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Inhibition of tyrosinase activity by a polyphenol esterase using selected phenolic substrates

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Abstract

The inhibition of tyrosinase activity by polyphenol esterase (PPE) fractions, recovered from the biomass of *Aspergillus niger*, was investigated using a wide range of mono- and di-phenols as substrates. An inhibitory effect on tyrosinase activity was exhibited by the partially purified PPE fraction FI with 3,4-dihydroxyphenylacetic acid (DHPAA), L-3,4-dihydroxyphenylalanine (L-DOPA), 4-methylcatechol, catechol and caffeic acid as substrates. No inhibitory effect on tyrosinase activity was detected using fraction FI with 4-hydroxyphenylpyruvic acid and *m*- and *p*-cresol, measured spectrophotometrically. The purification of fraction FI by ion-exchange chromatography resulted in a more purified one (FII), which markedly increased the inactivation of tyrosinase activity using all substrates indicated above. Purified PPE exhibited, using spectrophotometric analysis, an uncompetitive type of inhibition on tyrosinase activity with caffeic acid and 4-methylcatechol as substrates and a mixed one with DHPAA, catechol, L-DOPA, 4-hydroxyphenylpyruvic acid and *m*- and *p*-cresol. The results also indicated that, using the polarographic method, fraction FII reduced the rate of oxygen consumption with diphenols as substrates but not with monophenols.

Keywords: Tyrosinase; Inhibition; Polyphenol esterase; Phenolic substrates

1. Introduction

Polyphenol oxidase (PPO) catalyzes, in the presence of molecular oxygen, two successive reactions involving molecular oxygen; the hydroxylation of monophenols leads to the formation of *o*-diphenols and their subsequent oxidation into *o*-quinones, which in turn are polymerized into brown, red or black pigments (Mayer & Harel, 1979; Whitaker, 1985). The most important endogenous phenolic substrates for PPO in apple and potato sources are chlorogenic acid, catechol, (+)-catechin, caffeic acid, L-3,4-dihydroxyphenylalanine (L-DOPA), 4-methylcatechol, *p*-hydroxyphenylacetic acid (DHPAA), 4-hydroxyphenylpyruvic acid, *p*-coumaric acid and *m*- and *p*-cresol; the latter four are mainly concentrated in the potato and potato tuber (Ndubizu, 1976; Walker, 1964).

Polyphenolic compounds of apples and potatoes have been well investigated because of their contribution to the change in the color and flavor of processed foods (Spanos & Wrolstad, 1990; Spanos, Wrolstad & Heatherbell, 1990). Enzymatic browning has generally been reported to be responsible for these changes in apples and potatoes (Toribio & Lozano, 1984). Enzymatic browning is considered to affect the appearance, organoleptic properties and nutritional quality of many food products.

The control of enzymatic browning has always been a challenge to the food industry (Ponting, 1960). Beside halide salts, aromatic carboxylic acids are known to inhibit PPO (Rouet-Mayer & Philippon, 1986; Walker & Wilson, 1975). Certain reducing agents such as sulfites are also known as effective inhibitors

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for PPO (Sayavedra-Soto & Montgomery, 1986); however, the use of these compounds has been more restricted, due to the associated potential hazards (Taylor & Bush, 1986), thereby making the need for an alternative obvious.

Monohydroxylated cinnamic acids are considered to be appropriate inhibitors of PPO activity (Walker & McCallion, 1980). Walker (1969, 1970) suggested that the hydrolysis of cinnamoyl derivatives of quinic acid, catalyzed by the pectinolytic activity of *Penicillium expansum*, could result in the formation of phenolic acids which can act as competitive and non-competitive inhibitors for PPO activity, due to their structural similarities with phenolic substrates (Walker & McCallion, 1980; Walker & Wilson, 1975). Recently, our group (Madani, Kermasha, Goetghebeur & Tse, 1997) reported on the use of a polyphenol esterase (PPE), obtained from the biomass of *Aspergillus niger*, capable of generating caffeic acid from chlorogenic acid. The characterization of tyrosinase and polyphenol esterase-catalyzed end-products using selected phenolic substrates indicated that PPE inhibited tyrosinase activity by reacting with the phenolic substrates (Madani, Kermasha & Versari, 1999).

The aim of this work was to study the inhibitory effect of the partially purified and purified polyphenolic hydrolyzing esterase (PPE), obtained from the biomass of *A. niger*, on the tyrosinase activity using a wide range of phenolic substrates in a model system.

