JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Purification and Characterization of Latent Polyphenol Oxidase from Apricot (*Prunus armeniaca* L.)

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Supporting Information

ABSTRACT: Polyphenol oxidase from apricot (*Prunus armeniaca*) (*Pa*PPO) was purified in its latent form (L-*Pa*PPO), and the molecular weight was determined to be 63 kDa by SDS-PAGE. L-*Pa*PPO was activated in the presence of substrate at low pH. The activity was enhanced by $CuSO_4$ and low concentrations ($\leq$ 2 mM) of SDS. *Pa*PPO has its pH and temperature optimum at pH 4.5 and 45 °C for catechol as substrate. It showed diphenolase activity and highest affinity toward 4-methylcatechol (K_M = 2.0 mM) and chlorogenic acid (K_M = 2.7 mM). L-*Pa*PPO was found to be spontaneously activated during storage at 4 °C, creating a new band at 38 kDa representing the activated form (A-*Pa*PPO). The mass of A-*Pa*PPO was determined by mass spectrometry as 37 455.6 Da (Asp102 → Leu429). Both L-*Pa*PPO and A-*Pa*PPO were identified as polyphenol oxidase corresponding to the known *Pa*PPO sequence (UniProt O81103) by means of peptide mass fingerprinting.

KEYWORDS: *apricot, tyrosinase, polyphenol oxidase, protein purification, characterization, activation*

INTRODUCTION

Apricot (*Prunus armeniaca L.*) is one of the most delicious and important crops in the Mediterranean region and is consumed worldwide. Algeria is the leading apricot producer of Africa and the fifth largest producer in the world with an annual production of 286 000 tons (FAO, 2017). Apricot is considered as an excellent source of phenolic compounds, carotenoids, and vitamin C.[1] Apricot conservation is a challenge resulting in a limited period of storage (2 weeks), during which the fruit rapidly loses its attractive and pleasant orange color and becomes brownish,[2] a color which is not accepted by the market. This loss is mainly caused by enzymatic reactions that lead to browning.[3] Different preservation methods such as freezing, canning and drying are commonly applied to extend the availability of the fruits.[4] However, browning remains a problem in apricot handling and preservation and is believed to be a major factor of quality loss during processing.[5]

Most browning reactions in fruits are assumed to be a direct consequence of polyphenol oxidase (PPO) action on phenolic compounds. This reaction produces quinones, highly reactive compounds that can polymerize spontaneously to form brown pigments, which are responsible for the loss of quality of fresh-cut fruit and vegetable products.[5]

Polyphenol oxidases (catechol oxidases (EC 1.10.3.1) and tyrosinases (EC 1.14.18.1)) are oxidoreductases that activate molecular oxygen and catalyze the hydroxylation (tyrosinases only) and/or oxidation (both tyrosinases and catechol oxidases) of phenolic compounds. Although PPOs have been investigated intensively for many years (biochemically as well as structurally), the reasons for their divergent substrate specificity is still under debate[6,7] and the natural substrates of nearly all PPOs are still unknown. PPOs are widely distributed in plants, fungi, and bacteria.[8] Plant PPOs are mainly associated with enzymatic browning reactions and with the protection of organisms against biotic and abiotic stress.[8] Plant PPOs consist of three domains: a signal peptide (minority of plant PPOs) or a transit peptide containing a thylakoid transfer domain (majority of plant PPOs) (~4−9 kDa), a catalytically active domain (~40 kDa), and a C-terminal domain (~19 kDa). PPOs are expressed as ~64−68 kDa pro-enzymes (latent form) *in vivo*.[9] Apricot contains a gene coding for a PPO precursor polypeptide of 597 amino acids with a calculated molecular weight of 67.1 kDa; the mature protein (latent form) has 496 amino acids (Asp102 → Ser597) with a calculated molecular weight of 56.2 kDa.[10] The C-terminal domain shields the PPO's active site,[7,11,12] wherefore the enzymes possess either only very weak or even no enzymatic activity *in vitro*, unless the enzymes are activated artificially, for example, by proteases, acidic pH, fatty acids, or detergents (e.g., SDS).[13]

