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Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var. *capitata* L.)

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Abstract

Polyphenol oxidase (PPO) was isolated from butter lettuce (*Lactuca sativa* var. *capitata* L.) grown in Poland and its biochemical characteristic were studied. PPO from butter lettuce showed a higher affinity to 4-methylcatechol than to catechol. The K_M and V_{max} values were: 3.20 ± 0.01 mM and 4081 ± 8 U/ml min^{-1} for catechol and 1.00 ± 0.09 mM and 5405 ± 3 U/ml min^{-1} for 4-methylcatechol. The optimum pHs of the enzyme were found to be 5.5 using catechol and 6.8 using 4-methylcatechol as substrate. The enzyme had a temperature optimum of 35 °C. The enzyme was relatively stable at 30 °C and 40 °C. The times required for 50% inactivation of activity at 50 °C, 60 °C and 70 °C were found to be about 30, 20 and 5 min, respectively. Inhibitors used for investigation in this study were placed in relative order of inhibition: *p*-hydroxybenzoic acid > glutathione ≈ ascorbic acid > L-cysteine > EDTA > citric acid. The enzyme eluted in the chromatographic separations was analyzed electrophoretically under denaturating conditions. The analysis revealed a single band on the SDS–PAGE which corresponded to a molecular weight of 60 kDa.

Keywords: Polyphenol oxidase; Purification; Lettuce; *Lactuca sativa* var. *capitata* L.; Characterization; Inhibitors

1. Introduction

Polyphenol oxidase (PPO) is a common copper-containing enzyme which is responsible for melanization in animals and browning in plants. The enzyme catalyses two distinct reactions: the *o*-hydroxylation of monophenols to *o*-diphenols (acts like cresolase) (E.C. 1.14.18.1.) and the oxidation of *o*-diphenols to *o*-quinones (acts like catecholase) (E.C. 1.10.3.2.) (Rodríguez-López et al., 2001; van Gelder, Flurkey, & Wichers, 1997). Quinones are highly reactive electrophilic molecules that can polymerize, leading to the formation of brown or black pigments. The role of PPO in plants is not yet clear. It is suggested that it may be involved in immunity reactions and in biosynthesis of plant components, and it also may play the role of a scavenger of free radicals in photo-synthesizing tissues (Heimdal, Larsen, & Poll, 1994).

Enzymatic browning of fruit and vegetables after minimal processing is one of the most important causes of quality losses, resulting in rejection by consumers. The quality and shelf life of lettuce varies depending on cultivars, maturity, processing and storage conditions, although the information on tissue browning susceptibility is limited (Castaner, Gil, & Ruiz, 1999). Activity of PPO has been studied in apples (*Malus* sp.) (Espin, Morales, Varon, Tudela, & Garcia-Carnovas, 1995), pears (*Pyrus* sp.) (Hwang, Yoon, & Kim, 1996) broad beans (*Vicia faba* L.) (Ganesa, Fox, & Flurkey, 1992), potatoes (*Solanum tumerosum* L.) (Chen et al., 1992), artichokes (*Cynara scolymus* L.) (Leoni & Palmeri, 1990), lettuce (*Lectuca sativa* L.) (Heimdal et al., 1994), plums (*Prunus* sp.) (Siddiq, Sinha, & Cash, 1992), banana (*Musa cavendishii* L.) (Galleazi, Sgarbieri, & Constantinides, 1981), peppermint (*Mentha piperita* L.) (Kavrayan & Aydemir, 2001), coffee (*Coffea arabica* L.) (Mazzafera & Robinson, 2000) and seeds of

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field bean (*Dolichos lablab*) (Paul & Gowda, 2000). The main objective of this investigation was to characterize PPO from butter lettuce and to elucidate the mechanism of its inhibition by chosen chemical compounds.