2. Results and discussion

2.1. Enzymatic study with mushroom tyrosinase

Kinetic studies (data not shown), using polarographic and spectrophotometric methods, indicated that both the mono- and di-phenols were oxidized by mushroom tyrosinase. The kinetic studies also showed that *p*-coumaric acid was not a substrate for mushroom tyrosinase. Bajaj, Diez De Bethencourt, Junquera and Gonzalez-San José (1997) reported that *p*-coumaric acid, ferulic acid, phloridzin, naringin and procyanidin were not substrates for mushroom tyrosinase.

2.2. Effect of partially purified PPE on mushroom tyrosinase activity

The inhibitory effect of the partially purified PPE fraction FI on mushroom tyrosinase activity is reported in Fig. 1. The oxidation of DHPAA (Fig. 1A), L-DOPA (Fig. 1B), 4-methylcatechol (Fig. 1C), catechol (Fig. 1D) and caffeic acid (Fig. 1E), used as substrates, decreased with an increase in the concentration of PPE; however, using the spectrophotometric method, fraction FI showed no inhibitory effect on the tyrosinase oxidation of 4-hydroxyphenylpyruvic acid and *m*- and *p*-cresol. The experimental findings (not shown) indicated the absence of any inhibitory effect of PPE on tyrosinase activity with all the mono- and di-phenols, as substrates, when the enzymatic assays were determined by oxygen uptake using the polarographic method; however, these findings may be attributed to the lower sensitivity of this method.

The results (Table 1) show that fraction FI demonstrated a stronger inhibitory effect on the oxidation of caffeic acid by mushroom tyrosinase, as indicated by the lower I_{50} and K_i values in comparison to the other substrates. The I_{50} values, defined as mg protein of PPE needed to reduce tyrosinase activity by 50% for caffeic acid, were 4, 5, 3 and 2 times lower than those for DHPAA, L-DOPA, 4-methylcatechol and catechol, respectively. The K_i values, defined as the inhibition dissociation constant, varied between 0.78×10^{-3} and 5.40×10^{-3} mg protein per ml for DHPAA, L-DOPA, 4-methylcatechol, catechol and caffeic acid. With regard to caffeic acid, the strongest inhibitory effect of PPE on the rate of brown color formation may be explained by the fact that this compound can act as both substrate and inhibitor for mushroom tyrosinase (Ferrar & Walker, 1996; Kermasha, Goetghebeur, Monfette, Metche & Rovel, 1993).

The V_{max} values (Table 1), obtained from the Lineweaver–Burk plots (Fig. 2), for tyrosinase activity were determined to be between 0.39×10^{-1} and 1.39×10^{-1} μmol product per min per enzyme unit, using DHPAA, L-DOPA, 4-methylcatechol, catechol and caffeic acid as substrates. L-DOPA showed the highest affinity towards mushroom tyrosinase as indicated by the lower K_m value (Segal, 1976), followed by 4-methylcatechol, DHPAA, catechol and caffeic acid (Table 1). The K_m values for potato tuber PPO (Ndubizu, 1976) with L-DOPA, *p*-cresol, catechol and caffeic acid were 11.8, 0.67, 4.80 and 2.10 mM, respectively. Using caffeic acid as substrate, the K_m values were 0.63 mM for a PPO from apple chloroplast (Hyodo & Uritani, 1965; Walker, 1964) and 2.40 and 2.90 mM for those from two different potato sources (Ndubizu, 1976). The K_m values with catechol as substrate, using different enzymatic fractions from mushroom, were 2.5, 3.0, 4.0 and 22.0 mM (Sisler & Evans, 1958; Smith & Kruger, 1962). The variation in the K_m values may be due to the differences in the sources of PPO enzymes and the degree of purification; these variations may also be attributed to steric factors related to differences in protein structure (Vamos-Vigyazo, 1981).