PPOs are members of the type-3 copper enzyme family that contain a dinuclear copper site, in which both copper atoms are coordinated by three histidines each and can bind either molecular oxygen (side-on in a μ−η^2:η^2-peroxo geometry), hydroxide, or water molecules in a bridging mode as reviewed in ref 14. The distance between the copper atoms ranges from 2.8−4.6 Å, depending on the state of the active site.[14]

The characterization of PPO activity in apricot is of great interest to the food industry. Chevalier et al. (1999) reported the purification of (latent) apricot PPO to homogeneity, even if the biochemical characterization done was minimal.[10] Partial purification of a latent PPO from peach (*Prunus persica* L. Cv. Catherina) has been reported.[15] A better inhibition method against enzymatic browning may be developed through the understanding of the chemistry, structure, and mechanism of action of PPOs.[3] Thus, in this paper, we investigate both the

Received: July 12, 2017
Revised: August 16, 2017
Accepted: August 16, 2017
Published: August 16, 2017

DOI: 10.1021/acs.jafc.7b03210
J. Agric. Food Chem. 2017, 65, 8203−8212

molecular and kinetic properties of latent and active apricot (*Prunus armeniaca L.*) PPO.

MATERIALS AND METHODS

Plant Material. Fruits of *Prunus armeniaca L.* cv. Bulida were harvested from a local farm in the M'sila region of Algeria at commercial maturity in June 2016. The fruits were immediately transported to the laboratory and stored at 4 °C, where they were processed the same day. Apricots were rinsed with tap water, deseeded, frozen in liquid nitrogen, immediately broken into small pieces with mortar and pestle, lyophilized, and stored at −25 °C until use.

Extraction of Apricot PPO. Apricot PPO (*Pa*PPO) was extracted as described by Ünal and Şener[16] with some modifications. Using a prechilled blender, the lyophilized apricots (100 g) were homogenized for 2 min in 500 mL of cold acetone (−25 °C) containing 4 g of poly(ethylene glycol) (PEG 4000). The slurry was filtered through filter paper, and the residue was re-extracted several times with 300 mL of cold acetone until a white powder was obtained. The resultant acetone powder was dried overnight at room temperature and was homogenized in 1 L of cold sodium phosphate buffer (0.1 M) at pH 6.8 containing 30 mM sodium ascorbate, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 0.5% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was stirred for 45 min at 4 °C, then centrifuged at 30000 × *g* for 30 min at 4 °C. The supernatant was subjected to $(NH_4)_2SO_4$ precipitation (85% saturation). The precipitated fraction was separated from the supernatant by centrifugation at 30000 × *g* for 30 min at 4 °C. The precipitate was dissolved in 250 mL of 10 mM sodium phosphate buffer (pH 6.8) to obtain the crude extract.

Purification by Fast Protein Liquid Chromatography. The crude extract was dialyzed overnight at 4 °C against three changes of Tris-HCl buffer (10 mM, pH 8). The dialyzed suspension was centrifuged at 30000 × *g* for 30 min at 4 °C and filtered (through a 0.45 μm poly(ether sulfone) membrane) before being applied to an ÄKTA fast protein liquid chromatography system (FPLC). The protein solution was loaded onto an anion exchange column (Q-Sepharose FF, 20 mL) pre-equilibrated with 10 mM Tris-HCl, pH 8. Bound proteins were eluted with a linear gradient of sodium chloride (0 to 1 M) at a flow rate of 5 mL/min (Figure 1A). The fractions were monitored for protein content (280 nm) as well as for enzymatic activity (diphenolase activity). Fractions containing activity were pooled and concentrated by ultrafiltration (30 kDa molecular weight cutoff) driven by centrifugal force (3200 × *g*, 4 °C). The protein solution was then applied to a Mono S HR 5/50 GL column for cation exchange chromatography. The column was pre-equilibrated with 10 mM sodium acetate buffer, pH 5, and eluted with a linear gradient of sodium chloride (0 to 1 M) at a flow rate of 1 mL/min (Figure 1B). The active fractions were pooled, washed with 10 mM Tris-HCl buffer, pH 8, and concentrated by ultrafiltration. Enzyme activity during the purification was monitored as described below, using catechol as substrate, in 50 mM sodium citrate buffer (pH 6.5) containing 2 mM SDS as activator.

