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Food Chemistry 95 (2006) 503–508
Food Chemistry
www.elsevier.com/locate/foodchem

Purification and characterization of polyphenol oxidase from *Ferula* sp.

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Received 16 May 2004; received in revised form 14 January 2005; accepted 14 January 2005

Abstract

Polyphenol oxidase (PPO) of several *Ferula* sp. was extracted and purified through $(NH_4)_2SO_4$ precipitation, dialysis, and gel filtration chromatography. Leaf and stem extracts were used for the determination of enzyme properties. Optimum conditions, for pH, temperature, and ionic strength were determined. The best substrates of PPO were catechol for leaf and (−) epicatechin for stem samples. Optimum pH and temperature were determined. K_M and V_{max} values were 2.34×10^{-3} M and 8541 EU/ml for catechol, and 2.89×10^{-3} M and 5308 EU/ml for (−) epicatechin. The most effective inhibitor was sodium diethyl dithiocarbamate for leaf samples and sodium metabisulphite for stem samples. Both inhibitors indicated competitive reactions. PPO showed irreversible denaturation after 40 min at 60 °C.

Keywords: Polyphenol oxidase; *Ferula* sp.; Kinetics

1. Introduction

Polyphenol oxidase (PPO) is a major enzyme responsible for the browning reaction in damaged plant tissues and fruits (Mathew & Parpia, 1971; Ricard-Forget & Gauillard, 1997). Enzymatic browning occurs in plants via phenolic compounds which are oxidized to quinones in the presence of O_2 by PPO; then, the quinones are polymerized to pigments (Fraignier, Marques, Fleuriet, & Macheix, 1995; Lee, 1992; Macheix, Sapis, & Fleuriet, 1991; Mayer & Harel, 1981, 1991; Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994). PPO has monophenolase and diphenolase activities. Monophenolase activity is the hydroxylation of monophenols to *o*-diphenols, whereas diphenolase activity is the oxidation of *o*-diphenols to quinones (Mayer, 1987; Siddiq, Sinha, & Cash, 1992).

Although browning reactions, in some food products, result in good appearance in terms of colour, these kinds of reactions, in general, lead to undesirable results with respect to texture, sweetness, and overall flavour. Therefore, inhibition studies have gained more importance for these types of reactions in food and vegetable processing technology (Vamos-Vigyazo, 1981). PPO has been given more attention in food technology in this regard. Agricultural products also are harmed by enzymatic browning reactions during harvesting, storage, and processing. All these cause low-quality products and create economic losses (Cheynier, Osse, & Rigaud, 1988; Eskin, 1990; McEvily, Lyengar, & Otwell, 1992; Ricard-Forget & Gauillard, 1997). Some plants, for instance, *Ferula* sp., *Allium* sp. and *Thymus* sp., are used in significant amounts in herb cheeses, in Anatolia, Turkey (Arslan, Temur, & Tozlu, 1997). *Ferula* sp. in particular, are used much more than the others. These plants grow at high

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doi:10.1016/j.foodchem.2005.01.044

altitudes in rural areas (Davis, 1972). *Ferula* plants are harvested in May and June and only the body of the plant is of interest. The plants are boiled in water to rid them of bitter taste and, then, they are incubated in a glass jar containing 16% salt for 16–20 days (Arslan et al., 1997). At the end of incubation, the salty water is decanted and the plant parts are kept in the cold before use in herb cheeses or directly in cooking with eggs.

The final product, kasni, is made from *Ferula* sp. in Iran, India, and Afghanistan. This product has a pale yellow colour and pleasant odour of spice. It is said to help digestion and to be easy on the stomach and intestine.

Ferula plants have been used for medical purposes as well. When the root is powdered, and then mixed with honey, use of this mixture is claimed to help sexual powers and to cure diabetes (Baytop, 1999).