PPE exhibited a mixed inhibitory effect towards tyrosinase activity, using DHPAA (Fig. 2A), L-DOPA (Fig. 2B) and caffeic acid (Fig. 2E); these findings

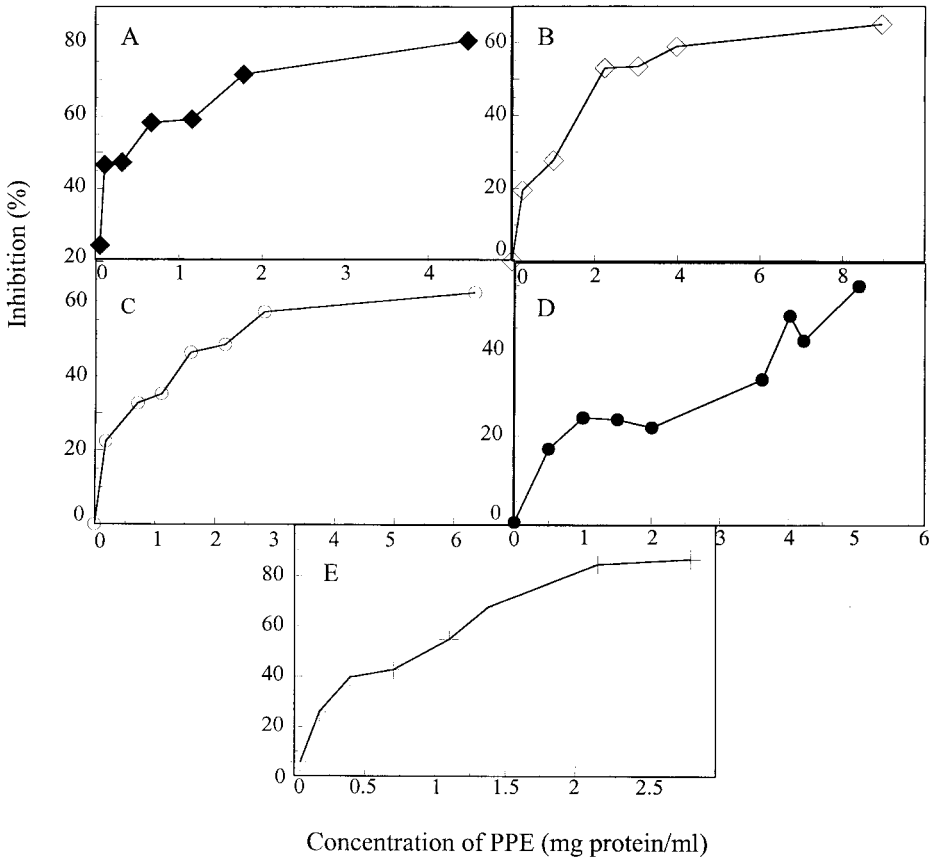

Fig. 1. Inhibitory effect of the partially purified polyphenol esterase (FI) on mushroom tyrosinase activity, using (A) 3,4-dihydroxyphenylacetic acid (◆), (B) L-DOPA (◇), (C) 4-methylcatechol (○), (D) catechol (●) and (E) caffeic acid (+) as substrates, using the spectrophotometric method.

suggest that PPE reduces the affinity of the substrate for PPO, yet it does not bind to the active site. These results may also indicate that the dissociation of the enzyme–substrate complex was prevented (Segal, 1976). PPE also showed a competitive type of inhibition with 4-methylcatechol (Fig. 2C) and an uncompetitive one with catechol (Fig. 2D), as indicated by their corresponding V_{maxapp} and K_{mapp} values (Table 1) of 1.42×10^{-1} to 1.23×10^{-1} μmol product per min per enzyme unit and 0.71–0.15 mM, respectively.

2.3. Effect of purified PPE on mushroom tyrosinase activity

The purified PPE fraction FII exhibited, using the spectrophotometric method, a higher inhibitory effect (Table 2) on tyrosinase activity compared to that of fraction FI (Table 1), as indicated by the lower I_{50} and K_i values. The I_{50} values for fraction FII (Table 2) were 106×10^2, 11×10^2, 16, 71 and 36 times lower than those obtained for fraction FI (Table 1), using

Table 1
Inhibitory effect of the partially purified PPE on tyrosinase activity, determined spectrophotometrically

Substrate	K_m[a] (mM)	V_{max}[a] (μmol/min/U) × 10^{-4}	K_{mapp}[b] (mM)	V_{maxapp}[b] (μmol/min/U) × 10^{-4}	I_{50}[c] (mg/ml)	K_i[d] (mg/ml)	Type of inhibition
3,4-Dihydroxyphenylacetic acid	0.402	0.630	0.638	0.300	35.10	4.206	mixed
L-DOPA	0.245	0.705	0.677	0.742	47.00	5.396	mixed
4-Methylcatechol	0.389	1.390	0.707	1.420	23.00	3.085	competitive
Catechol	0.419	1.377	0.152	1.230	21.00	3.439	uncompetitive
Caffeic acid	0.605	0.385	0.142	0.060	9.600	0.782	mixed

