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Polyphenol Oxidase from Yacon Roots (*Smallanthus sonchifolius*)

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Polyphenol oxidase (E.C. 1.14.18.1) (PPO) extracted from yacon roots (*Smallanthus sonchifolius*) was partially purified by ammonium sulfate fractionation and separation on Sephadex G-100. The enzyme had a molecular weight of 45 490 ± 3500 Da and K_m values of 0.23, 1.14, 1.34, and 5.0 mM for the substrates caffeic acid, chlorogenic acid, 4-methylcatechol, and catechol, respectively. When assayed with resorcinol, DL-DOPA, pyrogallol, protocatechuic, *p*-coumaric, ferulic, and cinnamic acids, catechin, and quercetin, the PPO showed no activity. The optimum pH varied from 5.0 to 6.6, depending on substrate. PPO activity was inhibited by various phenolic and nonphenolic compounds. *p*-Coumaric and cinnamic acids showed competitive inhibition, with K_i values of 0.017 and 0.011 mM, respectively, using chlorogenic acid as substrate. Heat inactivation from 60 to 90 °C showed the enzyme to be relatively stable at 60−70 °C, with progressive inactivation when incubated at 80 and 90 °C. The E_a (apparent activation energy) for inactivation was 93.69 kJ mol^{-1}. Sucrose, maltose, glucose, fructose, and trehalose at high concentrations appeared to protect yacon PPO against thermal inactivation at 75 and 80 °C.

KEYWORDS: Yacon roots; *Smallanthus sonchifolius*; polyphenol oxidase; purification; kinetics; inhibitors; thermal inactivation; sugar protection

INTRODUCTION

Yacon [*Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson] is an under-exploited root crop [fam. Asteraceae] originally cultivated in Andean South America. Like the related "Jerusalem artichoke", *Helianthus tuberosus*, the plant produces large tuberous roots similar to sweet potatoes in appearance, with a rather sweeter taste due to an abundance of soluble carbohydrates such as fructose, glucose, sucrose, and fructosylsucrose (FOS, GF_2-9) (*1*), but lacking starch, which makes it potentially beneficial in the diet of diabetics (*2*). The roots of yacon also contain considerable amounts of phenolic compounds (2030 mg/kg) with a predominance of chlorogenic acid (*2, 3*) and caffeic acid derivatives (*4*). As natural antioxidants, these compounds have an increasing importance to human health, as they may protect cell membranes against damage by oxygen radicals and its consequences in cardiovascular disease and cancer. Therefore, it is very important to avoid oxidation of phenols in yacon to maintain the dietary value of these roots. Yacon root is sometimes used in home cooking, but it is not a common foodstuff because of its poor shelf life, and the rapid browning of the juice or injured tissues. In many localities throughout the Andes, the fresh root is eaten like a "fruit", due to its juiciness, sweet taste, and relatively low energy value; but farmers in Peru, Brazil, and Japan produce a number of processed yacon products, such as air-dried tuber slices, unrefined yacon syrup, which has the consistency of honey and can be marketed as a dietetic sweetener, and other products (*1, 2, 5*). During the processing of these products, it is necessary to control enzymatic browning by thermal treatment, addition of antioxidant, or both.

Enzymatic browning of fruits and vegetables during postharvest handling and processing is one of the main causes of quality losses (*6, 7*). Browning is primarily related to the oxidation of phenolic endogenous compounds into highly unstable *o*-quinones, which are later polymerized to brown, red, and black pigments. The degree of browning depends on the nature and amount of endogenous phenolic compounds, on the presence of oxygen, reducing substances, metallic ions, pH, and temperature, and on the endogenous activity of polyphenol oxidase (PPO), the main enzyme involved in the reaction (*8*).

PPO (E.C. 1.14.18.1) is a copper-containing enzyme widely distributed in fruits and vegetables, which catalyzes the oxidation of monophenols, diphenols, and polyphenols to orthoquinones. PPO has been studied extensively for many years with respect to its physicochemical properties and response to inhibitors and substrates in a number of fruits and vegetables (*7, 8*). PPO is a plastid enzyme in the higher plants, generally located on the thylakoid membrane in chloroplasts (*7, 8*), requiring therefore a strong non-ionic detergent (Triton X-100) to achieve full extraction, although a readily soluble form of the enzyme has

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also been reported (*8*−*10*). Yoruk and Marshall (*8*) reported that the apparent presence of multiple forms of the enzyme in purification studies of various plant sources is due, at least in part, to artifacts of the extraction procedures, where the simultaneous presence of the phenolic substrate and enzyme can give rise to aggregated forms as a consequence of the browning reaction. Thus, the minimization of *o*-quinone formation during the extraction procedure is very important, to avoid these artifacts, and this can be achieved by adding phenol-scavengers such as polyethylene-glycol (PEG), insoluble polyvinyl(poly)pyrrolidone (PVPP), or one of several reducing agents as ascorbic acid, 2-mercaptoethanol, cysteine, and so on (*11*, *12*).

In particular, the yacon root darkens rapidly on storage, cutting, or during processing, and this tendency could be related to its phenolic content, especially to the levels of chlorogenic and caffeic acids (*2*, *3*), and the endogenous PPO activity. Although the presence of PPO in yacon roots has been demonstrated (*13*), there is no published study on the properties of the enzyme from this source.

Thus, the objective of this work was to investigate the properties of PPO from yacon root that catalyze the browning reaction during storage and handling following harvesting.

MATERIALS AND METHODS

Material. Yacon roots were obtained from the local market. They were washed with distilled water, dried on filter paper, and stored at 4 °C until use. The fresh roots were weighed and used for the preparation of the enzyme extracts.

Reagents. Catechol, 4-methylcatechol, L-ascorbic acid, 2-mercaptoethanol, DL-DOPA, 1,4-dithioerythritol (DTE), L-tyrosine, Triton X-100, chlorogenic, cinnamic, *p*-coumaric, ferulic, caffeic, protocatechuic, and benzoic acids, resorcinol, and pyrogallol were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were analytical reagent grade.

