Substrates and Carboxylic Acid Inhibitors of a Partially Purified Polyphenol Oxidase from Gum Arabic

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Polyphenol oxidase (PPO) was extracted from gum arabic, and two isoenzymes were partially purified by ammonium sulfate treatment and hydrophobic and ion-exchange chromatographies. Both fractions displayed an optimum at pH 5.3. PPOs showed activity toward *o*-diphenolic substrates but not on monophenols or *p*-diphenols. Activity was maximum with 4-methylcatechol (4MC) followed by the two catechins. Both enzymes showed apparent K_m of 0.8 mM for (+)-catechin and 2.4 mM for (−)-epicatechin and 4MC. Aromatic acids of the benzoic, cinnamic, and phenylalkanoic series and sorbic acid were mixed-type inhibitors. Benzoic acid was the most effective one (K_I = 0.44 mM and K'_I = 1.3 mM). Inhibition efficiency increased when pH was lowered indicating that both neutral (AH) and dissociated (A^-) forms are responsible for inhibition. For all compounds tested, AH forms were more potent inhibitors than A^- forms, and their affinity was higher for free enzyme than for the complex enzyme−substrate.

Keywords: *Gum arabic; polyphenol oxidase; PPO; enzymatic browning; phenolic substrates; aromatic carboxylic acid inhibitors*

INTRODUCTION

Although innocuous to consumers, the darkening of many food products is generally considered to affect food quality from both sensoric and nutritional points of view (Rouet-Mayer *et al.*, 1993). Further, the decrease in market value leading to economic loss is still of great concern to food technologists and processors. Polyphenol oxidase (1,2-benzenediol:oxygen oxidoreductase, EC 1.10.3.1), or PPO, is an ubiquitous copper-containing enzyme, responsible for the undesired browning reactions during handling, storage, and processing of damaged tissues of fresh fruits and vegetables, as well as in some animal products (Macheix *et al.*, 1990; Zawistowski *et al.*, 1991).

The presence of oxidases in gum exudates has been known for many years, (Glicksman and Schachat, 1959); among them, peroxidase and polyphenol oxidase activities have been shown to be present in varying amounts, depending on the gum sample (Marques and Xavier-Filho, 1991). Gum arabic, widely used as a food additive, is defined as a dried exudation obtained from the stems and branches of *Acacia senegal* (L) Willdenow or related species of *Acacia* (family Leguminosaea). On food labels, this hydrocolloid is referred to as stabilizer of food emulsions and dispersions, emulsifier and thickener, fixative for flavors, and adhesive in the confectionery industry (Phillips and Williams, 1993).

Enzymatic browning may occur at various steps of the gum processing (cleaning, dissolution, pasteurization, spray drying, and powdering). The endogenous enzyme PPO which catalyzes the initial phase of browning remains active throughout processing and causes a discoloration, resulting in poor acceptance of the finished product, which cannot comply with technical specifications and/or customer's requirements.

Enzymatic browning can be controlled by different ways (Mc Evily *et al.*, 1992; Nicolas *et al.*, 1994). In addition to heat treatment and acidification, a wide range of chemicals inhibit PPO activity, but only a limited number of them are considered acceptable for the sake of consumer safety and/or cost and could act as potential alternatives to sulfites which are very effective in controlling browning but subject to regulatory restrictions (Taylor, 1993). Among them, sorbic acid and some aromatic carboxylic acids of the benzoic, cinnamic, and phenylalkanoic series have been widely studied and proven to be inhibitors for the PPO activities of various origins (Pifferi *et al.*, 1974; Walker and Wilson, 1975; Walker, 1976; Walker and McCallion, 1980; Gunata *et al.*, 1987; Janovitz-Klapp *et al.*, 1990; Kermasha *et al.*, 1993).

Since little information is available on the purification and properties of PPO from gum arabic, the aim of this study was (i) to partially purify the enzyme from the crude extract, (ii) to characterize this oxidoreductase, and (iii) to assess the inhibitory potency of several carboxylic aromatic acids and sorbic acid on PPO activity and study the effect of pH on the inhibition, in order to prevent or weaken browning of the gum throughout the process and afford an effective long-term control of browning.

MATERIALS AND METHODS

Materials. Crude nodules of gum arabic were kindly supplied from Colloïdes Naturels International Co. (Rouen, France). They were stored at 4 °C, until used as an enzyme source. Their chemical characteristics obtained from manufacturer were as follows: arabinose, 45%; galactose, 39%; rhamnose, 4%; glucuronic acid, 6%; *O*-methylglucuronic acid, 6%; proteins, 1%. Phenyl-Sepharose CL4B was obtained from Pharmacia Co (Uppsala, Sweden); DEAE-trisacryl and CM-trisacryl were purchased from Sepracor SA (Villeneuve la Garenne, France). All other chemicals were of reagent grade from Sigma Chemical Co. (St. Louis, MO) and Prolabo (Paris, France).

Extraction Procedure. Raw nodules of gum arabic (5 g) were extracted for 30 min at room temperature by stirring with 100 mL of 0.1 M sodium acetate buffer at pH 4.5 (buffer A) containing 2% hydrated polyvinylpolypyrrolidone (PVPP) and

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15 mM ascorbic acid. The homogenate was centrifuged (30000*g*, 30 min) at 10 °C, and the supernatant was filtered through 10 μm nylon filter and used as the crude extract.