2. Materials and methods

2.1. Materials

Butter lettuce was purchased at commercial maturity from a local store. Catechol, 4-methylcatechol, polyvinylpyrrolidone (PVP 40), DEAE–Sephadex A-50, Sephadex G100, chlorogenic acid, caffeic acid, ferulic acid, tyrosine, vanilin, sodium salt of ethylenediaminetetraacetic acid (EDTA), *p*-hydroxybenzoic acid, L-cysteine, glutathione, and Bradford reagent were obtained from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

2.2. Enzyme extraction and partially purification

Ten grams of material were homogenized in 80 ml of 0.1 M sodium phosphate buffer pH = 6.8 containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone and extracted with the aid of a magnetic stirrer for 1 h. The crude extract samples were centrifuged at 32,000*g* for 20 min. The process was conducted at the temperature of 4 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% saturation. After an hour, the precipitated proteins were separated by centrifugation at 32,000*g* for 30 min. The precipitate was dissolved in a small amount of 5 mM phosphate buffer (pH 6.8) and dialyzed in the cellulose bag (MW cut off >12,000) at 4 °C in the same buffer for 24 h with four changes of the buffer during dialysis. In order to conduct further purification, the dialysate was transferred to a column filled with DEAE–Sephadex A-50 gel, balanced with 5 mM phosphate buffer, pH 6.8. The column was eluted with the same buffer at the flow rate of 30 ml/h and linear gradient of NaCl concentration from 0 to 1.0 M. Three millilitre fractions were collected in which the protein level and PPO activity towards catechol as substrate were monitored. The fractions which showed PPO activity were collected, concentrated and then dissolved in 2 ml of phosphate buffer, pH 6.8. The combined fractions were transferred to a glass column filled with Sephadex G100 gel. The column was eluted with the same buffer solution. 3-ml fractions were collected and the protein content and PPO activity towards catechol was monitored in them spectrophotometrically. The fractions showing PPO activity were combined and concentrated.

2.3. Enzyme assay

PPO activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 420 nm (Perkin–Elmer Lambda 40 UV–Vis spectrophotometer was used) (Wisserman & Lee, 1980). An increase in absorbance of 0.001 min^{-1} was taken as one unit of enzyme activity (Ho, 1999). The increase in absorbance was linear with time for the first 120 s. The sample cuvette contained 2.95 ml of substrate solution in 50 mM phosphate buffer (pH = 6.8) and 0.05 ml of the enzyme solution. The blank sample contained 2.95 ml of substrate solution and 0.05 ml of phosphate buffer. Information of substrates used is provided in the adequate sections.

2.4. Protein determination

Protein content was determined according to the dye-binding method of Bradford (1976) using bovine serum albumin as standard.

2.5. Determination of molecular weight

The molecular weight of the partially purified enzyme was estimated by SDS–PAGE. SDS–PAGE was performed according to the method of Laemmli (1970). Proteins were dissolved in a 12.5% polyacrylamide gel and visualized with colloidal Coomassie staining.

2.6. Characterization of PPO

2.6.1. Effect of pH on enzyme activity

PPO activity, as a function of pH, was determined under standard conditions using various buffers in the pH buffering range 2.0–12.0. The buffer systems were prepared according to Britton–Robinson (Kłyszejko-Stefanowicz, 2003). The optimum pH for the PPO was obtained using two substrates: 10 mM catechol and 10 mM 4-methylcatechol. The pH value corresponding to the highest enzyme activity was taken as the optimal pH.

2.6.2. Kinetic data analysis and substrate specificity

The specificity of lettuce PPO extract was investigated for seven commercial grade substrates (catechol, 4-methylcatechol, chlorogenic acid, caffeic acid, ferulic acid, tyrosine and vanilin) at concentrations 10 mM. PPO activity was assayed in triplicate. The activity of PPO extract as a function of the concentration of catechol and 4-methylcatechol was investigated. Michaelis constant (K_M) of the PPO was determined by Lineweaver–Burk's method.

2.6.3. Effect of inhibitors on PPO

The inhibitory effects of ascorbic acid, citric acid, EDTA (sodium salt of ethylenediaminetetraacetic acid), *p*-hydroxybenzoic acid, L-cysteine and glutathione on PPO activity were determined. Three different concentrations of these inhibitors (0.1, 1.0 and 10 mM) were tested using 10 mM of catechol substrate. The corresponding control contained the same concentration of enzyme, in the absence of inhibitor.

