities near pH 7.0 (Fig. 2) and were much more stable near neutral and alkaline pH values (Fig. 3). Although the diphenolase activity from *Morus alba* L. gradually increased with pH, the diphenolase from *Morus nigra* L. losses its activity by 50% when kept at pH 5.0 for 24 h at 4°C.

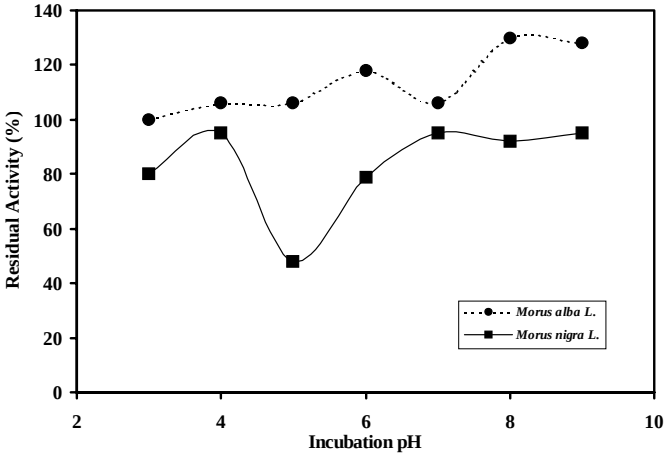


Fig. 3. pH stabilities of mulberry diphenolases from *Morus alba* L. and *Morus nigra* L. for DHPPA as a substrate

Thermal activity: Temperature-activity profiles of the diphenolases from the two mulberry fruits were shown in Fig. 4. It is clearly seen that both enzymes possess very high diphenolase activities at temperatures from 20 to 70°C. The enzyme from *Morus alba* L. showed a temperature optimum at 40°C whereas it is around 20°C for *Morus nigra* L. Similar temperature optima were also reported for dog-rose fruits in the presence of 4-methyl catechol[25] and for persimmon fruits in the presence of catechol or 4-methyl catechol substrates[10]. Although the diphenolase activity of mulberry fruit from *Morus nigra* L. decreased to less than 60 % upon temperature increases to 70°C, the enzyme from *Morus alba* L. was still 80 % active even at this temperature. It is clear from these data that the two mulberry cultivars possess extremely high diphenolase activities over a wide range of temperatures.

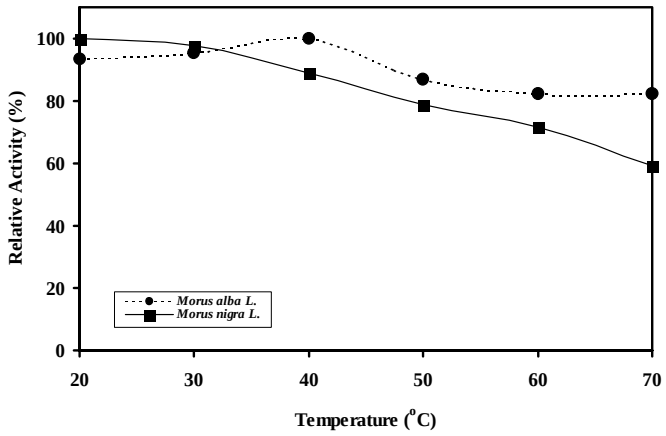
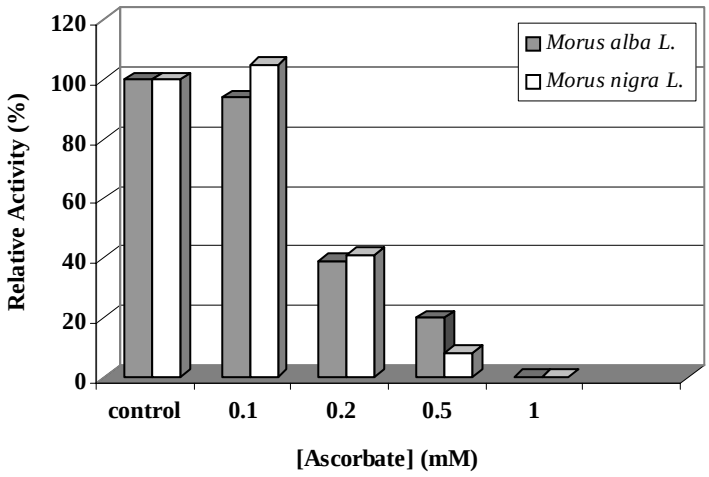