Extraction and Partial Purification of PPO from Yacon Root. Enzyme extracts were prepared so that PPO activity was as high as possible. Yacon roots (100 g) were peeled, cut into small pieces, and homogenized in an Ultra-turrax homogenizer (type TP 18-10, from Jankle & Kunkel, IKA, Germany) with 200 mL of 0.05 M phosphate buffer (pH 6.0) containing 5 mM ascorbic acid, 0.35 M KCl, 0.5% Triton X-100, and Polyclar aT (PVPP) (0.1 g/g), as phenolic scavenger. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10 000*g* for 40 min at 4 °C, and the supernatant was collected. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 20% saturation with gentle stirring. The resulting suspension was centrifuged at 10 000*g* for 40 min at 4 °C. The supernatant was then treated further with $(NH_4)_2SO_4$, and the protein fraction precipitating between 20% and 80% saturation after centrifugation was pooled. The precipitate was dissolved in 0.05 M phosphate buffer (pH 7.0) containing 3 mM ascorbic acid and desalted on a column of Sephadex G-25 eluted with the same buffer. The desalted sample was applied on Sephadex G-100 column (60 × 2.5 cm) equilibrated with 0.05 M phosphate buffer (pH 7.0), and fractions of 5.2 mL were collected. The PPO-active fractions were pooled and used in further experiments.

PPO Assay. PPO activity was determined by measuring the initial linear rate of quinone formation at 30 °C as indicated by an increase in the absorbance at 420 nm for 4-methylcatechol (4-methyl-1,2-benzenediol), catechol, and caffeic acid, and 410 nm for chlorogenic acid (*11*). An Ultrospec 2000 (Amersham Pharmacia Biotech, Upsala, Sweden) spectrophotometer was employed throughout. The reaction mixture contained the substrates in 0.05 M phosphate buffer, pH 6.0, and enzyme solution, for a total volume of 3 mL in the cuvette. The initial rate of the enzyme-catalyzed reaction was linear with time for 3 min. In all determinations, PPO activity was assayed in triplicate, and one unit was defined as a change of 0.001 absorbance per min at the appropriate wavelength for each substrate.

Protein Determination. Protein concentration was determined by the dye-binding method of Bradford (*14*) using bovine serum albumin as standard.

pH Optimum and pH Stability. PPO activity was determined as a function of pH with 4-methylcatechol, catechol, chlorogenic, and caffeic acid as substrates. Assays were run at the appropriate temperature for each substrate, in McIlvaine's buffer at various pH values (pH 3−8), with partially purified enzyme. All of the assays were performed in triplicate.

Optimum Temperature and Stability. PPO activity was determined as a function of temperature from 10 to 60 °C for various substrates. Heat inactivation studies were performed over the range of 60−90 °C. After being heated for a given period in the absence of substrate, the enzyme aliquot was cooled and immediately assayed as described above. To study the effect of additives on heat stability, PPO was incubated in the presence of various sugars, and the remaining activity was determined. The rate constant (k) for inactivation was calculated from the slope of the time course of denaturation, using the equation: $\log(A/A_o) = -(k/2.303)t$ or $\ln(A/A_o) = -kt$, where A_o is the initial enzyme activity and A is the activity measured at time t. The slopes of these plots were determined by linear regression, and the rate constants calculated were replotted. The apparent activation energies (E_a) were calculated from the slopes of the Arrhenius plots: $\ln k \times 1/T$, by applying the equation $\ln k = -E_a/RT$, where R is the gas constant (8.314 J mol^{-1} K^{-1}), and T is the temperature in kelvin. Slopes were calculated by linear regression. All of the assays were performed in triplicate.

Substrate Specificity and Kinetics Studies. The Michaelis−Menten constant (K_m) and maximum velocity (V_{max}) of yacon PPO were determined for various substrates (catechol, 4-methylcatechol, caffeic, and chlorogenic acid). For each substrate, data were plotted as $1/V \times 1/S$, where V is the reaction rate and S is the substrate concentration, according to the method of Lineweaver−Burk (*15*). All of the assays were performed in triplicate.

Effect of Inhibitors on PPO Activity. The effect of several inhibitors on yacon PPO activity was measured in the standard reaction medium in the presence and absence of various concentrations of inhibitors. Using two distinct concentrations of substrates, PPO activity was assayed at various concentrations of the inhibitors [i]. Values of $1/V \times$ [i] were plotted, and the K_i was determined by the method of Dixon (*16*). All of the assays were performed in triplicate.

Molecular Weight Determination. Molecular weight was determined by gel filtration on a Sephadex G-100 column, as described by Whitaker (*17*). The column (60 × 2.5 cm) was calibrated for molecular weight with standard proteins: cytochrome *c* (12.4 kDa), soybean trypsin inhibitor (21.5 kDa), ovalbumin (45 kDa), and bovine serum albumin (67 kDa).

RESULTS AND DISCUSSION

Extraction and Partial Purification. Several buffer compositions were employed for extracting PPO from yacon, and Polyclar aT, Triton X-100, and ascorbic acid in the extraction buffer gave the desired result. PEG-8000 (poly ethylene glycol), SDS (sodium dodecylsulphate), EDTA, L-cysteine, and 2-mercaptoethanol, at several concentrations in the buffer, alone or together with others, were not effective in the extraction of yacon PPO. The PPO extract was partially purified in various steps, including ammonium sulfate fractionation and gel filtration on Sephadex G-100. Only one peak with PPO activity was obtained from gel chromatography (**Figure 1**). From the total activity and protein applied to the column, 6.04% and 0.76% were recovered, respectively, in the peak. The enzyme after ammonium sulfate precipitation and elution on Sephadex G-25 was applied to a DEAE-cellulose column, revealing only one PPO form (data not shown). A summary of protein and activity data for each purification stage is given in **Table 1**. Polyacrylamide gel electrophoresis of the peak PPO activity eluted from the Sephadex G-100 column revealed only one band of enzyme activity with 4-methylcatechol as substrate (data not shown).