Purification Procedure. Ammonium sulfate (561 g L^{-1}, 80% saturation) was added to the crude extract, and the sample was kept at 20 °C for 2 h. After centrifugation (30000*g*, 30 min), inactive proteins were partially removed, and the resulting supernatant referred to as S 80 extract was filtered and submitted to hydrophobic chromatography. Aliquots of S 80 extract (90 mL) were loaded onto a 18 × 2.5 cm column of phenyl-Sepharose CL4B (90 mL bed vol) equilibrated with buffer A containing 4.25 M ammonium sulfate at a flow of 180 mL h^{-1} and at 20 °C. After elution of unbound proteins by 2 vol of the equilibration buffer, another inactive fraction was washed out from the column with 140 mL of buffer A enriched with 1.3 M ammonium sulfate. PPO activity was eluted by using 2 vol of buffer A but 0.8 M in ammonium sulfate. Proteins still bound to the gel were removed by H_2O and 50% ethylene glycol in H_2O. Absorbance at 280 nm and PPO activity were determined on each 10 mL fraction.

The active fractions referred to as HIC extract were pooled, dialyzed overnight at 4 °C against 0.05 M sodium acetate buffer at pH 5 (buffer B), and added to a 20 × 1.6 cm DEAE-trisacryl column (40 mL bed vol) previously equilibrated with buffer B at a flow rate of 100 mL h^{-1}. PPO-containing fractions not adsorbed on the column (F_1 fraction) were first eluted with the equilibration buffer B (1 vol bed gel). Bound PPO fractions (F_2 fraction) were then eluted with a linear gradient of ammonium sulfate (0−0.3 M) in buffer B. Proteins still bound to the gel were washed out with buffer B containing 2 M ammonium sulfate. Absorbance at 280 nm and PPO activity were determined on each 7 mL fraction.

F_2 fraction was collected and constitutes the DEAE extract. In order to concentrate active fractions unretained on DEAE-trisacryl gel, F_1 fraction was subsequently applied onto a CM-trisacryl (15 × 1.6 cm, 30 mL bed gel) column, equilibrated with buffer B. After elution of inactive proteins by 40 mL of buffer B, the enzyme active fractions were eluted with the above buffer but 0.3 M in ammonium sulfate (80 mL h^{-1} flow rate). Proteins still bound to the gel were removed by changing the mobile phase to 2 M ammonium sulfate. The eluent was collected, and UV absorbance was measured at 280 nm. Each 5 mL fraction was estimated for PPO activity; active fractions were pooled and constitute the CM extract.

Measurement of PPO Activity. PPO activity was determined polarographically at 30 °C in a 2 mL reaction cell containing 20 mM 4-methylcatechol (4MC) in air-saturated McIlvaine's buffer at pH 5.3 as the substrate extract. For inhibition studies by aromatic carboxylic and sorbic acids at pH 5.3, 4MC was varied from 1 to 20 mM in the control. Each inhibitor was added at two concentrations before the enzyme extract. When the pH was modified, the same procedure was used, except that McIlvaine's buffer was adjusted to the pH under study. Optimum pH was determined with 20 mM 4MC in 0.1 M sodium acetate buffer between pH 3 and 5 and McIlvaine's buffer between pH 5 and 7.5. Activity is expressed as nmol of oxygen consumed/s (nanokatals) under the assays conditions. All the determinations were performed in duplicate. Kinetics parameters K_m and V_m were determined by using a nonlinear regression data analysis software developed for IBM PC (Leatherbarrow, 1987). pK_a values of carboxylic acids were either measured or taken from the *Merck Index* (1989, 11th ed.).

Determination of Proteins. Protein content of extracts was determined by the dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Electrophoretic Methods. Isoelectric focusing (IEF) experiments were performed with a PhastGel system (Pharmacia Co., Uppsala, Sweden) using a homogeneous polyacrylamide gel and Pharmalyte carrier ampholytes, PhastGel 3-9. Four microliter aliquots of extracts were loaded, using sample applicators. The programmed method was used to separate proteins at 15 °C. Coomassie brilliant blue- and $AgNO_3$-staining conditions for the detection of proteins were those described by the manufacturer. Gels stained for PPO activity were immersed in the substrate solution supplemented with (0.05%) *p*-phenylenediamine. The p*I* of the proteins was estimated by using the broad p*I* calibration kit from Pharmacia (pH 3.5−9.3). IEF in liquid-phase medium was carried out using a Rotofor system (Bio-Rad SA, Ivry/Seine, France). The enzymatic extracts were dialyzed overnight against water and then diluted to *ca.* 50 mL with water, and ampholytes (pH 3.8−9.3) solution was added to a final concentration of 2%. The solution was loaded onto the Rotofor cell without further treatment. Focusing was carried out at 12 W constant power for 4 h at 4 °C. Recorded voltages varied between 200 and 670 V. Twenty fractions were collected and their pH values measured. PPO activity was detected using 4MC as substrate as described above.

The molecular weight of native enzymatic extracts was estimated by polyacrylamide gel electrophoresis (PAGE) and performed on a 8−25% polyacrylamide gradient gel zone (Phastgel 8-25), using a Phastsystem electrophoresis instrument (Pharmacia, Uppsala, Sweden) according to the standard procedure. Four microliter aliquots of enzymatic extracts were loaded on the cathodic side of the gel. α-Lactalbumin (M_r 14 400), soybean trypsin inhibitor (M_r 20 100), carbonic anhydrase (M_r 30 000), ovalbumin (M_r 43 000), bovine serum albumin (M_r 67 000), phosphorylase b (M_r 94 000), lactate dehydrogenase (M_r 140 000), catalase (M_r 232 000), ferritin (M_r 440 000), and thyroglobulin (M_r 669 000) were used as low and high molecular weight calibration standards. Staining of gels for the detection of proteins and PPO activity was performed as described above.