[a] Kinetic parameters for mushroom tyrosinase.
[b] Kinetic parameters for mushroom tyrosinase in the presence of PPE (0.013–4.437 mg protein).
[c] Concentration of PPE, considered as inhibitor, in mg protein required to reduce by 50% the activity of 5.3 units of tyrosinase.
[d] Inhibition dissociation constant.

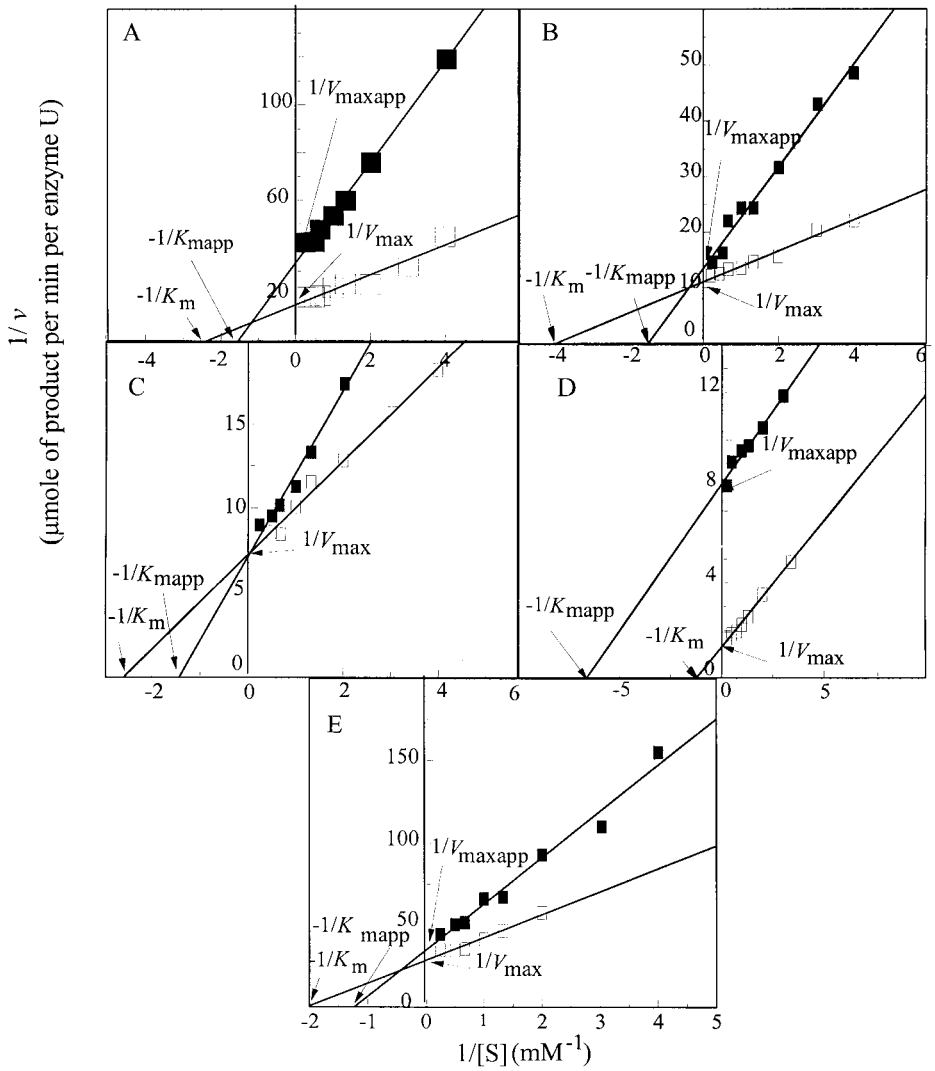

Fig. 2. Lineweaver–Burk plots of $1/v$ versus 1/[S] with (A) 3,4-dihydroxyphenylacetic acid, (B) L-DOPA, (C) 4-methylcatechol, (D) catechol and (E) caffeic acid. The enzymatic assay was performed with tyrosinase alone (□), and with both tyrosinase and partially purified polyphenol esterase (FI) (■), using the spectrophotometric method.