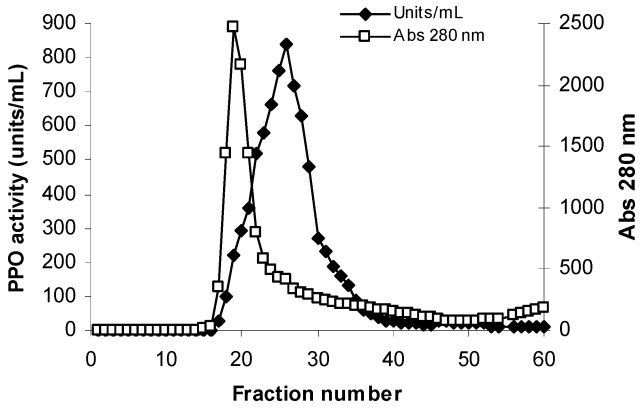

Figure 1. Chromatography on Sephadex G-100 for yacon PPO. Enzyme from $(NH_4)_2SO_4$ fractionation was applied on the Sephadex G-100 column (60 × 2.5 cm). Elution was carried out with 5 mM potassium phosphate buffer, pH 7.5. Activity: A_{420nm} min^{-1} mL^{-1}. Protein: A_{280nm}.

Table 1. Summary[a] of the Purification Stages of Yacon Root PPO

purification step	volume (mL)	activity (Units/mL/min)	protein (mg/mL)	specific activity[b]	purification fold
crude extract	182	80 990	61.6	1314.2	1
ammonium sulfate precipitation (20–80%)	26	101 400	42.1	2408.5	1.8
Sephadex G-100	4.8	4896	0.47	10 417.0	7.9

[a] Mean of four replicates. [b] Units/mg protein.

Thus, the procedure adopted provided a partially purified enzyme preparation. The molecular weight for yacon PPO determined from the Sephadex G-100 column was found to be 45.49 ± 3.50 kDa. The elution of the enzyme in a single peak indicated the presence of only one molecular species. While there can be uncertainty in the number, size, forms, and composition of PPOs from many plants, as a function of the development stage of the tissue, cellular localization, ripening, storage conditions, presence of proteases, and so on, the isolation and purification procedures can also produce artifacts that result in the modification of the native form of the enzyme (*7*, *8*). Thus, the extraction, isolation, and purification of yacon PPO were achieved in conditions that avoided the browning due to the oxidation of the phenolic compounds in the tissue and the possible attachment of its oxidation products to the native enzyme. Even so, the PPOs from many plants have been reported to vary in molecular mass mostly within the range of 35−100 kDa (*7*, *10*, *18*, *19*).

Effect of pH on PPO Activity. The pH optima of yacon PPO for various substrates are shown in **Figure 2**. When the enzyme was assayed with 4-methylcatechol as substrate, it displayed high activity between pH 6.0 and 6.5 and was optimally active at pH 6.2 with a rapid loss of activity above pH 6.8. With caffeic acid and catechol, the enzyme showed optimal activity at pH 6.6, more than 90% of this activity remaining between pH 6.0 and 7.0. It was also observed that approximately 85% of the optimal activity was retained at pH 4.5 and 5.8, with a maximum at pH 5.0, when chlorogenic acid was the substrate. The optimum pH of PPO varies with the source of enzyme and also depends on the phenolic substrate chosen for the assay, so that a range of values of maximum PPO activity have been reported in the literature between pH 4.0 and 7.0 for the enzyme from several sources with various substrates. It is reported that the optimum pH values of PPO are 5.0 and 5.7 for Victoria grape and egg-plant (*9*, *20*), 4.5−

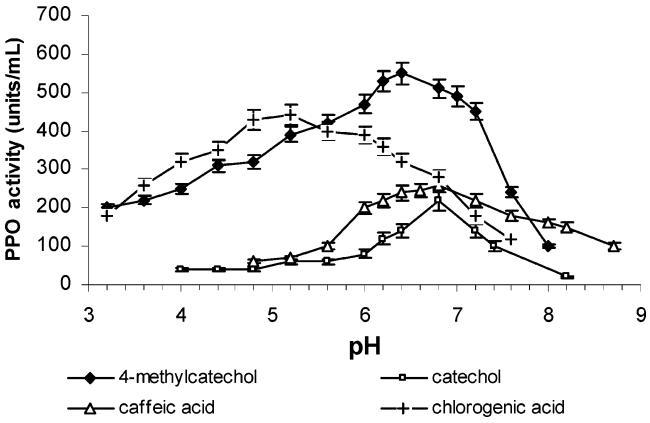

Figure 2. pH activity profile for yacon PPO with various substrates. The PPO activity was measured at 30 °C in McIlvaine's buffer at the indicated pH values.

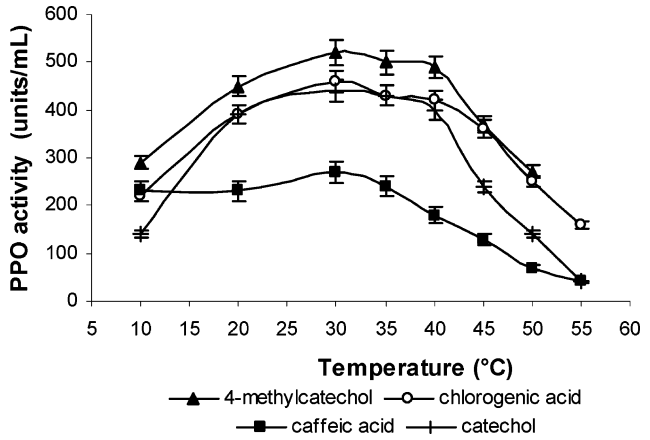

Figure 3. Temperature activity profiles for yacon PPO in McIlvaine's buffer with various substrates.