Enzyme Activity and Protein Concentration. *Pa*PPO activity measurements were performed at 25 °C in 200 μL of assay mixture by measuring the increase in absorbance at 410 nm using a microplate reader (Infinite M200, Tecan). The standard reaction mixture consisted of 0.5 μg of enzyme and 10 mM catechol in 50 mM sodium citrate buffer (pH 4.5). *Pa*PPO activity was determined from the slope of the initial linear part of the experimental curves (absorbance vs time) and expressed as U/mL. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol of quinones per minute (1 U = 1 μmol/min). The purified enzyme (L-*Pa*PPO) of 63 kDa was found to be activated in the reaction media with the tested diphenol without the addition of an external activator (e.g., SDS). All assays were performed in triplicate. Protein contents of the enzyme solutions were determined according to the Bradford method[17] using bovine serum albumin as standard.

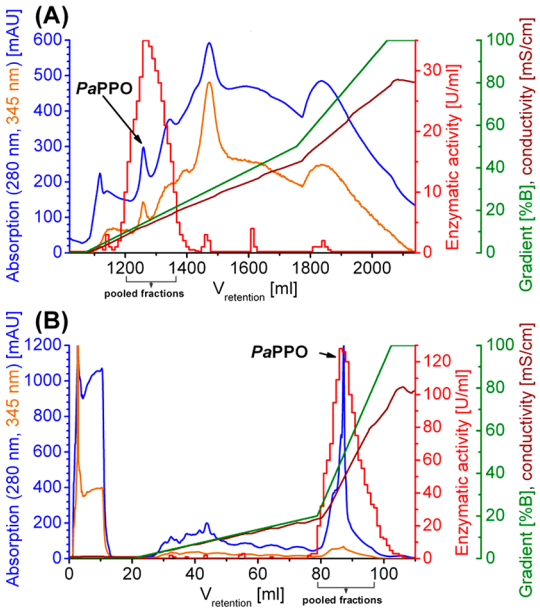

Figure 1. Chromatographic separation steps. (A) AEX chromatography using Q-Sepharose as the stationary phase. (B) CEX chromatography on MonoS. Legend: (blue) UV absorbance at 280 nm (mAU), (orange) UV absorbance at 345 nm (mAU), (red) PPO activity (U/mL), (green) elution gradient (% buffer B), (brown) conductivity (mS/cm).

Gel Electrophoresis. Denaturing SDS-PAGE was performed as described by Laemmli[18] in a mini gel apparatus (Mini-PROTEAN Tetra Cell, Bio-Rad). Enzyme solutions were denatured by heating at 99 °C (Thermomixer comfort, Eppendorf) for 5 min; the samples were applied under reducing conditions to 5% stacking and 9% resolving polyacrylamide gels, which were subsequently run at 120 V. Gels were stained with Coomassie Brilliant Blue G-250. Molecular weight was estimated by comparison to molecular weight markers (Precision Plus Protein Standard Dual Color, Bio-Rad). Target proteins (bands at 63 kDa (L-*Pa*PPO) and 38 kDa (A-*Pa*PPO)) were cut out and used for protein identification. To permit substrate staining, 9% partially denaturing SDS-PAGE was performed as described above with the two sole modifications of omitting the β-mercaptoethanol in the loading buffer and the skipping of the heating step prior to loading the samples on the gel. Activity staining for *Pa*PPO was done by immersing the gel in 50 mM sodium citrate buffer (pH 6.5) containing 0.2 M catechol at 25 °C. The in-gel activity staining was performed at pH 6.5 to avoid L-*Pa*PPO activation at low pH (optimum pH 4.5). Photographs of the gels were taken after 20 min of incubation on an orbital shaker.