Ferula is a genus of *umbelliferous* plants. The roots of the species *Ferula asafoetida*, *Ferula foetida*, *Ferula narthex*, and *Ferula scorodosma* yield *asafoetida (asafetida)*, a pungent resin extract, used as a culinary additive in some Indian and Near East cooking. *Ferula galbaniflua* and *Ferula rubricaulis* exudates yield galbanum, used as a flavour additive in candy and baked goods but mainly in perfumery (Bruneton, 1993; Evans, 1989).

Ferula plants contain much PPO. Since PPO causes undesirable results during processing of the plant, it has to be investigated with this in mind. In the literature, there is no study on purification and characterization of PPO from *Ferula* sp. This study covers an investigation of biochemical features and the purification of PPO from *Ferula* sp.

2. Materials and methods

2.1. Plant material

Ferula plants were harvested in the second half of May, in Erzurum, Turkey. The harvested plant material was separated into stems, roots and leaves. Each part was separately wrapped with aluminium foil, then frozen with liquid nitrogen, and then kept at −30 °C prior to use.

2.2. Enzyme extraction and purification

Two grams of each *Ferula* sample (stem and leaves) was frozen in liquid nitrogen and then powdered. Powdered material was added to 10 ml of extraction buffer, 0.05 M phosphate buffer, pH 5.2, containing 0.5% polyvinylpyrrolidone and 10 mM ascorbic acid. The crude extract samples were centrifuged at 48,000*g* for 30 min. at 5 °C. Several precipitations with solid $(NH_4)_2SO_4$, between 0–20%, 20–40%, 40–60%, 60–70%, and 70–80%, were tested to find the proper saturation point. The precipitated PPO was separated by centrifugation at 48,000*g* for 30 min. The precipitate was dissolved in a small amount of 5 mM phosphate buffer (pH 5.2) and dialyzed at 4 °C in the same buffer for 24 h with four changes of the buffer during dialysis.

For further purification of PPO, a dialyzed enzyme aliquot was fractionated by gel filtration chromatography. In gel filtration chromatography, a column with 100 ml bed volume was prepared using Sephadex G-100 and equilibrated with 0.05 M phosphate buffer (pH 5.2). The dialyzed enzyme solution was passed through the column. The elution rate was adjusted to 15–20 ml/h.

The eluates were collected in tubes as 4 ml volumes. The elution process was continued until 0 absorbance at 280 nm was obtained. Qualitative protein determination was done at 280 nm on the eluates obtained and PPO activity was measured in the eluates showing absorbance at 280 nm. Thus, values obtained were plotted against the tube number. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity before and after purification. For determining specific activity, PPO activity and quantitative protein measurements were carried out. Protein contents were determined by the protein dye-binding method (Bradford, 1976).

2.3. Determination of PPO activity

PPO activity was determined by measuring the increase in absorbance at 475 nm for L-dopa and dopamine and 420 nm for other substrates with a spectrophotometer (LKB Biochrom Ultraspec II). The sample cuvette contained 50 μl of the enzyme solution and 2.95 ml of substrate solution in various concentrations. The blank sample contained only 3 ml of substrate solution. The reaction was carried out at various temperatures and pH values with the substrates mentioned as follows. PPO activity was calculated from the linear portion of the curve (Wong, Zub, & Whitaker, 1971). One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min.

2.4. Characterization of PPO

2.4.1. Effect of pH

PPO activity was determined with 5 different substrates (catechol, 4-methylcatechol, (+) catechin, (−) epicatechin and chlorogenic acid) at a concentration of 10 mM; for (−) epicatechin concentration was 1.33 mM for leaves and 2.30 mM for stem. Appropriate buffers (0.1 M citrate/0.2 M phosphate for pH 4.0–5.5, 0.2 M phosphate for 5.5–7.0, and Tris–HCl for 7.0–10.0) were used for the determination of optimum pH

of PPO. The optimum pH values obtained from this assay were used in all subsequent experiments.

PPO activities were measured with 0.1 M phosphate buffer (pH 5.0–7.5) to determine the stability of PPO. The activity measurements were performed every 3 days by using catechol as substrate under optimum conditions.