6.5 for sweet potato (*10*), 4.5−6.6 for palm-heart (*11*), 5.0−7.0 for globe artichoke heads (*19*), 6.6−6.8 for potato (*18*, *21*), and 6.0−9.0 for sweet basil (*Ocimum basillicum* L.) (*22*). The optimum pH reported here for all substrates tested is within these reported values.

Effect of Temperature on PPO Activity. The temperature dependence of the activity of the partially purified PPO from yacon roots for various substrates is presented in **Figure 3**. Optimum temperature for the enzyme was near 30 °C, for all four substrates, and for caffeic acid and catechol there was a rapid decline up to 50−55 °C. PPO from Victoria grape (*9*), sweet potato (*10*), palm-heart (*11*), artichoke heads (*19*), potato (*18*, *21*), egg-plant (*20*), and persimmon fruits (*23*) has optimum temperatures between 10 and 60 °C, which depend on the substrate used. It is clear that in general the optimum temperatures for PPO are quite species- and substrate-dependent (*7*, *8*).

Substrate Specificity and Enzyme Kinetics. The PPO from yacon roots showed activity with various *o*-diphenols. The enzyme was unable to oxidize L-tyrosine, suggesting a possible absence of monophenol monooxygenase (cresolase) activity. Types and relative concentrations of natural phenols vary widely in different plant sources. Thus, it appears that substrate specificity of PPO similarly depends on the species and cultivar. The potential substrates resorcinol (400 nm), DL-DOPA (475 nm), pyrogallol (470 nm), protocatechuic (410 nm), *p*-coumaric (410 nm), ferulic (410 nm), and cinnamic acids (410 nm), catechin, and quercetin were not oxidized by yacon PPO, although these compounds have been shown to be substrates

Table 2. Kinetic Constants[a] (K_m and V_{max}) for Various Substrates of Yacon Polyphenoloxidase

substrate	K_m (mM)	V_{max} (Units/min)	V_{max}/K_m (Units/min/mM)	R^2
chlorogenic acid	1.14	1428.6	1253.2	0.989
4-methylcatechol	1.34	525.3	392.0	0.993
caffeic acid	0.23	494.4	1890.4	0.994
catechol	5.00	666.0	133.2	0.991

[a] Calculated by the method of Lineweaver–Burk from a plot of $1/V$ versus $1/[S]$.

for other plant PPO (*8*). The PPO extracted from various sources has shown varying substrate specificity in published reports (*11*, *19*, *24*).

The enzyme kinetic constants, K_m, and V_{max} for yacon PPO with various substrates are listed in **Table 2**. The K_m for caffeic acid was 0.23 mM, a value lower than those reported for potato (*21*) and sweet potato enzymes (*10*) at 2.3 and 5 mM, respectively, and comparable to the value of 0.590 mM for palm-heart PPO (*11*), indicating a high affinity of yacon PPO for this substrate. The K_m for 4-methylcatechol was lower than those reported for grape (*9*), artichoke heads (*19*), egg-plant (*20*), and pear (*25*). Lee et al. (*26*) found K_m values of 15.9 and 24.6 mM for DeChaumac grape with caffeic and 4-methylcatechol as substrate, respectively. Duangmal and Owusu-Apenten (*21*) reported that 4-methylcatechol was the preferred substrate for PPO from taro and potato with K_m values of 9.0 and 1.1 mM, respectively. When using catechol as substrate, the K_m for yacon PPO is close to the values of 6.25, 6.8, and 7.51 mM reported for peppermint (*27*), potato (*21*), and Victoria grape (*9*).

Maximum activity was observed with chlorogenic acid, followed by catechol, 4-methylcatechol, and caffeic acid; however, this does not define the order of substrate specificity, because the best substrate for each enzyme depends on two factors: strong substrate binding, indicated by a low K_m, and high catalytic efficiency, as expressed by a high V_{max} value. Thus, the criterion for choosing the best substrate is the highest V_{max}/K_m ratio. From the apparent V_{max}/K_m values, caffeic and chlorogenic acids (**Table 2**) were the best substrates for yacon PPO, while catechol was the worst. Rapeanu et al. (*9*) reported that Victoria grape PPO showed higher values of substrate specificity for chlorogenic, catechin, and 4-methylcatechol, in this order, while Duangmal et al. (*21*) showed the order for substrate specificity was 4-methylcatechol, caffeic acid, catechol, and chlorogenic acid for potato PPO. However, until now there are no data for yacon PPO in the literature.

It has been found that yacon contains considerable amounts of phenolic compounds (*3*); for that reason, it represents a rich source of phenolic acids and other radical scavenging compounds (*4*). Simonovoska et al. (*28*) detected chlorogenic, ferulic, and caffeic acids, quercetin, and unidentified flavonoids in yacon roots by thin-layer chromatography and HPLC/MS.

Effect of Inhibitors on PPO Activity. The behavior of yacon PPO toward inhibitors was examined with various compounds, including analogues of the substrates. The effect of these inhibitors on enzyme activity with chlorogenic acid, caffeic acid, and 4-methylcatechol as substrates is presented in **Tables 3** and **4**. The inhibition study appears to show stronger inhibition for yacon PPO by the compounds tested when using chlorogenic and caffeic acids as substrates as compared to 4-methylcatechol. It is important to note that the former are natural substrates present in yacon tissue (*2*, *3*, *28*). *p*-Coumaric, cinnamic, and ferulic acids strongly inhibited the enzyme with chlorogenic acid as substrate, as compared to caffeic acid and 4-methylcatechol.