RESULTS AND DISCUSSION

Enzyme Purification. The procedure used to purify PPO of gum arabic requires five steps: extraction of PPO, ammonium sulfate treatment, hydrophobic interaction chromatography, and ion-exchange chromatographies on DEAE-trisacryl and CM-trisacryl gels. Preliminary experiments have been performed in order to set the optimum conditions for PPO extraction. Thus, it was observed that extracted PPO activity only slightly changed when the pH of the extraction buffer was raised from 4.5 to 7.5. Substitution of acetate ions for citrate or phosphate did not substantially modify extracted PPO activity (data not shown). A readily soluble and active PPO was obtained when extracted in a sodium acetate buffer at pH 4.5, and in order to limit spontaneous phenolic oxidation at high pH, these conditions were chosen for the study. Since endogenous phenols were present in the crude extract, PVPP was added to the extraction medium to adsorb these contaminants and prevent irreversible binding with proteins (Loomis, 1974). Moreover, optimal concentration of 15 mM ascorbic acid was also added to the extraction medium to protect enzyme from inactivation by quinone polymerization. Since a nonionic detergent such as Triton X-100 has been shown to improve extraction of PPOs from various vegetal species (Janovitz-Klapp *et al.*, 1989; Ôba *et al.*, 1992; Zhou *et al.*, 1993), this compound was also tested for PPO in gum arabic. Loss of enzyme activity at pH 6.5 was 14% and 27% when the detergent was added at concentrations of 0.3% and 0.7%, respectively. At pH 7.5, losses reached 23% and 28%, respectively. Recovery of PPO activity in the supernatant after centrifugation of the crude extract suggests that the enzyme in gum arabic is not a membrane-bound protein but is in a soluble state.

After extraction, ammonium sulfate treatment (20−95% saturation) was investigated to partially remove inactive proteins. Whatever the $(NH_4)_2SO_4$ concentration tested, the bulk of PPO activity was retained in the supernatant, but optimal conditions of 80% saturation in $(NH_4)_2SO_4$ resulted in precipitation of some inactive proteins in the pellet and recovery of nearly all PPO

Table 1. Purification of PPO from Gum Arabic

step	total vol (mL)	total protein (mg)	total activity (μkat)	specific activity (μkat/mg)	yield (%)	purification factor
crude extract	100	5.26	4.17	0.79	100	
S 80 extract	115	2.32	4.2	1.81	100	2.3
phenyl-Sepharose						
pooled frs	36	0.35	1.93	5.51	46	7
most active fr	9.3	0.093	0.63	6.77	15	8.6
dialysis	41	0.35	1.57	4.49	38	5.7
DEAE-trisacryl						
pooled frs (F_2)	19.8	0.23	0.89	3.87	21	4.9
most active fr (F_2)	6.6	0.063	0.37	5.87	9	7.4
pooled frs (F_1)	50	0.05	0.28	5.6	6.7	7.1
CM-trisacryl						
pooled frs	9.4	0.039	0.25	6.41	6	8.1
most active fr	4.7	0.019	0.16	8.42	4	10.7

activity in the supernatant. This purification step allowed a more than 2-fold purification with no loss in the initial PPO activity (Table 1).

Phenyl-Sepharose CL4B chromatography has been successfully used for the purification of the PPO from various sources (Flurkey and Jen, 1980; Wisseman and Lee, 1980; Janovitz-Klapp *et al.*, 1989; Zhou *et al.*, 1993). Two peaks with PPO activity were separated by column chromatography, using a stepwise fractionation with $(NH_4)_2SO_4$. The major peak was eluted using 0.8 M $(NH_4)_2SO_4$, and a minor peak was washed out from the gel with water. The recovery of PPO after chromatography was 80% of the applied activity, leading to an overall purification factor of 7 for the eight pooled fractions and a yield close to 50%. The most active fraction accounted for 15% of the original crude extract and was purified 9-fold. In the phenyl-Sepharose chromatographic step, a large amount of nonactive protein was eluted in the void volume of the column, during washing of the matrix with the equilibrating buffer, and later with a decreased concentration (1.3 M) of $(NH_4)_2SO_4$, providing evidence of efficiency of this purification step. Although the purification factor appears lower than that reported by Janovitz-Klapp *et al.* (1989) for apple PPO, the HIC extract was adequately purified and stable enough for kinetic studies, and the four major PPO fractions were combined. Moreover, this purification step allows to eliminate the bulk of the polysaccharides, a component which is not taken in account in the purification factor.

Chromatography on DEAE-trisacryl column was effective in separating two isoenzymes. A first peak of PPO activity (F_1 fraction) passed out of the column unadsorbed during HIC extract application and subsequent washing of the column, representing 20% of the recovered PPO activity. The major peak (F_2 fraction) was eluted at the beginning of the linear gradient of $(NH_4)_2SO_4$, and 80% of the recovered activity was associated with this fraction. An overall recovery of 73% was obtained over the applied enzyme activity for the pooled fractions, and a more than 7-fold purification was obtained for the most active fraction F_2. Although this purification step resulted in a loss of activity with no apparent purification (Table 1), it allowed the separation of two peaks of PPO activity with different p*I*s. Active proteins of the highly diluted F_1 fraction were combined and applied to a CM-trisacryl column. All PPO activity was retained on the matrix with a yield of total activity close to 100% and eluted at 0.3 M $(NH_4)_2SO_4$. This step was found to be effective in concentrating the PPO activity (3-fold), and a more than 10-fold purification was achieved for the most active fraction (Table 1).