Table 2
Inhibitory effect of the purified PPE on tyrosinase activity, determined spectrophotometrically

Substrate	K_m[a] (mM)	V_{max}[a] (μmol/min/U) × 10^{-4}	K_{mapp}[b] (mM)	V_{maxapp}[b] (μmol/min/U) × 10^{-4}	I_{50}[c] (mg/ml)	K_i[d] (mg/ml)	Type of inhibition
3,4-Dihydroxyphenylacetic acid	0.318	0.637	0.469	0.359	0.33	0.03×10^{-3}	mixed
L-DOPA	0.245	0.647	0.295	0.244	4.32	5.87×10^{-1}	mixed
4-Methylcatechol	0.400	1.286	0.148	0.233	140.90	0.95	uncompetitive
Catechol	0.419	1.377	0.631	0.674	29.80	3.70	mixed
Caffeic acid	0.605	0.385	0.142	0.060	26.87	7.12	uncompetitive
4-Dihydroxyphenylpyruvic acid	1.217	0.846	1.080	0.440	21.50	20.00×10^{2}	mixed
m-Cresol	0.484	0.317	0.506	0.194	0.37	1.62×10^{2}	mixed
p-Cresol	0.248	0.425	0.380	0.129	1.40	14.30×10^{2}	mixed

[a] Kinetic parameters for mushroom tyrosinase.
[b] Kinetic parameters for mushroom tyrosinase in the presence of PPE ($0.251–14.00 \times 10^{2}$ mg protein) × 10^{-2}.
[c] The concentration of PPE, considered as inhibitor, in mg protein required to reduce by 50% the activity of 5.3 and 2.1 units of tyrosinase for the di- and mono-phenols, respectively.
[d] Inhibition dissociation constant.