Table 3. Effect of Various Inhibitor Substances on Yacon PPO Activity[a]

substance	concentration in the assay (μmol)	% inhibition (substrates) chlorogenic acid	caffeic acid	4-methyl catechol
control		0	0	0
p-coumaric acid	0.5	95.00	24.75	9.80
cinnamic acid	0.5	76.25	21.00	3.28
ferulic acid	0.5	68.75	42.45	1.60
pyrogallol	2.0	62.50		5.02
protocatechuic acid	2.0	41.25	22.04	3.30
resorcinol	2.0	36.25	31.70	60.72
phenylalanine	2.0	13.75	43.50	18.00

[a] Chlorogenic, caffeic acids, and 4-methylcatechol were used as substrates as described in Material and Methods.

Table 4. Effect of Inhibitor Substances on Yacon PPO Activity[a]

substance	concentration in the assay (μmol)	% inhibition (substrates) chlorogenic acid	caffeic acid	4-methyl catechol
control		0	0	0
salicylic	1.2	3.5	23.8	0
oxalic acid	1.2	62.2		
	2.0	81.3	7.5	6.6
metabisulphite	0.15	100	21.5	44.6
	0.30	100	32.3	100
cysteine	0.15	56.5	16.9	34.6
	0.30	73.1	62.3	58.4
DTE	0.15	48.2		38.7
	0.30	81.3	23.3	73.1
ascorbic acid	0.06	10.4	8.5	8.4
	0.30	19.2	22.5	30.3
	0.60	31.1	28.7	77.3
benzoic acid	2.0	38.8	23.4	0
	3.0	43.0	23.8	4.20

[a] Chlorogenic, caffeic acids, and 4-methylcatechol were used as substrates as described in Material and Methods.

Only resorcinol exerted stronger inhibition with 4-methylcatechol, while phenylalanine showed strong inhibition with caffeic acid. The inhibition by *p*-coumaric and cinnamic acids with chlorogenic acid as substrate was shown to be competitive, with K_i values of 0.017 and 0.011 mM, respectively, calculated from Dixon plots (*16*). Ferulic acid was the phenolic acid that inhibited both chlorogenic and caffeic acids efficiently and showed uncompetitive inhibition with chlorogenic acid. Oxalic and benzoic acids showed relatively poor inhibition (**Table 4**), while the thiol reagents dithioerythritol (DTE), sodium metabisulfite, and cysteine at the concentrations indicated were effective inhibitors of yacon PPO, in contrast to ascorbic acid, which showed low inhibition for all substrates. The thiol reagents and ascorbic acid produced a lag period, before any changes in absorbance were observed during the measurements of PPO activity. This effect increased with inhibitor concentration for all substrates in a variable manner between 2 and 5 min. This apparent lag has also been observed by other authors (*7*, *9*, *10*) for thiols and ascorbic acid with PPO from other sources. The lack of color development observed at the start of the reaction in the presence of these inhibitors is attributed to the inhibitor being involved in reducing the quinones formed back to the colorless *o*-dihydroxyphenols.

Thermal Inactivation Kinetics of Yacon PPO. From the log-linear plots of residual yacon PPO activity against inactivation time at constant temperature, it can be concluded that the thermal inactivation of the enzyme is adequately described by

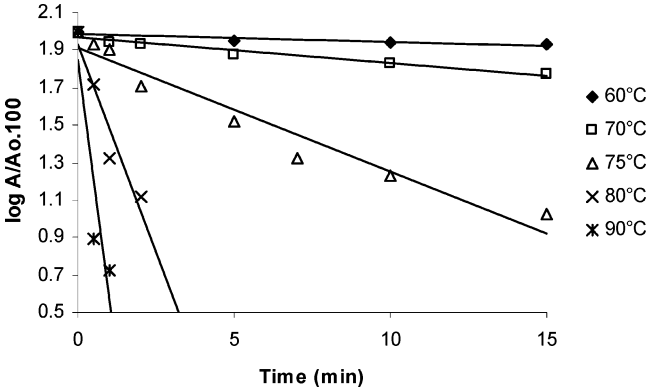

Figure 4. Heat inactivation plots of yacon PPO at different temperatures. Enzyme aliquots in 5 mM potassium phosphate buffer, pH 7.5, were incubated at the indicated temperatures. The remaining activity was determined with chlorogenic acid as substrate. A_0 and A, initial and residual activity at the time measured, respectively. The rate constants (k) for inactivation were determined from the slopes of the logarithmic plot of activity against time: $\log(A/A_0 \times 100) = -(k/2.303)t$.

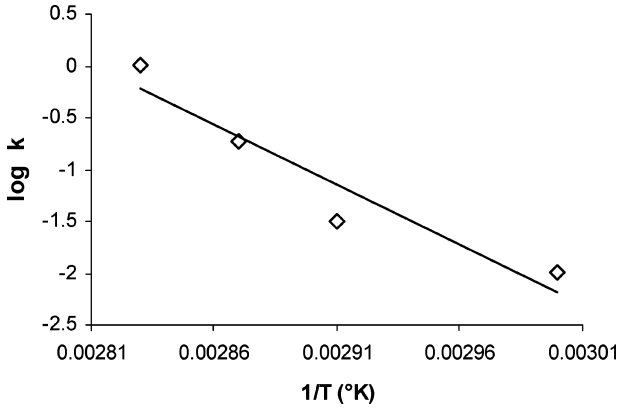

Figure 5. Heat inactivation of yacon PPO. The apparent activation energy (E_a) was calculated from the slope of the Arrhenius plot according to the equation $\ln k = -E_a/RT$, where k is the rate of inactivation at T, R is the gas constant (8.314 J mol^{-1} K^{-1}), and T is the temperature in kelvin. Slopes were calculated by linear regression.

a first-order model in the temperature range tested (**Figure 4**). As expected, at higher temperatures, inactivation proceeded faster. It can be seen in **Figure 4** that the heat inactivation of PPO between 80 and 90 °C was dramatically rapid. The enzyme activity determined with chlorogenic acid as substrate at pH 5.0 was reasonably stable at 60 °C; however, an increase from 60 to 70 °C inactivated PPO rather more quickly, with 50% reduction after 20 min of heating at 70 °C. Above 75 °C, the loss of activity became very rapid, whereas 2 min at 90 °C fully inactivated the enzyme. In contrast to peroxidases, PPO is not a heat-stable enzyme (*7*, *29*, *30*). The half-life of yacon PPO at 70 °C was similar to ones from other sources, such as grape (*9*), taro, and potato (*18*, *21*), and the enzyme was less resistant to heating than the PPO from sweet potato (*10*), palm-heart (*11*), persimmon (*23*), and peppermint (*27*).