2.4.2. Effect of temperature

For determining optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the range 0–60 °C using the five different substrates, as indicated above. The desired temperatures were provided by using an ice bath for temperatures under 20 °C and a constant-temperature circulator for temperatures above 20 °C.

2.4.3. Effect of ionic strength

Ionic strength effect on the enzyme was studied at 10 mM substrate concentration using different concentrations of buffers.

2.4.4. Heat stability

The denaturation property of the enzyme was determined by measuring PPO activity at different temperatures in the range 30–60 °C for 1 h duration at certain intervals using catechol. Renaturation property of the enzyme was investigated by measuring the activity within certain time intervals at lowered room temperature.

2.4.5. Enzyme kinetics and substrate specificity

For determining of Michaelis constant (K_M) and maximum velocity (V_{max}) values of the enzyme, PPO activities were measured with the 5 substrates at varying concentrations (2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 15.0 mM) under optimum conditions of pH, ionic strength, and temperature. K_M and V_{max} values of PPO, for each substrate, were calculated from a plot of $1/V$ against 1/[S] by the method of Lineweaver and Burk (1934).

2.4.6. Effect of inhibitors

Inhibitor effects on PPO activity were studied by using the following inhibitors: sodium chloride, ethylene glycol, thiodiglycol, dithioerithritol, cysteine chloride, ascorbic acid, sodium metabisulfite, β-mercaptoethanol, and sodium diethyl dithiocarbamate (DIECA) at five different concentrations of inhibitors with 10 mM catechol as substrate at pH 7.0. Percent activity graphs were drawn from these results to find both I_{50} values. Later, using five different concentrations of the substrates, PPO activities were measured at three constant inhibitor concentrations with the inhibitors indicated above. $1/V$ and 1/[S] values obtained from these activity measurements were used for drawing Lineweaver–Burk graphs. Finally, K_i constant values were found from the graphs.

3. Results and discussion

3.1. Extraction and purification of PPO enzyme

PPO activity of the precipitate of 0–70% $(NH_4)_2SO_4$ saturation was found to be the highest, and this saturation point was used for all the extraction processes. Polyvinylpyrolidine was used during extractions to bind the phenols which could inactivate the PPO. It is well documented that oxidation of phenolics by PPO produces quinones which would inhibit PPO (Walker, 1964). Therefore, ascorbic acid was also used to reduce quinones to phenolic substrates during extraction.

After ammonium sulfate precipitation, the PPO activity level in the dialyzed enzyme extract decreased to 49.41% from 100%. This situation was probably due to loss of copper ions from the medium during the dialysis (Isbiguro & Anyama, 1970). Since activity of the enzyme decreased, undialyzed samples (from after ammonium sulfate precipitation) were used in the PPO activity studies.

Results on the purification of PPO are given in Table 1. When the purification degrees were compared, the purification degree was 1.44 for leaves and 1.30 for stem after ammonium sulfate purification, and it was 43.3 for leaves and 25.8 for stem after gel filtration chromatography. Results of the purification of PPO by gel filtration chromatography for *Ferula* leaves and stem

Table 1
Purification of polyphenol oxidase from *ferula*

Purification steps	Total volume (ml)	Activity (EU/ml)	Total activity (EU)	Protein (mg/ml)	Total protein (mg)	Specific activity (EU/mg of protein)	Yield (%)	Purification (*n*-fold)
(a) Leaf samples								
Crude extract	18	1290	23220	1.34	24.12	0.96	100.0	1.00
$(NH_4)_2SO_4$ precipitation and dialysis	3.5	5800	20300	4.20	14.7	1.38	87.4	1.44
Gel filtration chromatography	9	2080	18720	0.05	0.45	41.6	80.6	43.33
(b) Stem samples								
Crude extract	16	638	10208	1.25	20.0	0.51	100.0	1.00
$(NH_4)_2SO_4$ precipitation and dialysis	3	2718	8154	4.10	12.3	0.66	85.0	1.30
Gel filtration chromatography	10	789	7890	0.06	0.6	13.15	77.3	25.78