2.6.4. Thermal stability of PPO

Thermal stability of partially purified PPO was investigated at pH 6.8 at various constant of temperatures from

25 °C to 70 °C using incubation time from 10 to 60 min. Residual PPO activity was then measured using 10 mM catechol substrate.

3. Results and discussion

3.1. Extraction and partial purification of PPO

A 19.4-fold purification of PPO relative to protein a yield of 5.8% was achieved (Table 1), and the elution profile of the PPO on DEAE Sephadex A-50 and Sephadex G 100 is shown in Fig. 1.

The technique of gel filtration is widely used in enzyme preparation. Chazarra, Garcia-Carmona, and Cabanes (2001) purified PPO from iceberg lettuce and achieved purification fold 44.8 along the protein recovery 31.3, Selles-Marchart, Casado-Vela, and Bru-Martinez (2006) purified polyphenol oxidase obtained from *Eriobotrya japonica* Lindl. and achieved the purification fold of 39.9 with the protein recovery of 15%. Xu, Zheng, Meguro, and Kawachi (2004) purified PPO from Henry chestnuts (*Castanea henryi*) and achieved purification fold of 36.87 with the protein recovery of 12.94%.

3.2. Enzyme kinetics and substrate specificity

There are a number of compounds such as dopamine, catechol, catechin, chlorogenic acid, pyrogallol, caffeic acid, tyrosine, 4-methylcatechol used as substrates for polyphenol oxidase in the literature (Dogan & Dogan, 2004). In this study, we selected the most frequently used substrates: catechol, 4-methylcatechol, chlorogenic acid, caffeic acid, ferulic acid and vaniline. The greatest activity was detected with catechol, followed by 4-methylcatechol and

Table 1
Purification of PPO from butter lettuce

Purification step	Total activity	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude extract	125,132	93.8	1354	1.0	100
$(NH_4)_2SO_4$	97,764	24.0	4073	3.0	78
DEAE	26,204	2.4	10,916	8.0	20.9
G-100	3875	1.6	26,285	19.4	5.8