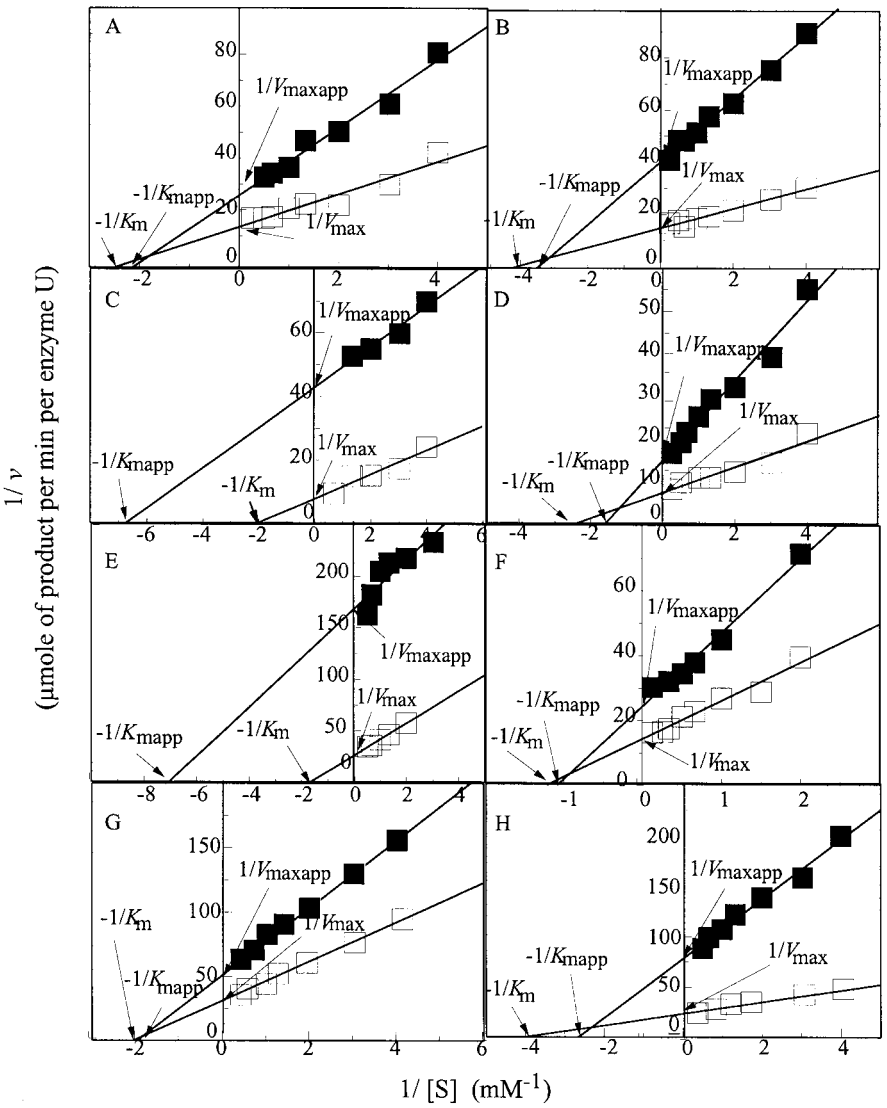

Fig. 3. Lineweaver–Burk plots of $1/v$ versus 1/[S] with (A) 3,4-dihydroxyphenylacetic acid, (B) L-DOPA, (C) 4-methylcatechol, (D) catechol, (E) caffeic acid, (F) 4-hydroxyphenylpyruvic acid, (G) *m*-cresol and (H) *p*-cresol. The enzymatic assay was performed with tyrosinase alone (□), and with both tyrosinase and purified polyphenol esterase (FII) (■), using the spectrophotometric method.

DHPAA, L-DOPA, 4-methylcatechol, catechol and caffeic acid, respectively. The K_i values obtained with FII were 140×10^4, 92, 33, 9 and 11 times lower than those obtained for FI, using DHPAA, L-DOPA, 4-methylcatechol, catechol and caffeic acid, respectively. These findings indicate that the inhibitory effect of PPE on tyrosinase activity increased with the purification of PPE. The results (Table 2) also show that the purified PPE fraction FII showed an inhibitory effect towards 4-hydroxyphenylpyruvic acid and *m*- and *p*-cresol as substrates, as indicated by the I_{50} and K_i values. These findings suggest that a more purified enzyme is required to inhibit the oxidation of the monophenol substrates.

A mixed type of inhibition was observed with the purified PPE fraction FII, using DHPAA (Fig. 3A), L-DOPA (Fig. 3B) and catechol (Fig. 3D) as substrates; the trend of these results is in agreement with that obtained with the partially purified PPE fraction FI, except for catechol. The purified PPE fraction FII also exhibited an uncompetitive type of inhibition with 4-methylcatechol (Fig. 3C) and caffeic acid (Fig. 3E). Fraction FII also showed a mixed type of inhibition with the monophenols including, 4-hydroxyphenylpyruvic acid (Fig. 3F) and *m*- and *p*-cresol (Fig. 3G and H). The V_{maxapp} and K_{mapp} values for tyrosinase (Table 2) were found to vary from 0.06×10^{-1} to 0.36×10^{-1} μmol product per min per enzyme unit and 0.14–0.61 mM, for DHPAA, L-DOPA, 4-methylcatechol, catechol, caffeic acid and *m*- and *p*-cresol, respectively.

The purified PPE fraction FII reduced the rate of oxygen consumption with diphenols and not with

Table 3
Inhibitory effect of the purified PPE on tyrosinase activity, determined by the polarographic method

Substrate	K_m[a] (mM)	V_{max}[a] (μmol/min/U) × 10^{-4}	K_{mapp}[b] (mM)	V_{maxapp}[b] (μmol/min/U) × 10^{-4}	I_{50}[c] (mg/ml)	K_i[d] (mg/ml)	Type of inhibition
3,4-dihydroxyphenylacetic acid	0.565	4.132	0.146	5.435	4.13	4.79	competative
L-DOPA	0.858	2.740	1.641	0.781	0.08	1.59	mixed
4-methylcatechol	1.218	4.180	0.921	2.817	2.30	4.20	uncompetitive
Catechol	0.609	6.99	1.055	4.608	2.25	8.90	mixed
Caffeic acid	0.473	2.198	0.535	0.855	0.90	0.12	competative

[a] Kinetic parameters for mushroom tyrosinase.
[b] Kinetic parameters for mushroom tyrosinase in the presence of PPE (0.08–4.13 mg protein).
[c] Concentration of PPE, considered as inhibitor, in mg protein required to reduce the activity by 50% of 5.3 units of tyrosinase.
[d] Inhibition dissociation constant.

monophenols as substrates. Table 3 shows that the I_{50} values, for the diphenols as substrates, are higher when the enzymatic assays were determined by oxygen uptake than those performed spectrophotometrically.