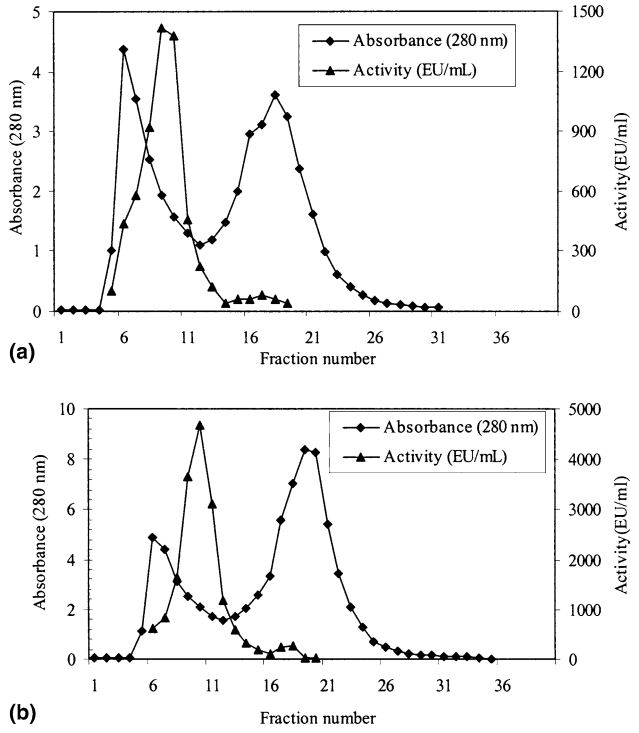

Fig. 1. Purification of PPO from *Ferula* leaves (a) and stems (b) by gel filtration chromatography.

are shown in Fig. 1. Active fractions in 5–15 eluates (Fig. 1(a)) for *Ferula* leaves and in 5–12 eluates (Fig. 1(b)) for *Ferula* stems were collected for the determination of purification degree.

3.2. Characterization of ferula PPO

The enzyme samples obtained by ammonium sulfate precipitation were used for characterization of the PPO.

3.2.1. Substrate specificity

PPO showed activity with the diphenols and polyphenolic substrates but not with the monophenolic substrates used in the study (Table 2). The best substrates of PPO were found to be catechol for leaf samples and (−) epicatechin for stem samples.

3.2.2. Effect of pH

Activities of *Ferula* leaf and stem samples were measured with 5 different substrates to determine optimum pH for each substrate (Table 3). Optimum pH values for the leaves and stems were found to be 7.0 with catechol, 6.5 with (+) catechin, and 6.0 with 4-methyl catechol, chlorogenic acid and (−) epicatechin.

The stability of the enzyme under different pH conditions (5.00, 5.20, 5.40, 5.50, 5.60, 5.80, 6.00, 6.20, 6.50, 7.00, and 7.50) and at certain time intervals was investigated by using catechol. *Ferula* PPO was more stable at pH 5.20 than at other pH values.

Table 2
Substrate specificity of *ferula* PPO

Substrate	Concentration (mM)	Leaf enzyme activity (EU/ml)	Stem enzyme activity (EU/ml)
Catechol	10	9400	4860
4-Methylcatechol	10	2940	2000
(+)-Catechin	0.99	3820	2900
(−)-Epicatechin	1.33	5160	6540
Chlorogenic acid	1.33	3560	1120
Dopamine	10	560	200
L-Dopa	10	100	80
Gallic acid	10	60	160
Pyrogallol	10	300	400
L-Tyrosine	2.5	0.0	0.0