The temperature dependence of the rate constant (k) for thermal inactivation of partially purified yacon PPO was evaluated from the Arrhenius equation, as shown by **Figure 5**. The calculated E_a (activation energy) for enzyme inactivation was 93.69 kJ mol^{-1}, higher than for PPO from potato (*21*) and lower than E_a for taro, Victoria grape, and DeChaunac grape (*9*, *21*, *26*). The possible involvement of peroxidase (POD) in the enzymatic browning has been reported (*32*), and this fact is

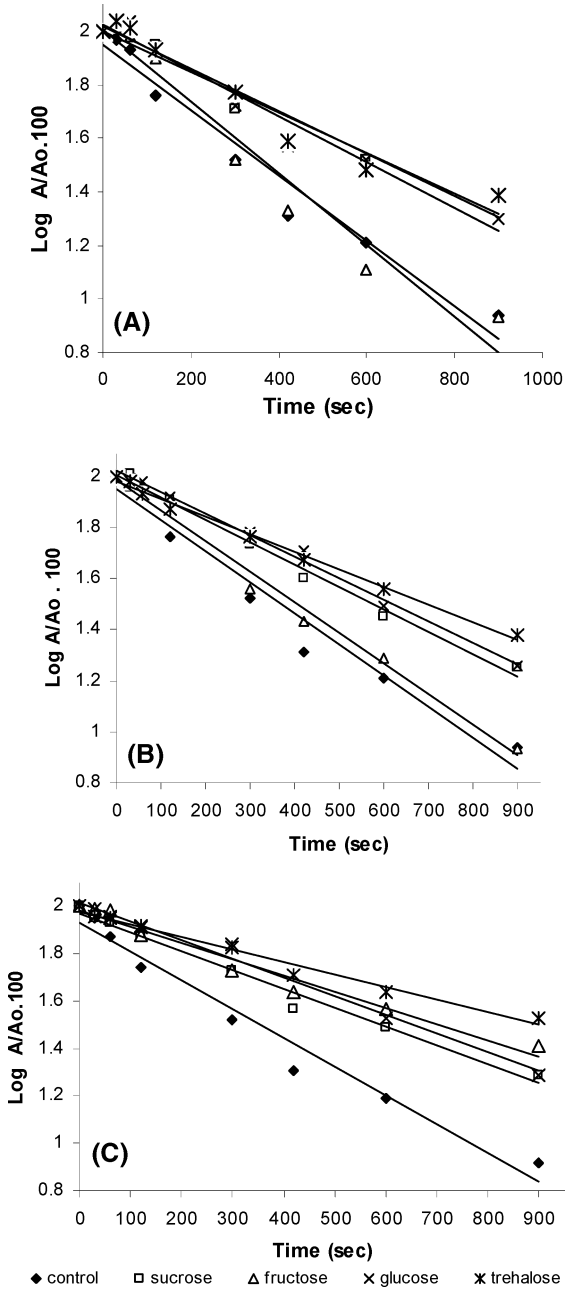

Figure 6. Heat inactivation curves of PPO in the presence of sugars at 75 °C. Enzyme aliquots in 5 mM potassium phosphate buffer, pH 7.5, were incubated in the presence of sugars at 10% (**A**), 20% (**B**), and 30% (**C**) concentration (w/w %), and the remaining activity was determined with chlorogenic acid as substrate. A_0 and A: initial and residual activity at the time measured, respectively.

important for the effective control of these undesirable reactions in processed fruits and vegetables; moreover, POD is an oxidative enzyme with an apparently higher resistance to heating than PPO, demonstrated by its higher values of E_a for inactivation in a number of plant sources (*7*, *30*, *31*).

Effect of Sugars on Heat Stability. It is well established that polyhydric compounds are among the most useful molecules for stabilizing the native conformation of globular proteins in solution, when added in high concentrations (*33*). Several theories into the mechanism of action for these solvent additives, also named cosolvents, appear to indicate that they are preferentially excluded from the surface of the native protein and cause a preferential protein−water interaction as a function of the cosolvent concentration in the system (*34*). Arakawa and