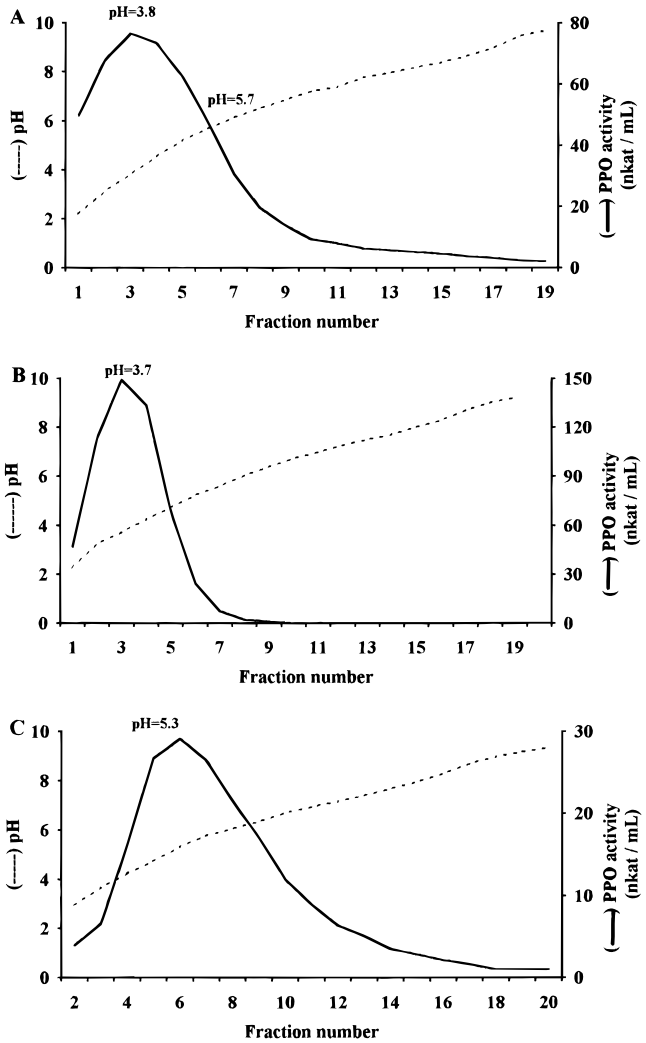

Figure 1. Preparative isoelectric focusing analysis of enzymatic extracts from gum arabic. PPO activity (—) and pH (– – –) were measured from Rotofor fractions following focusing of proteins from HIC extract (A), DEAE extract (B), and CM extract (C).

Estimation of Isoelectric Point and M_r of the Enzyme. HIC, DEAE, and CM extracts were subjected to IEF in liquid medium with a 3.8−9.3 pH gradient (Figure 1). The presence of a broad peak of PPO activity whose maximum was at pH 3.8, accompanied by a shoulder in the region of pH 5.7, was observed in HIC extract (Figure 1A). In DEAE and CM fractions, PPO activity was also revealed by a single peak whose maximum was at pH 3.7 (Figure 1B) and pH 5.3 (Figure 1C), respectively. In the HIC extract, the apparent lack

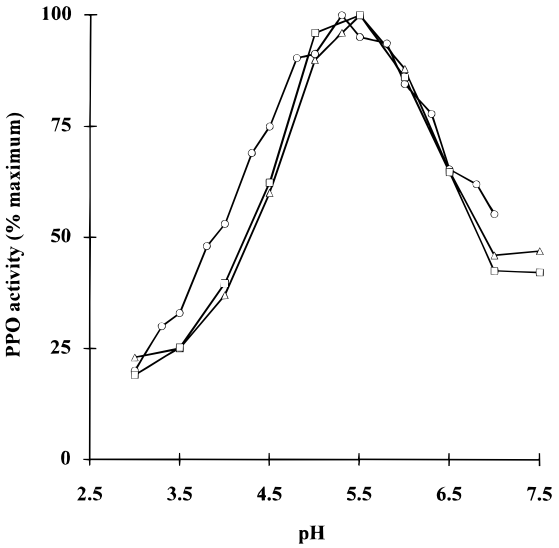

Figure 2. Optimum pH of enzymatic extracts from gum arabic. PPO activity was measured using 4-methylcatechol as the substrate: (○) HIC extract, (△) DEAE extract, and (□) CM extract.

of a peak of activity at pH 5.3 can be explained by the low relative amount of activity of the CM fraction (*ca.* 20%) in the sample. IEF of enzymatic fractions from crude, S 80, and HIC extracts was also performed on PhastGel 3-9. Two major bands migrated to the anodic end of the gel exhibited p*I* values of 3.5 (band 1) and 5.5 (band 2), confirming the presence of two acidic isoforms. They were accompanied with minor faint bands with p*I* values close to 7. Band 1 was also evidenced in the DEAE extract, and band 2 was only observed in the CM extract (data not shown). The p*I* values of various plant PPOs vary within the range of 4.0−10.0, and acidic PPOs have already been identified in apple (4.5 and 4.8) (Janovitz-Klapp *et al.*, 1989), Jerusalem artichoke (4.5) (Zawistoski *et al.*, 1987), iceberg lettuce (3.6) (Heimdal *et al.*, 1994), and edible burdock (3.5) (Murao *et al.*, 1993).

Estimation of the molecular mass of purified enzyme in HIC and DEAE extracts by PAGE provided M_r values of 50 000 and 90 000. In the CM extract a single band at M_r 90 000 was found (data not shown). M_r values of 30 000−40 000 were most commonly cited in the literature for plant PPOs (Vamos-Vigyazo, 1981), but a wider range of M_r has recently been reported (Marquès *et al.*, 1995).

Effect of pH and Temperature. Assays of PPO activities between pH 3 and 7.5 for the purified enzyme preparations showed an optimum pH of 5.3 for HIC extract and 5.5 for DEAE and CM extracts, with 4MC as the substrate (Figure 2). At pH values below 4.5 and above 6.5, PPO activity decreased sharply, but the enzyme was still active at pH as low as 3.0, with a relative activity close to 20%. At pH values above 7, a more than 40% relative activity was observed in DEAE and CM extracts. Similar optimum pHs have been obtained for PPOs from different origins (Vamos-Vigyazo, 1981).