Table 3
Optimum pH and temperature, and K_M and V_{max} alues of PPO in *ferula*

Substrate	Optimum pH	Optimum temperature (°C)	K_M (M)	V_{max} (EU/ml min)
(a) Leaf samples				
Catechol	7.0	12	2.34×10^{-3}	8541
4-Methylcatechol	6.0	25	6.58×10^{-3}	3502
Chlorogenic acid	6.0	20	7.64×10^{-4}	5866
(+)-Catechin	6.5	30	5.54×10^{-4}	3372
(−)-Epicatechin	6.0	15	7.98×10^{-4}	6426
(b) Stem samples				
Catechol	7.0	12	2.64×10^{-3}	4389
4-Methylcatechol	6.0	25	6.78×10^{-3}	2950
Chlorogenic acid	6.0	20	1.07×10^{-3}	1764
(+)-Catechin	6.5	30	3.06×10^{-4}	4823
(−)-Epicatechin	6.0	15	2.89×10^{-3}	5308

3.2.3. Effect of temperature

The temperature effects on *Ferula* PPO activity were studied between 5 and 70 °C, with the substrates used in the experiments (Table 3). As seen in the table, optimum temperatures were the same for leaf and stem samples in the case of each substrate.

Heat stability of the *Ferula* PPO was also investigated between 30 and 60 °C only for leaf samples, with a 60-min duration, using catechol as the substrate. A decrease in PPO activity of 40%, 65% and 100% was found at 30, 40 and 50 °C in 60 min, respectively. Moreover, the enzyme activity at 60 °C was found to be completely exhausted in 40 min (Fig. 2).

In addition, renaturation of the *Ferula* PPO was also studied by lowering the temperatures to ambient. The renaturation feature was not observed for the enzyme.

3.2.4. Enzyme kinetics

K_M and V_{max} values were calculated from the Lineweaver–Burk graphs for six different substrates and are shown in Table 3. As seen in the Table, the *Ferula* PPO had a great affinity for (+)-catechin when both leaf and stem samples were used. When the V_{max} values

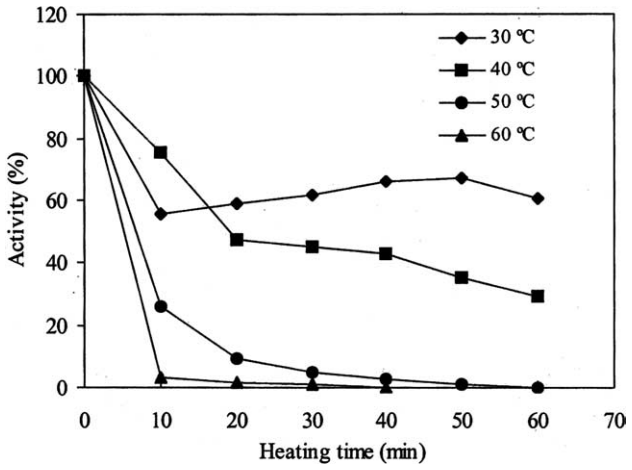

Fig. 2. Effects of different temperatures on PPO activity from *Ferula species*.

Table 4
I_{50} values for 5 inhibitors for PPO

Inhibitors	Leaf samples I_{50} (M)	Stem samples I_{50} (M)
Ascorbic acid	8.15×10^{-5}	7.18×10^{-5}
β-Mercaptoethanol	1.38×10^{-4}	6.32×10^{-5}
Sodium metabisulfite	1.19×10^{-4}	2.59×10^{-5}
Sodium diethyl dithiocarbamate	6.45×10^{-5}	4.54×10^{-5}
L-Cysteine chloride	1.26×10^{-4}	1.16×10^{-4}

of substrates for the leaf and stem samples were compared, the V_{max} values for leaf samples were higher than for stem samples with the same substrate. In contrast, the V_{max}value of catechin for stem samples was higher than that for leaf samples. Clearly, *Ferula* PPO had a great affinity for diphenolic subtrates in this study. This observation was similar to that of the work on dog-rose