The I_{50} values for DHPAA, L-DOPA, 4-methylcatechol, catechol and caffeic acid determined by the polarographic method were 12, 2, 1.6, 8 and 3 times greater than those obtained by spectrophotometric measurement. The results (Table 3) also demonstrate higher K_i values; these findings indicate that the spectrophotometric method showed a higher sensitivity than the polarographic one.

The findings also show that the purified PPE fraction FII exhibited a competitive inhibitory effect on PPO activity using DHPAA (Fig. 4A), a mixed one with L-DOPA (Fig. 4B), catechol (Fig. 4D) and caffeic acid (Fig. 4E), and an uncompetitive one using 4-methylcatechol (Fig. 4C). These results indicate that the degree and type of inhibition of mushroom PPO activity are dependent on the method used.

Partially purified PPE reduced the intensity of brown color formation by mushroom tyrosinase activity using the selected diphenols as substrates. The inhibitory effect of PPE on tyrosinase activity increased with the degree of purification; only purified PPE exhibited, spectrophotometrically, an inhibitory effect on the enzymatic oxidation of monophenols. The purified PPE fraction also reduced the rate of oxygen consumption, determined by the polarographic method, with diphenols but not monophenols as substrates. In conclusion, the overall results indicate that the degree and type of inhibition of tyrosinase activity depend on the nature of substrate and method used for the determination of PPE activity.

3. Experimental

3.1. Enzyme sources

A commercially purified mushroom tyrosinase (Sigma Chemical Co., St-Louis, MO), with an activity of 3400 units/mg solid, was used throughout this study; one unit of enzyme activity was defined as an increase in absorbance of 0.001 per min at pH 6.5 and 25°C. Partially purified and purified PPE, obtained from the culture of *A. niger,* were used throughout this study as inhibitors of PPO activity.

The partial purification of the crude enzymatic extract was carried out according to the procedure described previously by Madani et al. (1997). The partially purified enzymatic fraction FI was further purified by preparative ion-exchange chromatography on a Source 15Q HR 16/10 preparative column (Pharmacia, LKB Biotechnology, Uppsala, Sweden) using the fast-protein liquid chromatography (FPLC) system. The column was equilibrated with two column volumes of Tris–HCl buffer solution (20 mM, pH 8.0). A sample of 2 ml of the enzyme solution (50 mg protein) was injected. A gradient elution system was used consisting of eluent (A) Tris–HCl buffer solution (20 mM, pH 8.0) and eluent (B) the same buffer solution as in A but containing 1 M NaCl. The elution was performed at a flow rate of 2.8 ml per min for 52 min with a gradient system starting with 100% of eluent A followed by an increase in eluent B up to 100% within 35 min, followed by an isocratic elution of 100% of eluent B for 9 min. The eluted protein fractions were monitored at 280 nm and collected in fractions of 5 ml per tube.

3.2. Protein determination

The protein content of the enzyme fractions was determined according to a modification of Lowry method (Hartree, 1972), using bovine serum albumin (Sigma Chemical Co.) as a standard for the calibration curve.

3.3. PPO assay

The PPO assay was performed spectrophotometrically according to a modification of the method described previously by Kermasha et al. (1993). Each assay, carried out in triplicate, contained 500 μl of 10