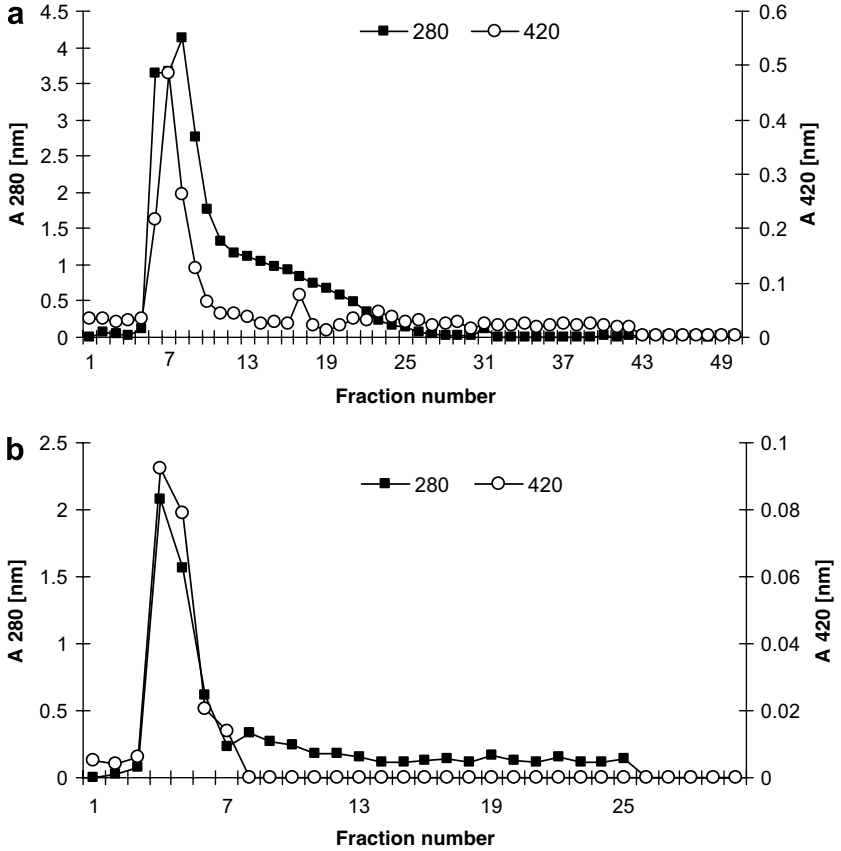

Fig. 1. Purification of PPO from butter lettuce by (a) separation on DEAE Sephadex A-50 and (b) Sephadex G-100 chromatography. Proteins were monitored by UV absorbance at $\lambda = 280$ nm and PPO activity by absorbance at $\lambda = 420$ nm as described in Section 2.

Table 2
Relative activity at 10 mM of substrate concentration

Substrate	Relative activity (%)
Catechol	100.0 ± 0.1
4-Methylcatechol	88.50 ± 0.07
Chlorogenic acid	53.40 ± 0.5
Caffeic acid	2.30 ± 0.04
Ferulic acid	0.20 ± 0.03
Vanillin	0
Tyrosine	0

chlorogenic acid measured at 10 mM substrate concentration. The butter lettuce PPO showed no activity towards vaniline and tyrosine at the same concentration (Table 2). The results obtained in the present study correspond well to those obtained by Xu et al. (2004), concerning PPO from Henry chestnuts (*C. henryi*). The enzyme catalysed the oxidation of catechol and pyrogallic acid, but had no effect on cresol or tyrosine. This suggests that the enzyme lacks cresolase activity. Catechol and 4-methylcatechol are the substrates that are usually chosen for determining the activity of polyphenol oxidase isolated from food of plant origin (Cho & Ahn, 1998; Heimdal et al., 1994; Lourenco, Neves, & da Silva, 1992; Zhou, Smith, & Lee, 1993). The analysis of the Lineweaver–Burk curves revealed that PPO from butter lettuce showed a higher affinity to 4- methylcatechol than to catechol. The K_M and V_{max} values were: 3.20 ± 0.01 mM and 4081 ± 8 U/ml min^{-1} for catechol and 1.00 ± 0.09 mM and 5405 ± 4 U/ml min^{-1} for 4-methylcatechol (Fig. 2).

The K_M values obtained for PPO towards catechol from various plant sources are: 3.13 mM from spinach, 10.5 mM from beans, 4 mM from Jerusalem artichoke (*Helianthus tuberosus*) and 18 mM from thyme. When 4-methylcatechol was used as the substrate for PPO, the following K_M values were obtained: 9.8 mM for PPO from thyme, 10 mM from strawberry, 3.1 mM from Amasya apples, 94.3 mM from beans and 0.2 mM for peach (Dogan & Dogan, 2004). Michaelis constant for the purified enzyme from mulberry were 19.81 and 9.18 mM with catechol and 4-methylcatechol substrate, respectively. The Km value of PPO with catechol substrate was 5 mM from olive enzyme (Arslan, Erzengin, Sinan, & Ozensoy, 2004).