Effect of pH. The *Pa*PPO activity as a function of pH was determined under the same assay conditions as described above, over the pH range from 2.0 to 9.5 with two kinds of buffer solutions: 50 mM sodium citrate buffer for the pH range of 2.0 to 7.0 and 50 mM Tris-HCl buffer for the pH range of 7.0 to 9.5. *Pa*PPO activity was calculated in the form of percent residual *Pa*PPO activity at the optimum pH. The optimum pH obtained was used in all other studies (except for in-gel activity staining).

Effect of Temperature. The effect of temperature on purified *Pa*PPO activity was estimated over a temperature range of 5 to 90 °C, using a Shimadzu UV spectrophotometer (UV-1800) connected to a thermostat (Julabo F25) through a circulating water bath in order to control and maintain the desired temperature. The optimum temperature for *Pa*PPO activity was determined in 600 μL of assay mixture, containing 1.5 μg of enzyme and 10 mM catechol in 50 mM citrate buffer (pH 4.5). The substrate and buffer were incubated for 10 min at the appropriate temperature using a water bath (Julabo F25), prior to the addition of the enzyme solution. *Pa*PPO activity is

DOI: 10.1021/acs.jafc.7b03210
J. Agric. Food Chem. 2017, 65, 8203−8212

presented in the form of percent residual activity relative to the *Pa*PPO activity at the optimum temperature.

Thermal Inactivation. To study the thermal inactivation of *Pa*PPO, the enzyme (0.5 μg) was incubated in 50 mM citrate buffer (pH 4.5), for various times (2, 5, 10, 20, and 30 min) at temperatures between 50 and 100 °C in a thermomixer (Thermomixer comfort, Eppendorf), rapidly cooled in an ice bath for 10 min, and warmed up to 25 °C, and then the residual enzyme activity was analyzed with 10 mM of catechol in 200 μL of assay mixture. The maximum activity at optimum temperature was set as 100%.

Substrate Specificity and Kinetic Parameters. The substrate specificity of *Pa*PPO was determined with different substrates: monophenols (phenol and tyramine), diphenols (L-dopa, caffeic acid, chlorogenic acid, catechol, 4-methylcatechol, dopamine), and a triphenol (pyrogallol) at 10 mM concentration, except for L-dopa and caffeic acid (1 mM) due to low substrate water solubility. The results are expressed relative to the activity on catechol (considered as 100%). Substrates that showed high activity were selected to calculate the kinetic parameters. In order to determine the Michaelis constant (K_M) and maximum velocity (V_{max}), *Pa*PPO activities were determined under the assay conditions described above using catechol, 4-methylcatechol, chlorogenic acid, and pyrogallol as substrates at various concentrations (0–160 mM). K_M and V_{max} values of the enzyme were calculated by nonlinear regression. The maximal turnover rate (k_{cat}) was calculated by dividing total substrate converted per min by total molecules of *Pa*PPO in the reaction mixture. Molar absorption coefficients in 50 mM sodium citrate at pH 4.5 were determined by quantitative oxidation of small quantities of the respective diphenol by an excess of sodium periodate as described by Muñoz et al.[19]