Table 5
K_i values and inhibition modes for 5 inhibitors for PPO

Inhibitors	[I] (M)	K_i (M)	Average values of K_i (M)	Type of inhibition
(a) Leaf samples				
Ascorbic acid	7.0×10^{-5}	7.09×10^{-5}	4.70×10^{-5}	Non-competitive
	8.0×10^{-5}	3.94×10^{-5}		
	9.0×10^{-5}	3.07×10^{-5}		
β-Mercaptoethanol	1.2×10^{-4}	4.45×10^{-9}		
	1.6×10^{-4}	4.35×10^{-9}	3.68×10^{-9}	Competitive
	2.0×10^{-4}	2.25×10^{-9}		
Sodium metabisulfite	1.0×10^{-4}	5.87×10^{-9}		
	1.2×10^{-4}	4.88×10^{-9}	4.85×10^{-9}	Competitive
	1.4×10^{-4}	3.82×10^{-9}		
Sodium diethyl dithiocarbamate	6.0×10^{-5}	3.52×10^{-9}		
	7.0×10^{-5}	1.14×10^{-9}	1.76×10^{-9}	Competitive
	8.0×10^{-5}	6.42×10^{-10}		
L-Cysteine chloride	4.0×10^{-5}	1.56×10^{-9}		
	5.0×10^{-5}	1.00×10^{-9}	1.08×10^{-9}	Competitive
	6.0×10^{-5}	6.81×10^{-10}		
(b) Stem samples				
Ascorbic acid	6.0×10^{-5}	2.85×10^{-5}	2.90×10^{-5}	Non-competitive
	7.0×10^{-5}	3.63×10^{-5}		
	8.0×10^{-5}	2.21×10^{-5}		
β-Mercaptoethanl	6.0×10^{-5}	3.41×10^{-9}		
	8.0×10^{-5}	2.71×10^{-9}	2.48×10^{-9}	Competitive
	1.0×10^{-4}	1.34×10^{-9}		
Sodium metabisulfite	2.0×10^{-5}	8.49×10^{-10}		
	3.0×10^{-5}	6.88×10^{-10}	6.53×10^{-10}	Competitive
	4.0×10^{-5}	4.22×10^{-10}		
Sodium diethyl dithiocarbamate	2.0×10^{-5}	7.79×10^{-10}		
	3.0×10^{-5}	8.98×10^{-10}	7.91×10^{-10}	Competitive
	4.0×10^{-5}	6.98×10^{-10}		
L-Cysteine chloride	4.0×10^{-5}	2.40×10^{-9}		
	5.0×10^{-5}	1.64×10^{-9}	1.70×10^{-9}	Competitive
	6.0×10^{-5}	1.06×10^{-9}		

PPO (Sakiroglu, Küfrevioglu, Kocacaliskan, Oktay, & Onganer, 1996) and Amasya apple PPO (Oktay, Küfrevioglu, Kocacaliskan, & Sakiroglu, 1995).

3.2.5. Effect of inhibitors

I_{50} values are shown in Table 4 for each inhibitor. K_i values and inhibition modes for 5 inhibitors are given in Table 5. From the K_i constants, it was concluded that the inhibition mode of ascorbic acid is non-competitive for both leaf and stem samples, and the other inhibitors are competitive. The strongest inhibitors were found to be sodium metabisulfite for stem samples and L-cysteine chloride for leaf samples. On the other hand, thiodiglycol, ethylene glycol and sodium chloride were investigated and their inhibition effects were found to be weaker, even though higher concentrations of them were employed. Thus, K_i values of these inhibitors were not calculated.

3.2.6. Activities of ferula parts

To determine which part (root, stem and leaf) of *Ferula* plants had the highest enzyme activity, catechol and 4-methyl catechol substrates at optimum conditions were used for enzyme activity assays. Enzyme activities for catechol substrate were 6520, 4680, and 9400 EU/ml for root, stem, and leaf samples, respectively. In the case of 4-methyl catechol, these values were 2480, 2000, and 2940 EU/ml for root, stem, and leaf samples, respectively. As a result, the highest activity values for both substrates were observed when leaf samples were employed.

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