When stored at pH 4.5, at 4 °C as well as at 20 °C, PPO activity from HIC extract remained stable, and the enzyme retained most of its activity (80% of the maximum at 20 °C) for more than 40 days. PPO activity of DEAE and CM extracts stored at pH 5.0 remained stable over a period of 30 days, in the same range of temperatures.

Table 2. Substrate Specificity for Some *o*-Dihydroxy Substrates of Partially Purified PPO Preparations from Gum Arabic (HIC, DEAE, and CM extracts)[a]

	V_{mapp} (%)			K_{mapp} (mM)		
	HIC	DEAE	CM	HIC	DEAE	CM
4-methylcatechol	100	100	100	2.4	2.4	2.5
chlorogenic acid	51	47	44	5.2	5.1	4.7
(+)-catechin	65	67	59	0.8	0.8	0.8
(−)-epicatechin	77	80	75	2.4	2.4	2.3

[a] Determined in air-saturated McIlvaine buffer solution at pH 5.3 and 30 °C. Values of V_m are given in percent of the value obtained for 4-methylcatechol. The standard assay conditions were used with the following substrate concentrations: 1−20 mM for 4-methylcatechol, 1−10 mM for chlorogenic acid, 0.2−5 mM for (+)-catechin, and 0.5−10 mM for (−)-epicatechin. All substrates gave Michaelis−Menten kinetics in the concentration ranges used.

Table 3. Kinetic Parameters of Some Phenolic Substrates of PPO from Gum Arabic (HIC Extract)[a]

substrate	V_{mapp} (%)	K_{mapp} (mM)	V_{mapp}/K_{mapp}
p-cresol	*b*		
tyrosine	*b*		
4-methylcatechol	100	2.4	41.7
pyrocatechol	49	12.6	3.9
chlorogenic acid	51	5.2	9.8
(+)-catechin	65	0.8	81.3
(−)-epicatechin	77	2.4	32.1
caffeic acid	14	1.5	9.3
o-dihydroxyphenylacetic acid	36	15.7	2.3
hydrocaffeic acid	85	8.1	10.5
protocatechuic acid	*b*		
gallic acid	10	2.2	4.5
hydroquinone	*b*		
p-phenylenediamine	*b*		

[a] Determined in air-saturated McIlvaine buffer solution at pH 5.3 and 30 °C. Values of V_{max} are given in percent of the value obtained for 4-methylcatechol. [b] No significant activity was measured.

Kinetic Properties of PPO. The substrate specificity of PPO in HIC, DEAE, and CM extracts and kinetic parameters of the enzyme against four phenolic substrates were first determined (Table 2). Relative activities of PPO were calculated using 4MC as the reference, and maximum activity was detected toward 4MC followed by (−)-epicatechin, (+)-catechin, and chlorogenic acid, at optimum pH of PPO activity. Similar activities against *o*-dihydroxy substrates were found for purified PPO from the three extracts. In the same way, Michaelis constants (K_{mapp}) for these four substrates were found to be independent of the enzymatic fractions tested. The highest affinity was found for (+)-catechin (0.8 mM) followed by 4MC and (−)-epicatechin with a K_{mapp} of 2.4 mM and chlorogenic acid (4.7−5.2 mM).

From these results, purified PPO from HIC extract was selected for subsequent kinetic studies, and various compounds were tested as substrates (Table 3). No detectable monophenolase activity was found with *p*-cresol and tyrosine, and purified PPO was also devoid of laccase activity, as indicated by the lack of reaction with *p*-dihydroxyphenol and *p*-phenylenediamine. PPO in gum arabic displayed activity for all *o*-dihydroxyphenols tested, except for protocatechuic acid which was not oxidized by the enzyme. Among these compounds, 4MC exhibited the highest activity toward PPO. The triphenol gallic acid was oxidized at a lower rate than the *o*-dihydroxyphenols by the enzyme.

Regarding the phenolic substrates, K_m values were ranging from 0.8 to 16 mM, and the highest affinity was found for (+)-catechin. Moreover, the affinity was 3-fold

higher with (+)-catechin than with (−)-epicatechin, indicating a stereospecificity toward the flavan-3-ol derivatives. Among the *o*-dihydroxyphenols, the acceptability of some substrates was dependent on the nature and position of the substituent group on the aromatic ring, as well as on the length of the side chain. When the methyl group of the 4MC molecule was substituted for a carboxylic group (protocatechuic acid), a loss of PPO activity was observed. Nevertheless, activity which was partially recovered with an additional methylene group (*o*-dihydroxyphenylacetic acid) became almost completely restored when a second methylene group was added between the benzene ring and the carboxylic group (*o*-(dihydroxyphenyl)propionic acid). The presence of a conjugated double bond between the ring and the carboxylic group (caffeic acid) markedly reduced the rate of oxidation by PPO, while esterification of the carboxylic function of caffeic acid with quinic acid (chlorogenic acid) resulted in an increase in PPO activity. The results also showed that PPO affinity for phenolic substrates was enhanced by the presence of a carboxylic group conjugated to the benzene nucleus, but its esterification or addition of a methylene group greatly lowered PPO affinity for the substrate.

This substrate specificity is in accordance with most reported PPOs, which markedly oxidize 4MC (Park and Luh, 1985; Janovitz-Klapp *et al.*, 1989; Siddiq *et al.*, 1992; Robinson *et al.*, 1993; Heimdal *et al.*, 1994). Purified PPO from gum arabic exhibits affinities for different substrates of higher or of similar order of magnitude than data reported for PPO in several fruits and vegetables, using chlorogenic acid or 4MC as the substrate (Zawistowski *et al.*, 1988; Janovitz-Klapp *et al.*, 1989; Lourenco *et al.*, 1992; Heimdal *et al.*, 1994). (+)-Catechin, which is a natural phenolic compound present in gum arabic, represents the best substrate for purified PPO, as assessed by its catalytic efficiency (Table 3).