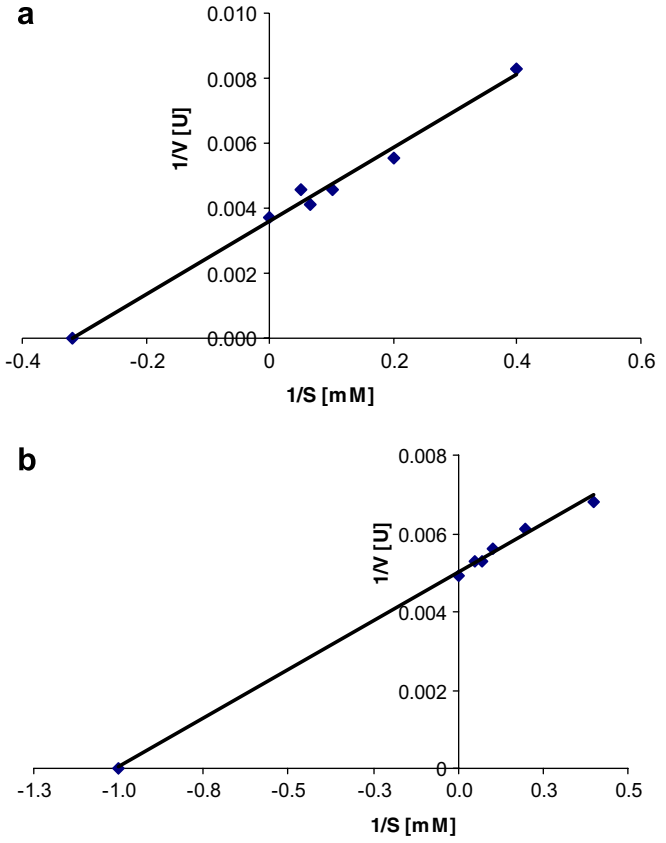

Fig. 2. The Lineweaver–Burk plots of enzyme activity measured with catechol (a) and 4-methylcatechol (b).

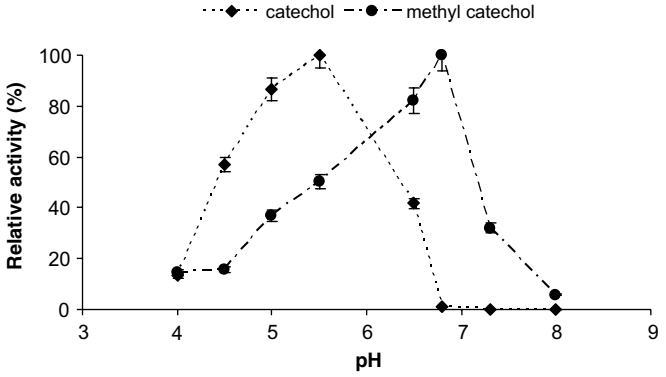

Fig. 3. Determination of optimum pH of butter lettuce PPO activity measured with catechol or methylcatechol substrate.

3.3. Effect of pH

The activity of PPO was measured at different pHs and temperatures using catechol and 4-methylcatechol as substrates. As seen in Fig. 3. the optimum pHs of the enzyme were found to be 5.5 using catechol and 6.8 using 4-methylcatechol as substrate.

In general, most plants show maximum PPO activity at or near neutral pH values. Different optimum pHs for PPO obtained from various sources are reported in the literature. For example, it is reported that optimum pH values are 5.5 for strawberry, 6.0 for DeChaunac apple, 7.0 for aubergine, 7.5 for *Allium* and 8.5 for Dog rose using catechol as substrate and 4.5 for strawberry, 6.0 for aubergine, 8.5 for Dog rose and 9.0 for Amasaya apple using 4-metchylcatechol as substrate (Dogan & Dogan, 2004).

3.4. Optimum temperature and thermal stability

Thermal activity of butter lettuce PPO is presented in Fig. 4. The enzyme had a temperature optimum of 35 °C. Similar result is obtained by Dincer, Colak, Aydin, Kadioglu, and Guner (2002) in investigations on PPO from med-

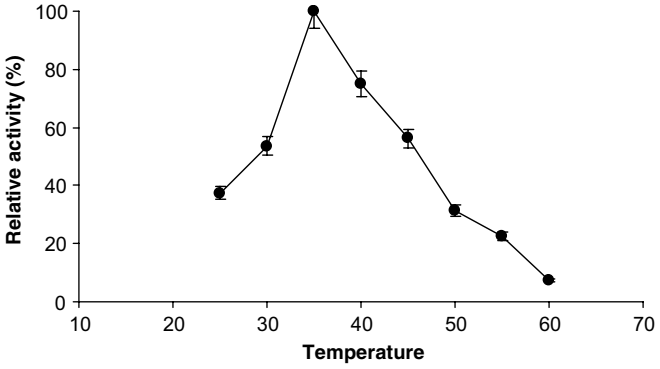

Fig. 4. Determination of optimum temperature of butter lettuce PPO activity.