Effect of SDS, Inhibitors and Metal Ions on Enzyme Activity. The effects of different concentrations of SDS (0–10 mM) on *Pa*PPO activity were studied, as well as the effects of a wide range of inhibitors (4-hexylresorcinol, ascorbic acid, benzoic acid, citric acid, kojic acid, succinic acid, EDTA, glutathione (reduced), L-cysteine, and sodium metabisulfite) for inhibitor concentrations of 0.1, 1, 5, and 10 mM. The effects of various metal ions ($MgSO_4$, $CaCl_2$, $FeSO_4$, KCl, NaCl, $ZnSO_4$, $CuSO_4$, $MnCl_2$, $AlCl_3$, and $NiCl_2$) on enzyme activity were also investigated for two concentrations of metal ions, 1 and 10 mM, respectively. The enzymatic activity of the control mixture without SDS, inhibitors, and metal ions was taken as 100%, and then compared to the other treatments. The activity was determined as described above.

Storage Stability of the Purified *Pa*PPO. In order to assess the behavior of *Pa*PPO during storage at 4 °C, enzyme activity, SDS-PAGE, and activity gels were assayed weekly over 4 weeks. The enzyme activity was tested without and with SDS (0.5 mM) and reported relative to the initial *Pa*PPO activity without SDS (set as 100%).

Molecular Mass Determination. Electrospray ionization liquid chromatography mass spectrometry was performed in an ESI–LTQ–Orbitrap Velos (Thermo Fisher Scientific Bremen, Germany) with a mass range of 200–4000 m/z and a mass accuracy close to 3 ppm with external calibration. Prior to MS measurements, the purified *Pa*PPO solution was ultrafiltered by centrifugation, and the buffer system was changed to 5 mM ammonium acetate, pH 5, in order to reduce the concentration of nonvolatile salts to a minimum. Afterward a protein solution with a concentration of approximately 0.53 g/L was diluted 100 times in a mixture of 80% (v/v) acetonitrile and 0.1% (v/v) formic acid.

Protein Identification and Sequence Confirmation. The gel pieces containing the bands at 63 and 38 kDa obtained by denaturing SDS-PAGE (20 and 4 μg of protein, respectively) were used. Tryptic digestions of *Pa*PPO were analyzed by nanoUHPLC–ESI–MS/MS using a high-resolution Orbitrap mass spectrometer (Dionex Ultimate 3000 RSLCnano, Q Exactive Orbitrap, Thermo Scientific). The data analysis was performed with Proteome Discoverer 1.4 by searching against the *P. armeniaca* entries from the UniProt database (containing all listed proteins from *P. mume*, *P. persica*, and *P. armeniaca*). Peptide mass tolerance was 5 ppm, and the fragment mass tolerance was 0.5 Da. Variable modifications allowed were oxidation of methionines and carbamidomethylation of cysteines.

Statistical Analysis. All experiments were carried out in triplicate; arithmetic mean and standard deviation are reported.

RESULTS AND DISCUSSION

Extraction and Purification of L-*Pa*PPO. To extract L-*Pa*PPO, lyophilized apricot was subjected to acetone extraction

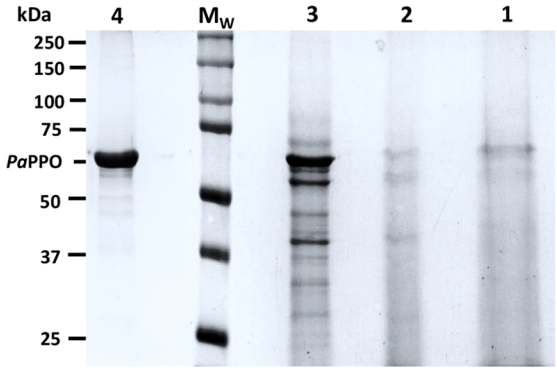

Figure 2. SDS–PAGE of L-*Pa*PPO purification steps: (1) crude extract, (2) dialysis, (3) anion exchange on Q-Sepharose, (M_w) molecular weight marker (values are given in kDa), (4) cation exchange on MonoS.

to eliminate most interfering compounds.[20] PVPP was used during extraction to avoid interaction of phenolic compounds with PPO.[21] Triton X-100 is widely used in the recovery of membrane-bound PPOs as it provides a mild, nondenaturing solubilization of proteins.[21] Ascorbic acid was also included to reduce quinones during PPO extraction and initial purification.[22] PMSF was added to the extraction buffer as a serine protease inhibitor.[23] After centrifugation, the supernatant was subjected to ammonium sulfate precipitation at 85% saturation.