Inhibition Effect of Carboxylic Acids. Inhibitory properties of partially purified PPO preparation (HIC extract) by carboxylic acids were first investigated at the optimum pH of PPO activity (pH 5.3) with 4MC as the substrate. Increasing amounts of sorbic acid and selected derivatives from the benzoic acid, cinnamic acid, and phenylalkanoic acid series were studied. In each case, the type of inhibition was deduced from Lineweaver−Burk double-reciprocal plots. A typical example is shown in Figure 3 for benzoic acid. The results lead to a series of lines which intersect to the left of the vertical axis and above the horizontal axis, with a decrease in V_{mapp} and, conversely, an increase in K_{mapp}, indicating that all aromatic carboxylic acids tested were mixed-type inhibitors.

If we consider the simplest mixed system as a mixture of competitive and noncompetitive inhibition, the corresponding equilibria can be written:

$$\begin{array}{ccccc} E + S & \Leftrightarrow & ES & \rightarrow & E + P \\ + & & + & & \\ I & & I & & \\ K_I \downarrow\uparrow & & \downarrow\uparrow K'_I & & \\ E.I + S & \Leftrightarrow & ES.I & & \end{array}$$

From rapid equilibrium assumptions, the velocity equation for this type of mixed inhibition is (Segel, 1975):

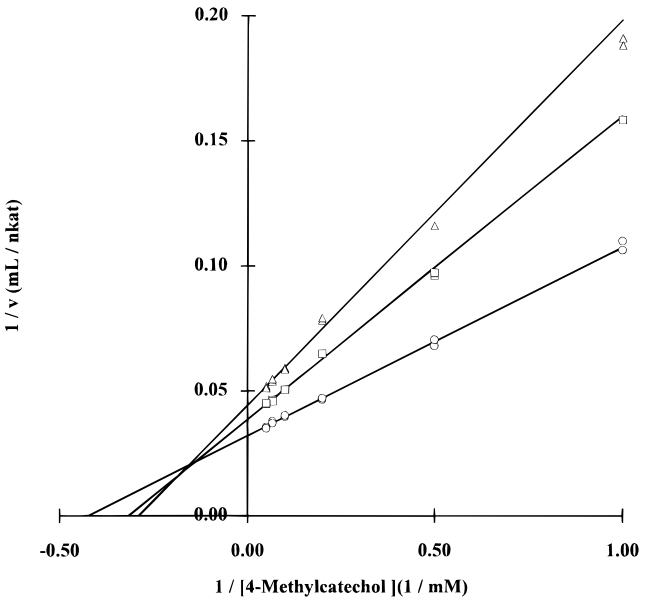

Figure 3. Inhibition of partially purified PPO from gum arabic (HIC extract) by benzoic acid, at pH 5.3 and 30 °C, with 4-methylcatechol as the substrate: (○) control, (□) 0.25 mM benzoic acid, and (△) 0.50 mM benzoic acid.

$$\frac{v}{V_m} = \frac{S/K_m}{1 + S/K_m + I/K_I + (S/K_m)(I/K'_I)} \quad (1)$$

Thus,

$$\frac{V_{mapp}}{V_m} = \frac{1}{1 + \frac{I}{K'_I}} \quad (2)$$

and

$$\frac{K_{mapp}}{K_m} = \frac{1 + \frac{I}{K_I}}{1 + \frac{I}{K'_I}} \quad (3)$$

The two inhibition constants, apparent K_I and K'_I, given in Table 4, were obtained by fitting the experimental data to eqs 2 and 3. For all the tested acids, the affinity was higher for the free form of the enzyme than for its complexed form ($K_I < K'_I$). When values of K_I and K'_I were of the same order of magnitude, the mixed-type system could be considered to be a noncompetitive inhibition, whereas it could be compared to a competitive inhibition when $K_I \ll K'_I$. In this respect, cinnamic and phenylalkanoic acids behaved as quasi-competitive inhibitors since the K_I values were about 10 times lower than the K'_I values, whereas benzoic acids exhibited a quasi-noncompetitive inhibition with K_I values 3−5 times lower than K'_I values. Gentisic and sorbic acids were found to have an intermediate effect.

Even though some authors have found a competitive inhibitory effect on PPO, using 4MC as the substrate (Walker and Wilson, 1975; Gunata *et al.*, 1987; Janovitz-Klapp *et al.*, 1990), differences in type and degree of inhibition of various PPOs were reported (Pifferi *et al.*, 1974; Kermasha *et al.*, 1993). Among the carboxylic acids tested, the strongest inhibition of PPO from gum arabic was observed with benzoic acid at pH 5.3 (K_I = 0.44 mM). At this pH value, the acids of the benzoic series were more potent inhibitors than those of the

Table 4. Inhibition Effects of Carboxylic Acids on PPO from Gum Arabic with Respect to Their Structures at pH 5.3[a]

	substitution[b]					
inhibitor	*ortho* (2)	*meta* (3)	*meta* (5)	*para* (4)	K_{Iapp} (mM)	K'_{Iapp} (mM)
benzoic acid series						
benzoic acid					0.44	1.3
p-hydroxybenzoic acid				OH	0.65	3.3
α-resorcylic acid		OH	OH		0.71	3.4
β-resorcylic acid	OH			OH	1.3	5.5
protocatechuic acid		OH		OH	1.5	7.5
salicylic acid	OH				2.9	11
gentisic acid	OH		OH		3.7	32
cinnamic acid series						
t-cinnamic acid					4.4	43
p-coumaric acid				OH	2.6	33
sorbic acid					3.1	19
phenylalkanoic acid series						
phenylacetic acid					11	>100
phenylpropionic acid					29	>100
p-(hydroxyphenyl)propionic acid				OH	15	>100

[a] Assays were performed in air-saturated McIlvaine buffer solution at 30 °C and pH 5.3, with 4-methylcatechol as the substrate (K_{mapp} = 2.4 mM). [b] The carboxyl group was numbered in position 1 on the aromatic ring.