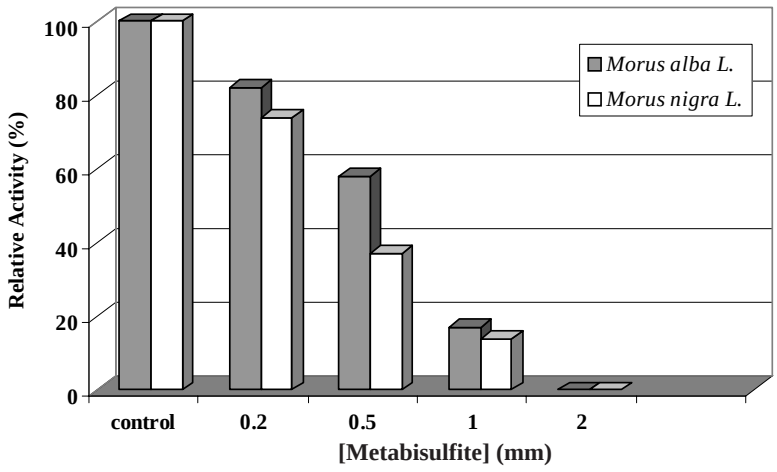
Fig. 4. Temperature optima of both mulberry fruits *Morus alba* L. and *Morus nigra* L. for DHPPA as a substrate

Effect of inhibitors: The effects of general PPO inhibitors, namely ascorbic acid (0.1-1 mM), *L*-cysteine (0.05-20 mM) and sodium metabisulfite (0.2-2 mM) on crude diphenolases of mulberry fruits from the two cultivars were examined (Fig. 5). The diphenolases from both cultivars were completely inhibited by ascorbic acid and metabisulfite (Figs. 5A and 5B). It seems that high concentrations of *L*-cysteine are required for the complete inhibition of the DHPPA oxidation by crude mulberry diphenolases (Fig. 5C). Only 30-40 % inhibition of DHPPA oxidation was achieved in the presence of 20 mM *L*-cysteine indicating that this compound was not a good inhibitor for both mulberry enzymes. The potentials of the examined inhibitors for the inhibition of the DHPPA oxidation by mulberry diphenolases from the two cultivars were also expressed as IC_{50} values. Ascorbic acid with IC_{50} of 0.18 mM for both mulberry diphenolases was the most effective inhibitor followed by

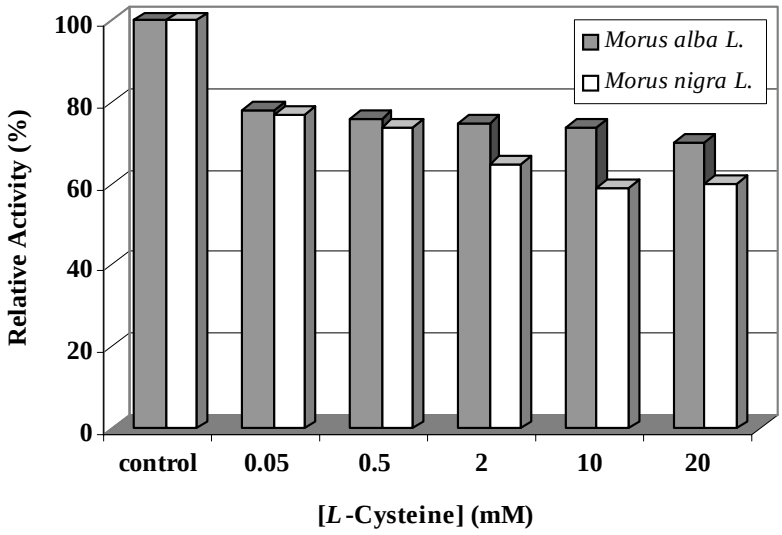
metabisulfite and both compound exhibited complete inhibition of DHPPA oxidation at a range of 1-2 mM (Figs. 5A and 5B). The inhibition of mulberry diphenolases by metabisulfite and ascorbate is consistent with the previous reports indicating that these compounds are potent inhibitors of PPOs[11,21,26-29]. The inhibition profiles of each individual inhibitor towards both mulberry diphenolases were almost very similar. It is known from the previous data that the chemical nature of the compound may effect its inhibitory action on diphenolases[24,26,28,30].