After ammonium sulfate precipitation, the L-*Pa*PPO was purified by a three-step procedure (SDS page shown in Figure 2 and summarized in Table 1) involving dialysis, anion exchange chromatography (Figure 1a), and cation exchange chromatography (Figure 1b). The crude extract was dialyzed against Tris-HCl-buffer, and the resulting protein solution was applied to an anion-exchange column using a FPLC system (Figure 1A). Five major protein peaks were eluted in which *Pa*PPO activity was found. The highest activity was present in the fractions that were recovered from the second peak eluted at ∼9 mS/cm (1210–1390 mL) (Figure 1A). Negligible *Pa*PPO activity occurred in some of the other eluting fractions. However, there was a little activity with the unbound proteins present in the flow-through of the column (data not shown), which induced a loss of activity after this step of purification (Table 1). Fractions possessing activity were pooled, concentrated, and then further purified by cation exchange on a MonoS column, which eluted the protein into a single peak of L-*Pa*PPO activity at ∼18.5 mS/cm (78–96 mL) (Figure 1B). L-*Pa*PPO was successfully purified 23.2-fold with 51.0% recovery and a specific activity on 10 mM catechol of 459 U/mg (see Figure 2 and Table 1).

Effect of pH on *Pa*PPO Activity. The activity of purified *Pa*PPO was measured at different pH values, ranging from 2.0 to 9.5, using catechol as substrate (Figure 3a). The enzyme remains active from pH 2.5 (38%) to pH 9 (17%) with a pH optimum of maximal activity at pH 4.5 (100%). The enzymatic

DOI: 10.1021/acs.jafc.7b03210
J. Agric. Food Chem. 2017, 65, 8203–8212

Table 1. Purification of Polyphenol Oxidase from Apricot

purification stage	volume (mL)	total protein (mg)	total activity[a] (units)	specific activity[a] (units/mg protein)	purification (fold)	yield (%)
crude extract: 85% $(NH_4)_2SO_4$ precipitation	250	130	2570	19.8	1	100
dialysis against Tris-HCl	300	69.0	2312	33.5	1.7	89.0
anion exchange: Q-Sepharose FF 20 mL column	1.23	12.1	1506	125	6.3	58.6
cation exchange: MonoS 5/50 GL column	1.00	2.9	1311	459	23.2	51.0

[a]Enzymatic activity was determined on 10 mM catechol in 50 mM citrate buffer, pH 4.5.