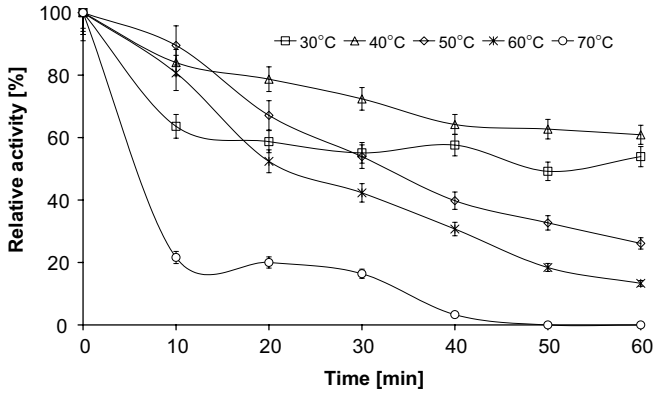

Fig. 5. Thermal stability of butter lettuce PPO.

lar fruits. It has been reported that the optimum temperatures for PPO of peach, grape and plum were 20, 25 and 37 °C respectively (Dincer et al., 2002). PPO from thymus had a optimum temperature of 25 °C for catechol and 4-methylcatechol (Dogan & Dogan, 2004). It is reported, that optimum temperature values are 23 °C for DeChaunac grape (Lee, Smith, & Pennesi, 1983), 30 °C for aubergine (Dogan, Arslan, & Dogan, 2002). The optimum temperature for mulberry PPO activity has been found regarding to vary the substrate of the enzyme. Whereas the optimum temperature of enzyme for 4-methylcatechol and pyrogallol oxidation was 20 °C, for catechol it was 45 °C. (Arslan et al., 2004).

The thermal stability profile of butter lettuce PPO, presented as residue activity after preincubation at the specified temperature, is shown in Fig. 5. The enzyme was relatively stable at 30 °C and 40 °C over a 60 min preincubation time period. The times required for 50% inactivation of activity at 50 °C, 60 °C and 70 °C were found to be about 30, 20 and 5 min, respectively. The enzyme from medlar fruits was stable for 30 min at 60 °C (Dincer et al., 2002). In the case of mulberry PPO the times required for 50% inactivation of activity at 50, 60 and 80 °C were found about 50, 30 and 20 min respectively (Arslan et al., 2004). It has been reported that *Allium* sp. PPO was stable at 40 °C for 30 min, stanley plum PPO at 70 °C, and banana, at 70 °C were stable for 30 min, and Jerusalem architoke PPO at 60 °C for 30 min (Aydemir, 2004).

Table 3
Effect of various compounds on activity of butter lettuce PPO measured with 10 mM catechol as substrate

Compound	C [mM]	Inhibition [%]	I_{50} [mM]
p-Hydroxybenzoic acid	0.1	9.1 ± 0.3	
	1.0	25.1 ± 0.1	4.54 ± 0.07
	10	100.0 ± 0.4	
EDTA	0.1	2.50 ± 0.08	
	1.0	11.5 ± 0.2	26.4 ± 0.1
	10	19.4 ± 0.5	
Ascorbic acid	0.1	13.30 ± 0.06	
	1.0	25.5 ± 0.1	4.61 ± 0.04
	10	96.6 ± 0.3	
Citric acid	0.1	8.0 ± 0.2	
	1.0	6.8 ± 0.1	62.91± 0.05
	10	11.50 ± 0.03	
Glutathione	0.1	7.80 ± 0.08	
	1.0	22.90 ± 0.06	4.70 ± 0.02
	10	98.1 ± 0.1	
L-cysteine	0.1	7.00 ± 0.05	
	1.0	16.80 ± 0.07	10.39 ± 0.02
	10	47.70 ± 0.06	

3.5. Effect of inhibitors

The effects of various inhibitors at 0.1, 1.0 at 10 mM on the partially purified butter lettuce PPO activity measured with 10 mM of catechol substrate are shown in Table 3. The relative inhibition studies indicates that the most potent inhibitors for lettuce PPO were *p*-hydroxybenzoic acid, ascorbic acid and glutathione, since these compounds induced a high degree of inhibition, even at the 0.1 mM concentration. Inhibitors used for investigation in this study were placed in relative order of inhibition: *p*-hydroxybenzoic acid > glutathione ≈ ascorbic acid > L-cysteine > EDTA > citric acid.