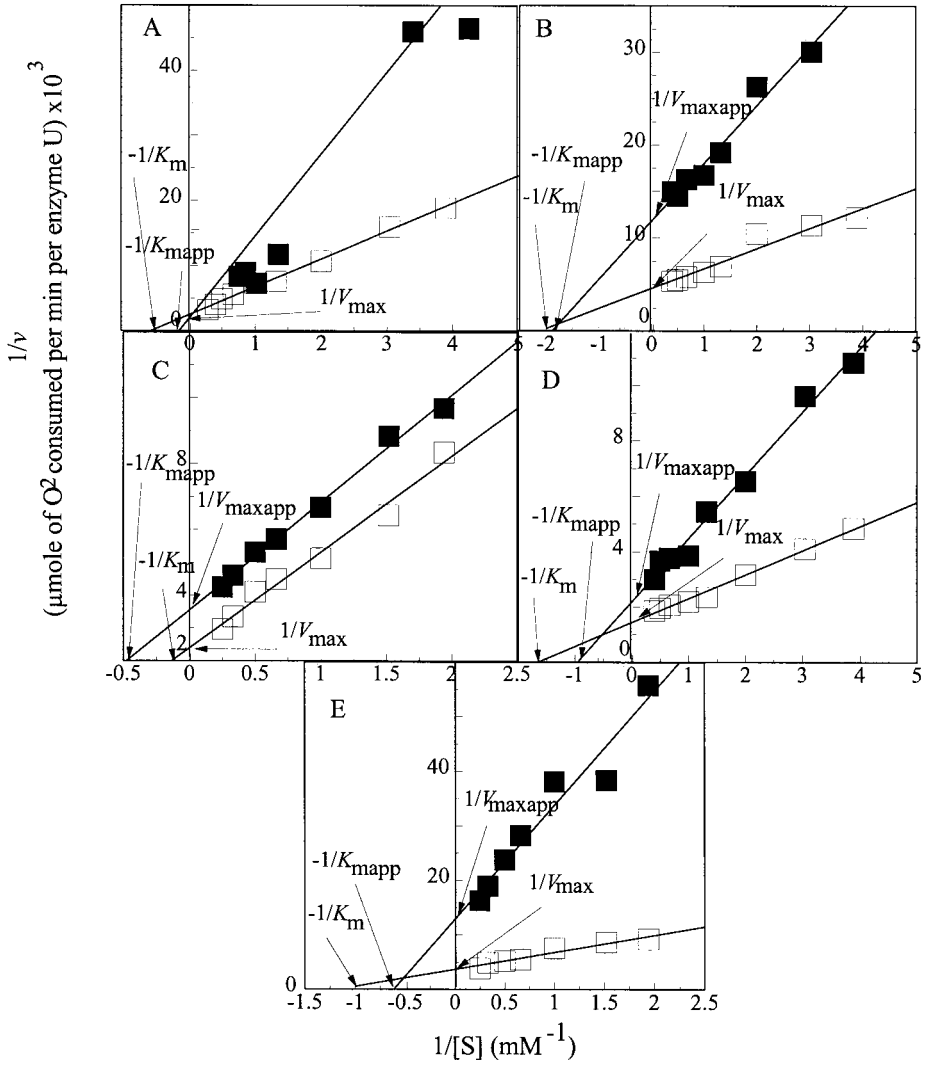

Fig. 4. Lineweaver–Burk plots of $1/v$ versus $1/[S]$ with (A) 3,4-dihydroxyphenylacetic acid, (B) L-DOPA, (C) 4-methylcatechol, (D) catechol and (E) caffeic acid. The enzymatic assay was performed with tyrosinase alone (□), and with both tyrosinase and purified polyphenol esterase (FII) (■), using the polarographic method.

mM substrate and mushroom tyrosinase, at a concentration of 5.3 units for DHPAA, L-DOPA, 4-methylcatechol, catechol, caffeic acid and 2.1 units for 4-hydroxyphenylpyruvic acid and *m*- and *p*-cresol. The total volume was then adjusted with phosphate citrate buffer solution (0.1 M, pH 6.0) to 1 ml. The absorbance was monitored at 400 nm for catechol, 4-methyl catechol, DHPAA, *m*- and *p*-cresol, and at 420 nm for caffeic acid and L-DOPA. The specific activity was expressed as µmol product per min per enzyme unit.

In addition, the PPO activity was also assayed at 25°C according to a modification of the method described previously by Kermasha et al. (1993), using a Gilson oxygraph equipped with a Clark electrode. The enzymatic reaction mixture was prepared in the same conditions as for the spectrophotometric assay but the final volume was 1.75 ml. The enzyme activity was monitored by the oxygen uptake and expressed as µmol of oxygen consumed per minute per enzyme unit.

To study the inhibitory effect of PPE on tyrosinase, different concentrations of PPE were added to the above-described reaction. The PPE inhibitory effect was assayed in triplicate. The results are reported as average percent inhibition of PPO activity in comparison to those obtained with controls without inhibitor.

3.4. PPE assay

PPE activity was assayed by measuring the concentration of caffeic acid, produced from the incubation of chlorogenic acid with PPE, using high-performance liquid chromatography, according to the procedure described previously by Madani et al. (1997). The

specific activity of the PPE was defined as μmol of caffeic acid produced per mg protein per min.

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