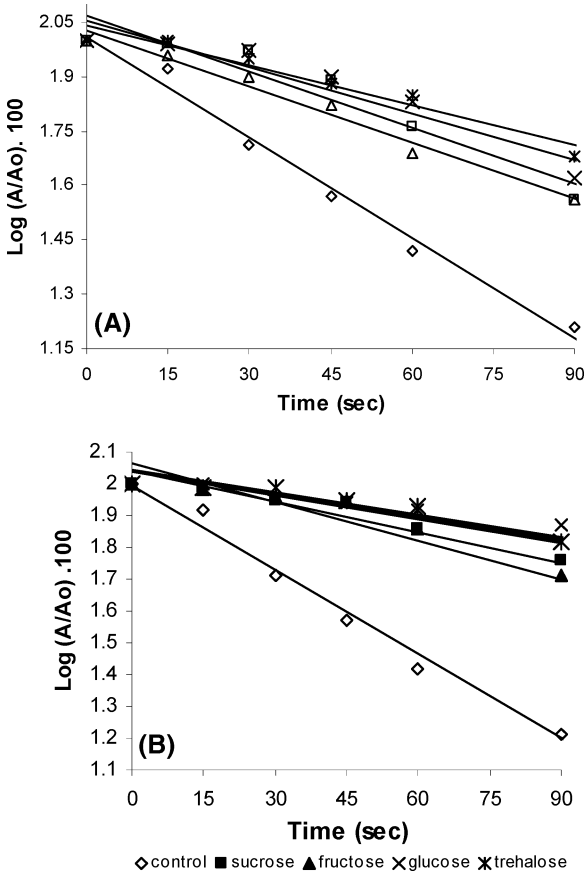

Figure 7. Heat inactivation curves of PPO in the presence of sugars at 80 °C. Enzyme aliquots in 5 mM potassium phosphate buffer, pH 7.5, were incubated in the presence of sugars at 20% (**A**) and 30% (**B**) concentration (w/w %), and the remaining activity was determined with chlorogenic acid as substrate. A_0 and A: initial and residual activity at the time measured, respectively.

Timashef (*33*) and Back et al. (*35*) showed evidence for the protein−solvent relationship acting as a stabilizing factor for the protein structure. The authors observed higher values for activation energy (E_a) of denaturation, with an increase on cosolvent concentration, for various enzymes, and they suggested that some physicochemical alterations of the system could be occurring, in particular related to the structure of the water. Investigations of the protective action of these agents against heat denaturation have been carried out with a number of proteins and enzymes from different sources. Although sugars (mainly glucose, sucrose, lactose, and trehalose) have been used to stabilize the biological activity of protein molecules, there are few reports of their effect on heat inactivation of plant enzymes (*10*, *29*, *30*).

The PPO from yacon was heated at 75 and 80 °C in the presence and absence of sugars at various concentrations (w/w) in the incubation mixture. **Figure 6A−C** shows the effect of these sugars on thermal inactivation of yacon PPO at 75 °C. The figures show a weak protective effect that increases as the sugar concentration rises from 10% to 30%. Although little difference between sugars was observed, the values indicated that the yacon PPO activity was more stable when the enzyme was heated in the presence of trehalose. At 30% trehalose (w/w), the enzyme still retained up to 43.5% of the original activity after 10 min heating at 75 °C, as compared to 16% value in its absence (**Figure 6C**). The heat inactivation of PPO at 80 °C in the presence of the sugars at various concentrations also showed that the enzyme was protected against inactivation, as compared to the control without sugar (**Figure 7**). Values of 67%, 71%, 51%, and 58% of the original enzyme activity were obtained for 30% (w/w) of trehalose, glucose, fructose, and sucrose in the medium, after 90 s, respectively, as compared to 16% for the control (**Figure 7B**).

Sola-Pena and Meyer-Fernandes (*36*) also observed a stronger stabilizing effect for some enzymes with trehalose relative to other sugars such as maltose, sucrose, glucose, and fructose. The authors suggested that the large hydrated volume of trehalose as compared to other sugars has a more pronounced size-exclusion effect and that all sugars have similar effects if the concentrations are corrected for the volume occupied by each one. These observations corroborate the idea of the preferential hydration of proteins, as suggested by Timasheff (*34*).

Various oxidative enzymes, such as plant peroxidases, have been shown to be less susceptible to thermal inactivation when heated in sugar solutions at various concentrations, and this variable susceptibility was reflected in alterations of E_a for thermal inactivation (*29*, *30*). According to our results and those of other authors, enzymes from different sources appear to behave differently in the presence of sugars on heating. Kavrayan and Aydemir (*27*) observed, for peppermint PPO, results contrary to those for yacon PPO, in which the sucrose solutions at 20% and 40% (w/w) did not protect the enzyme against heat inactivation. Lourenço et al. (*10*) reported a higher stability for sweet potato PPO in the presence of 20% and 40% (w/w) sucrose. However, there are only few data on the activation energy in the presence of sugars for inactivation of PPO from other sources.

LITERATURE CITED

(1) Grau, A.; Rea, J. Yacon [*Smallanthus sonchifolia* (Poepp. Et Endl.) H. Robinson]. Andean roots and tuberous roots: ahipa, arracacha, maca and yacon. In *Promoting the Conservation and Use of Underutilized Crops*; Hermann, M., Heller, J., Eds.; IPK, Gatersleben/IPGRI: Rome, 1997; Vol. 174, pp 199−256.

(2) Lachman, J.; Fernandez, E. C.; Orsák, M. Yacon [*Smallanthus sonchifolius* (Poepp. Et Endl.) H. Robinson] chemical composition and use − A review. *Plant Soil Environ.* **2003**, *49*, 283−290.

(3) Yan, X.; Suzuki, M.; Ohnishi-Kameyama, M.; Sada, Y.; Nakanishi, T.; Nagata, T. Extraction and identification of antioxidants in the roots of yacon (*Smallanthus sonchifolius*). *J. Agric. Food Chem.* **1999**, *47*, 4711−4713.

(4) Takenaka, M.; Yan, X.; Ono, H.; Yoshida, H.; Nagata, T.; Nakanishi, T. Caffeic acid derivatives in the roots of yacon (*Smallanthus sonchifolius*). *J. Agric. Food Chem.* **2003**, *51*, 793−96.

(5) Manrique, I.; Parraga, A.; Hermann, M. Yacon syrup: Principles and processing. Series: Conservacion y uso de la biodiversidad de raíces y tubérculos andinos: Uma década de investigación para el desarrolo (1993−2003). 8B. International potato center Univesidad Nacional Alcides Carrión. Erbacher Foundation. Swiss Agency for Development and Cooperation: Lima, Peru, 2005; 31 pp.

(6) Martinez, M.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.