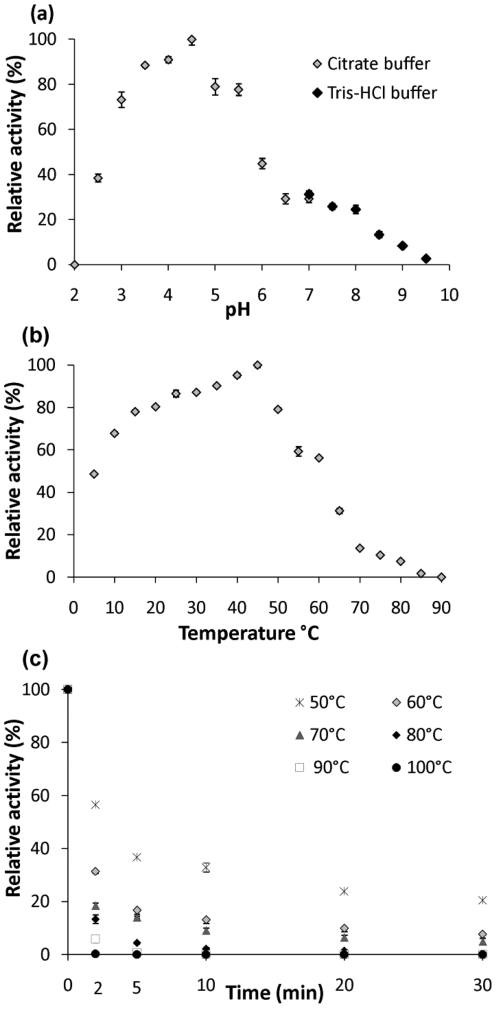

Figure 3. Effects of pH and temperature on the activity of *Pa*PPO. (a) pH optimum of *Pa*PPO activity, (b) optimal temperature for *Pa*PPO activity, (c) thermal inactivation of *Pa*PPO activity. The enzyme activity was measured using 10 mM catechol as substrate. The experiment was repeated three times, and each value is given as the mean value ± standard deviation.

activity of *Pa*PPO was reduced to < 50% at pH > 6 and no enzyme activity was detected at pH 2 and below. The pH-optimum reported here for *Pa*PPO is even lower than the one found by Ünal and Şener[16] and Fraignier et al.[24] who reported the optimum pH for *Pa*PPO at pH 5−5.8 and 5−5.5 using catechol and 4-methylcatechol as substrate, respectively. Arslan et al.[25] reported an optimum activity at pH 8.5 for PPO from Malatya apricot. Similarly low pH optima around pH 4.5 have been reported for PPOs from peach (cultivar Catherina) using 4-*tert*-butylcatechol as substrate.[15]

Table 2. Substrate Specificity of Purified *Pa*PPO[a]

substrate	concn (mM)	λ (nm)	$\varepsilon(\lambda)$ (M^{-1} cm^{-1})	relative activity (%)
		Monophenols		
phenol	10	426	4300	0.00 ± 0.0
tyramine	10	395	1500	0.00 ± 0.0[b]
		Diphenols		
chlorogenic acid	10	475	511	738 ± 5.4
4-methylcatechol	10	400	1720	378 ± 12
catechol	10	426	4300	100 ± 0.71
caffeic acid	1	481	591	336 ± 0.65
L-dopa	1	392	1240	5.47 ± 0.33
dopamine	10	395	1500	8.85 ± 0.80
		Triphenol		
pyrogallol	10	457	926	181 ± 3.9

[a]All measurements were performed in triplicate. Data is presented as mean ± standard deviation. [b]After storage A-*Pa*PPO showed activity (0.0724% ± 0.0010%) on 4 mM tyramine (sodium citrate buffer, pH 4.5; Figure S2).

Table 3. Kinetic Parameters of Purified *Pa*PPO

substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
catechol	5.3 ± 0.60	210 ± 30	40 ± 10
chlorogenic acid	2.7 ± 0.16	1400 ± 210	500 ± 80
4-methylcatechol	2.0 ± 0.26	700 ± 110	340 ± 70
pyrogallol	11 ± 1.5	590 ± 90	50 ± 10

Effect of Temperature on *Pa*PPO Activity. The temperature effect on the *Pa*PPO activity was studied over a range from 5 to 90 °C. The results are depicted in Figure 3b. The enzyme is active from 5 to 85 °C, showing maximum activity at 45 °C. A decrease of *Pa*PPO activity was observed at temperatures ≥ 50 °C, and the enzyme lost 45%, 86% and 92% of its maximal activity at 60, 70, and 80 °C, respectively, until all activity was lost at 90 °C, due to thermal inactivation. Ünal and Şener[16] reported a lower value for the temperature optimum of apricot (cultivar Alyanak) PPO activity (30−40 °C) using catechol as substrate. Thus, PPOs from three apricot cultivars (Kabaasi, Çataloglu and Hacihaliloglu) showed optimal activity at 35, 40, and 45 °C respectively.[26] An identical temperature optimum at 45 °C has been reported for litchi pericarp PPO using (−)-epicatechin as substrate.[27] The optimum temperature for PPO activity generally ranged between 30 and 40 °C.[28,29] A PPOs temperature optimum usually depends on the environmental conditions where the fruit grows, the plant species and variety as well as the substrate used in the assay.[13]