Table 5. Effect of pH on the Inhibition Constants of Carboxylic Acids Acting on PPO from Gum Arabic[a]

	K_{Iapp} (mM)		K'_{Iapp} (mM)	
inhibitor	pH 4.5	pH 5.3	pH 4.5	pH 5.3
Benzoic Acid Series				
benzoic acid	0.15	0.44	0.37	1.3
p-hydroxybenzoic acid	0.22	0.65	0.89	3.3
α-resorcylic acid	0.30	0.71	2.3	3.4
β-resorcylic acid	0.42	1.3	1.5	5.5
protocatechuic acid	0.48	1.5	2.5	7.5
salicylic acid	0.87	2.9	2.0	11
gentisic acid	1.1	3.7	5.1	32
Cinnamic Acid Series				
p-coumaric acid	1.5	2.6	11	33
t-cinnamic acid	1.6	4.4	18	43
sorbic acid	1.6	3.1	7.3	19
Phenylalkanoic Acid Series				
phenylacetic acid	5.9	11	79	>100
phenylpropionic acid	15	29	>100	>100
p-(hydroxyphenyl)propionic acid	11	15	>100	>100

[a] Assays were performed in air-saturated McIlvaine buffer solution at 30 °C, with 4MC as the substrate, at pH 4.5 and 5.3.

cinnamic and phenylalkanoic series. When the carboxyl group was separated from the benzene nucleus, the inhibition was markedly reduced (K_I > 10 mM for the phenylalkanoic series). In the benzoic acid series, addition of another phenolic group always resulted in a decrease of the inhibition which was more marked for substitution in *ortho* position (salicylic acid) than in *para* position (*p*-hydroxybenzoic acid). Regarding the diphenolic acids, the inhibition was higher when the two phenolic groups were in *meta* (3 and 5 or 2 and 4) position (α- and *β*-resorcylic acids), whereas it decreased for substitutions in *ortho* (3 and 4) position (protocatechuic acid) and *para* (2 and 5) position (gentisic acid). Sorbic acid, in which the benzene nucleus is substituted for conjugated double bonds, exhibited an inhibitory effect similar to that of cinnamic acids. Thus, the presence of the aromatic structure is not absolutely required for the inhibitory effect. Variations in the inhibitory effect on oxidation of phenolic substrates by benzoic, cinnamic, and phenylalkanoic series have already been observed for PPO from various origins (Pifferi *et al.*, 1974; Gunata *et al.*, 1987; Janovitz-Klapp *et al.*, 1990; Kermasha *et al.*, 1993).

The effect of pH on the inhibition by carboxylic acids was further studied by lowering the pH value to 4.5. In the absence of inhibitor and using 4MC as substrate, the decrease of pH from 5.3 to 4.5 resulted in an increase of K_{mapp} from 2.4 to 4.2 mM and a decrease of V_{mapp} from 100% to 80%. All the carboxylic acids tested still exhibited a mixed-type inhibition at pH 4.5 but with an increased affinity as shown by the lower values of inhibition constants (Table 5). If we consider that pH directly affects the carboxylic acid, both neutral and dissociated forms (respectively AH and A^-) of the compound are responsible for inhibition by binding to free and complexed enzyme with four different affinity constants (K_{A^-}, K_{AH}, K'_{A^-}, and K'_{AH}). The equilibria describing this type of inhibition are shown below:

$$\begin{array}{ccc} E.A^- + S & \Leftrightarrow & ES.A^- \\ \mathbf{K}_{A^-} \downarrow\uparrow & & \downarrow\uparrow \mathbf{K'}_{A^-} \\ A^- & & A^- \\ + & & + \\ E + S & \Leftrightarrow & ES \rightarrow E + P \\ + & & + \\ AH & & AH \\ \mathbf{K}_{AH} \downarrow\uparrow & & \downarrow\uparrow \mathbf{K'}_{AH} \\ E.AH + S & \Leftrightarrow & ES.AH \end{array}$$

By analogy with eq 1, we obtain:

$$\frac{v}{V_m} = \frac{\frac{S}{K_m}}{1 + \frac{S}{K_m} + \frac{AH}{K_{AH}} + \frac{A^-}{K_{A^-}} + \frac{S}{K_m} \times \left[\frac{AH}{K'_{AH}} + \frac{A^-}{K'_{A^-}}\right]} \quad (4)$$

with $K_{AH} = (E)(AH)/(E{\cdot}AH)$, $K_{A^-} = (E)(A^-)/(E{\cdot}A^-)$, $K'_{AH} = (ES)(AH)/(ES{\cdot}AH)$, and $K'_{A^-} = (ES)(A^-)/(ES{\cdot}A^-)$.

Then,

$$\frac{V_{mapp}}{V_m} = \frac{1}{1 + \frac{AH}{K'_{AH}} + \frac{A^-}{K'_{A^-}}} \quad (5)$$

and

$$\frac{K_{mapp}}{K_m} = \frac{1 + \frac{AH}{K_{AH}} + \frac{A^-}{K_{A^-}}}{1 + \frac{AH}{K'_{AH}} + \frac{A^-}{K'_{A^-}}} \quad (6)$$