(7) Vámos-Vigyázo, L. Polyphenoloxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr*. **1981**, *15*, 49−127.

(8) Yoruk, R.; Marshall, M. R. Physicochemical properties and function of plant polyphenoloxidase: A Review. *J. Food Biochem.* **2003**, *27*, 361−422.

(9) Rapeanu, G.; Van Loey, A.; Smout, C.; Hendrickx, M. Biochemical characterization and process stability of polyphenoloxidase extracted from Victoria grape (*Vitis vinifera ssp. Sativa*). *Food Chem.* **2006**, *94*, 253−261.
(10) Lourenço, E. J.; Neves, V. A.; Da Silva, M. A. Polyphenoloxidase from sweet potato: Purification and properties. *J. Agric. Food Chem.* **1992**, *40*, 2369−2373.
(11) Lourenço, E. J.; Leão, J. S.; Neves, V. A. Heat inactivation and kinetics of polyphenoloxidase from palmito (*Euterpe edulis*). *J. Sci. Food Agric.* **1990**, *52*, 249−259.
(12) Sojo, M. M.; Nunez-Delicado, E.; Garcia-Carmona, F.; Sanchez-Ferrer, A. Partial purification of a banana pulp polyphenol oxidase using Triton X-100 and PEG 8000 for removal of polyphenols. *J. Agric. Food Chem.* **1998**, *46*, 4924−4930.
(13) Yoshida, M.; Ono, H.; Mori, Y.; Chuda, Y.; Mori, M. Oxigenation of bisphenol A to quinones by polyphenol oxidase in vegetables. *J. Agric. Food Chem.* **2002**, *50*, 4377−4381.
(14) Bradford, M. M. A. rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(15) Lineweaver, H.; Burk, D. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **1934**, *56*, 658−66.
(16) Dixon, M. The determination of enzyme inhibition constants. *Biochem. J.* **1953**, *55*, 170−171.
(17) Whitaker, J. R. Determination of molecular weights of proteins by gel filtration on sephadex. *Anal. Chem.* **1963**, *35*, 1950−1953.
(18) Cho, Y. K.; Ahn, H. K. Purification and characterization of polyphenol oxidase from potato: I. Purification and properties. *J. Food Biochem.* **1999**, *23*, 577−592.
(19) Dogan, S.; Turan, Y.; Ertürk, H.; Arslan, O. Characterization and purification of polyphenol oxidase from Artichoke (*Cynara scolymus L.*). *J. Agric. Food Chem.* **2005**, *53*, 776−785.
(20) Concellón, A.; Añón, M.; Chaves, A. Characterization and changes in polyphenoloxidase from eggplant fruit (*Solanun melongena L.*) during storage at low temperature. *Food Chem.* **2004**, *88*, 17−24.
(21) Duangmal, K.; Owusu-Apenten, R. K. A. comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum*, var Romano). *Food Chem.* **1999**, *64*, 351−359.
(22) Dogan, S.; Turan, P.; Dogan, M.; Arslan, O.; Alkan, M. Purification and characterization of *Ocimum basillicum* L. Polyphenol oxidase. *J. Agric. Food Chem.* **2005**, *53*, 10224−10230.
(23) Nunez-Delicado, E.; Mar Sojo, M.; Garcia-Carmona, F.; Sanchez-Ferrer, A. Partial purification of latent persimmon fruit polyphenol oxidase. *J. Agric. Food Chem.* **2003**, *51*, 2058−2063.
(24) Dogan, S.; Arslan, O.; Ozen, F. Polyphenol oxidase activity of oregano at different stages. *Food Chem.* **2005**, *91*, 341−345.
(25) Gauillard, F.; Richard-Forget, F. Polyphenoloxidases from Williams pear (*Pyrus communis* L., cv. Williams): Activation, purification and some properties. *J. Sci. Food Agric.* **1997**, *74*, 49−56.
(26) Lee, C. Y.; Smith, N. L.; Pennesi, A. P. Polyphenoloxidase from De Chaunac grapes. *J. Sci. Food Agric.* **1983**, *34*, 987−991.
(27) Kavrayan, D.; Aydemir, T. Partial purification and characterization of polyphenoloxidase from peppermint (*Mentha piperita*). *Food Chem.* **2001**, *74*, 147−154.
(28) Simonovska, B.; Vovk, I.; Adrensek, F.; Valentová, K.; Ulrichová, J. Investigation of phenolic acids in yacon Smallanthus sonchifolius leaves and tubers. *J. Chromatogr., A* **2003**, *1016*, 89−98.
(29) Neves, V. A.; Lourenço, E. J. Peroxidase from peach fruit: Thermal stability. *Braz. Arch. Biol. Technol.* **1998**, *41*, 179−186.
(30) Chang, B. S.; Park, K. H.; Lund, D. B. Thermal inactivation kinetics of horseradish peroxidase. *J. Food Sci.* **1988**, *53*, 920−923.
(31) Hemeda, H. M.; Klein, B. Inactivation and regeneration of peroxidase activity in vegetables extracts treated with antioxidants. *J. Food Sci.* **1991**, *56*, 68−72.
(32) Richard-Forget, F. C.; Gauillard, F. A. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* Cv. Williams) polyphenol oxidase and peroxidase: A possible involvement of peroxidase in enzymatic browning. *J. Agric. Food Chem.* **1997**, *45*, 2472−2476.
(33) Arakawa, T.; Timasheff, S. N. Stability of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536−6544.
(34) Timasheff, S. N. Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry* **2002**, *41*, 13473−13482.
(35) Back, J. F.; Oakenful, O.; Smith, M. B. Increased thermostability of proteins in the presence of sugars and polyols. *Biochemistry* **1979**, *18*, 5191−5200.
(36) Sola-Penna, M.; Meyer-Fernandes, J. R. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars? *Arch. Biochem. Biophys.* **1998**, *360*, 10−14.

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