Enzymatic browning of plants may be delayed or eliminated by removing the reactants, such as oxygen and phenolic compounds, or by using PPO inhibitors. Complete elimination of oxygen from plants is difficult, because oxygen is ubiquitous (Dogan & Dogan, 2004). There are a number of inhibitors used by researches to prevent enzymatic browning (Dogan et al., 2002; Sakiroglu, Kufrevioglu, Kocacaliskan, Oktay, & Onganer, 1996; Yang et al., 2001) Inhibition by thiol compounds is attributed to either the stable colourless products formed trough an additional reaction with *o*-quinones or binding to the active centre of PPO, like metabisulfite. Ascorbate acts more as an antioxidant that as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions which lead to browning. Ascorbic acid has also been reported to cause irreversible inhibition (Aydemir, 2004).

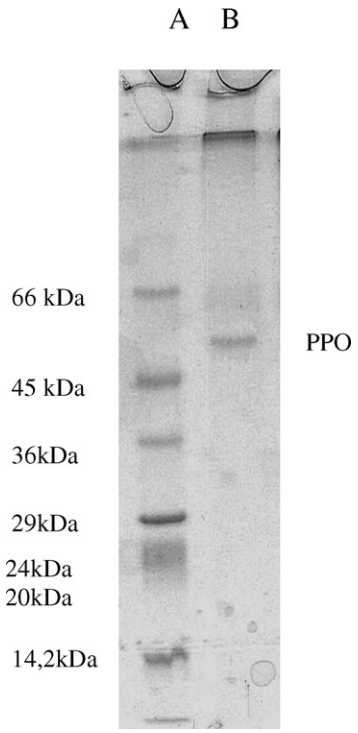

Fig. 6. SDS–Page of purified PPO from butter lettuce. Lane A contained the molecular weight markers, lane B contained purified PPO.

3.6. Determination of molecular weight of butter lettuce PPO

The enzyme eluted in the chromatographic separations was analyzed electrophoretically under denaturating conditions. The analysis revealed only one major protein band detected under the staining condition specified on the SDS–PAGE which corresponded to a molecular weight of 60 kDa (Fig. 6). We tentatively assignment this protein as the PPO from butter lectuce.

The results obtained in the present study correspond well to those obtained by Chazarra et al. (2001) concerning purification and characterization of PPO from iceberg lettuce (*L. sativa* L.). Molecular mass of PPO determined by SDS–PAGE was 60 kDa. Other researchers, indicate that the molecular weight of polyphenol oxidase ranges from 45 to 67 kDa (de Fatima Pereira Goulart, Alves, Magalhaes, de Oliveira Lima, & Meyer, 2003; van Gelder et al., 1997; Weemaes, Ludikhuyze, Van den Broeck, & Hendrickx, 1998). It has been shown that PPO is synthesized as a 60–65 kDa protein, which can be converted to a 40-45 kDa form by proteolysis as has been found in peaches, apricots, plums and cherries. Thus, PPO has been purified to apparent homogeneity from several species and a molecular mass of 45 kDa has been frequently reported (Chazarra et al., 2001).

It can be concluded that PPO isolated from butter lettuce have an activity very similar to that of other plants. The enzyme is a catecholase, active toward diphenols, and has the greatest substrate specificity towards catechol and 4-methylcatechol among the substrates tested. The pH optimum for the enzyme was 5.7. Moreover the butter lettuce PPO activity was sensitive to some of general PPO inhibitors, especially to *p*-hydroxybenzoic acid, glutathione and ascorbic acid. Molecular mass determined by denaturing SDS–PAGE of butter lettuce PPO was 60 kDa.

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