Inserting the expressions 5 and 6 into eqs 2 and 3 gives the following system of equations:

$$\frac{I}{K'_{Iapp}} = \frac{AH}{K'_{AH}} + \frac{A^-}{K'_{A^-}} \quad (7)$$

$$\frac{I}{K_{Iapp}} = \frac{AH}{K_{AH}} + \frac{A^-}{K_{A^-}} \quad (8)$$

with $I = AH + A^-$.
Using $\beta = AH/(AH + A^-)$ (nonionized fraction of the carboxylic acid), it becomes

$$\frac{1}{K'_{Iapp}} = \beta\left[\frac{1}{K'_{AH}} - \frac{1}{K'_{A^-}}\right] + \frac{1}{K'_{A^-}}$$

and

$$\frac{1}{K_{Iapp}} = \beta\left[\frac{1}{K_{AH}} - \frac{1}{K_{A^-}}\right] + \frac{1}{K_{A^-}}$$

The β value can be calculated at any pH provided the pK_a value of the carboxylic function is known. By plotting $1/K_{Iapp}$ and $1/K'_{Iapp}$ versus β for different pH values, a straight line was obtained, the extrapolations of which at $\beta = 0$ and 1 allowed the determination of K_A- and K_{AH} on the one hand and of K'_A- and K'_{AH} on the other hand. The results are shown in Figure 4 with benzoic acid between pH 4 and 5.3. Values of 0.07, 0.46, 0.15, and 3.34 mM were obtained for K_{AH}, K_A- (Figure 4A), K'_{AH}, and K'_A- (Figure 4B), respectively. Using this method, inhibition constants for the other carboxylic acids have been calculated from the results obtained at pH 4.5 and 5.3 (Table 6). The results indicate that for all the tested compounds, the AH form of the carboxylic group is mainly responsible for the inhibition. Moreover, affinity of carboxylic acid is always stronger for the free enzyme than for the complexed enzyme by its substrate ($K_{AH} < K'_{AH} < K_A- < K'_A-$). In the benzoic acid series, the highest affinities of the protonated carboxylic acids were obtained for the acids with the lowest pK_a values. Therefore, compared to the apparent K_I and K'_I at pH 4.5, further acidification would result in a large increase of inhibition for these three acids (β-resorcylic, salicylic, and gentisic acids), whereas for the other acids, the increase in inhibition would be less important. These results are in agreement with those obtained by Pifferi *et al.* (1974) and Janovitz-Klapp *et al.* (1990) who also showed that PPO inhibition by carboxylic acids was mainly associated with the protonated form.

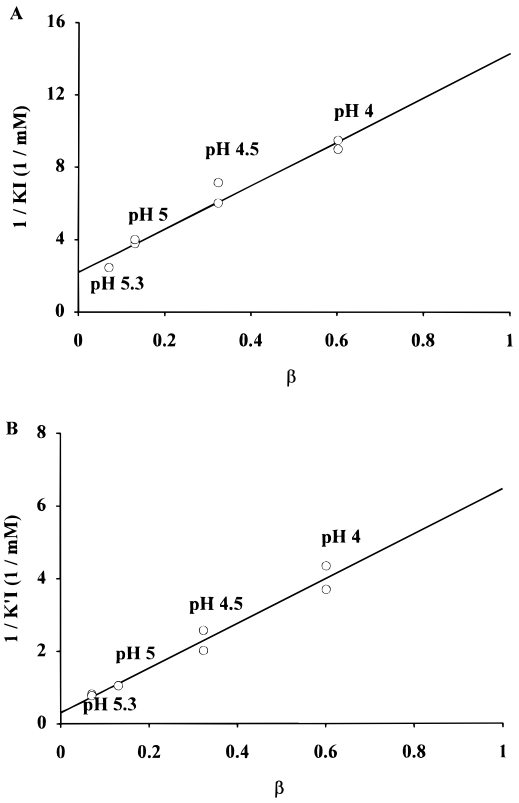

Figure 4. Effect of pH on the inhibition constants K_I and K'_I of purified PPO from gum arabic (HIC extract), in the presence of benzoic acid. β was calculated using a pK_a value of 4.18 for benzoic acid ($\beta = 1/[1 + 10^{pH-pK_a}]$). Assays were performed at 30 °C, with 4-methylcatechol as the substrate. K_{A^-}, K_{AH} (A), and K'_{A^-}, K'_{AH} (B) were determined at $\beta = 0$ and 1, respectively.

Table 6. Inhibition Constants of Carboxylic Acids Acting on PPO from Gum Arabic[a]

inhibitor	pK_a	K_{AH} (mM)	K_{A^-} (mM)	K'_{AH} (mM)	K'_{A^-} (mM)
Benzoic Acid Series					
β-resorcylic acid	2.94	0.012	2.5	0.042	13
salicylic acid	2.97	0.029	5.3	0.056	89
gentisic acid	2.97	0.036	6.9	0.15	>100
benzoic acid	4.18	0.055	0.46	0.16	2.8
α-resorcylic acid	4.04	0.073	1.4	0.51	5.0
p-hydroxybenzoic acid	4.48	0.11	2.2	0.43	>100
protocatechuic acid	4.48	0.24	6.3	1.2	31
Cinnamic Acid Series					
t-cinnamic acid	4.43	0.81	11	9.2	82
p-coumaric acid	4.50	0.93	3.5	5.9	>100
sorbic acid	4.76	1.1	6.5	4.7	>100
Phenylalkanoic Acid Series					
phenylacetic acid	4.31	3.1	14	50	>100
phenylpropionic acid	4.66	10	48	>100	>100
p-(hydroxyphenyl)-propionic acid	4.44	8.5	18	>100	>100

[a] Inhibition constants were determined using the pK_a value for each carboxylic acid. These values were calculated from the assays performed in air-saturated McIlvaine buffer solution at 30 °C, with 4-methylcatechol as the substrate, at pH 4.5 and 5.3.

In conclusion, among all the tested carboxylic acids, benzoic acid is the best inhibitor of PPO in gum arabic for moderate acid pH. Therefore, provided the pH conditions are adequately defined, it could be considered as a potential alternative to sulfites in controlling enzymatic browning.

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