(A)



(B)



(C)

Fig. 5. Dependence of DHPPA oxidation by crude diphenolases from mulberry fruits (*Morus alba* L. and *Morus nigra* L.) on general polyphenol oxidase inhibitors: (A) ascorbate, (B) sodium metabisulfite and (C) *L*-cysteine. Control contained all the reagents except inhibitor

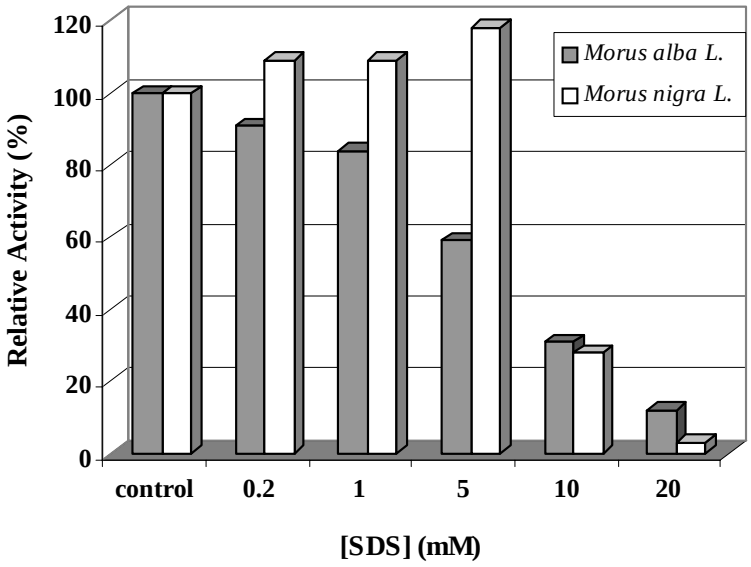


Fig. 6. Behaviour of crude diphenolases from mulberry fruits (*Morus alba* L. and *Morus nigra* L.) in the presence of sodium dodecyl sulfate as a detergent. Control contained all the reagents except inhibitor

Effect of sodium dodecyl sulfate: The effect of sodium dodecyl sulfate (SDS, 0.2-20 mM) as a detergent, on crude diphenolases of mulberry fruits from the two cultivars was examined (Fig. 6). About 20 % increase in the activity was observed for the *Morus nigra* L. diphenolase in the presence of 5 mM SDS. The activation of the polyphenol oxidases up to a specific concentration of SDS is also common for other plant polyphenol oxidases and the behaviour of the enzyme towards SDS has been attributed to the critical micelle formation by the detergent which results a conformational change in the enzyme structure[23,31,32]. Over 5 mM SDS concentrations significantly inhibited the diphenolases from both mulberry diphenolases at 70 % levels. Similar observations for polyphenol oxidases towards SDS were also reported for vanilla[32], banana[33], persimmon[34] and broad bean[35].

Conclusion

It can be concluded from the present study that the crude enzymes prepared from the ripe mulberry fruits of two cultivars possess diphenolase activities sharing very similar functional properties. The enzymes from both *Morus alba* L. and *Morus nigra* L. cultivars are very active against DHPPA at acidic pH values with temperature optima of 40 and 20ºC, respectively. The crude diphenolases from both mulberry cultivars were highly sensitive towards ascorbate and metabisulfite as reported for other plant polyphenol oxidases. A specific concentration of SDS is needed for the formation a more active diphenolase from mulberry cultivars.

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