Thermal Inactivation of *Pa*PPO. The thermal inactivation profile of purified *Pa*PPO, presented as the residual activity after preincubation at various temperatures from 50 to 100 °C for 2 to 30 min, is shown in Figure 3c. *Pa*PPO exhibited a

DOI: 10.1021/acs.jafc.7b03210
J. Agric. Food Chem. 2017, 65, 8203−8212

Table 4. Effect of Various Inhibitors on *Pa*PPO Activity[a]

inhibitor	relative activity (%)			
	0.1 mM	1 mM	5 mM	10 mM
4-hexylresorcinol	34 ± 1.4	11.0 ± 0.66	0.31 ± 0.05	0.0 ± 0.0
ascorbic acid	89 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
benzoic acid	95 ± 2.2	41 ± 1.2	8.3 ± 0.11	5.4 ± 0.49
citric acid	100 ± 1.5	93 ± 1.5	88 ± 2.1	80.6 ± 0.87
kojic acid	65 ± 2.2	28 ± 1.1	7.3 ± 0.47	4.1 ± 0.52
succinic acid	99 ± 1.6	94 ± 1.6	90.9 ± 0.80	89.1 ± 0.53
EDTA	99.7 ± 0.45	93.7 ± 0.59	90.3 ± 0.43	88 ± 1.5
glutathione, reduced	84.3 ± 0.67	2.5 ± 0.21	0.0 ± 0.0	0.0 ± 0.0
L-cysteine	89.3 ± 0.45	0.65 ± 0.07	0.0 ± 0.0	0.0 ± 0.0
sodium metabisulfite	72 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]All measurements were performed in triplicate. Data is presented as mean ± standard deviation.

Table 5. Effect of Metal Ions on *Pa*PPO Activity[a]

salt	relative activity (%)	
	1 mM	10 mM
$MgSO_4$	102 ± 1.7	107 ± 1.8
$FeSO_4$	50 ± 2.6	34 ± 1.9
$ZnSO_4$	104 ± 1.8	108 ± 1.9
$CuSO_4$	108 ± 1.5	134 ± 1.1
KCl	87 ± 1.4	45 ± 2.8
NaCl	94 ± 1.5	56 ± 1.2
$CaCl_2$	78 ± 2.5	23 ± 1.4
$MnCl_2$	85 ± 0.99	23 ± 1.3
$NiCl_2$	84 ± 2.3	15 ± 1.4
$AlCl_3$	80 ± 2.0	3.67 ± 0.31

[a]All measurements were performed in triplicate. Data is presented as mean ± standard deviation.

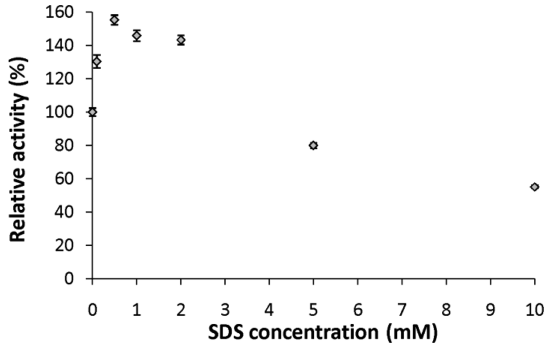

Figure 4. Effect of SDS on *Pa*PPO activity.

reduction in catalytic activity as the temperature and duration of incubation increased.

The enzyme retained 56%, 31%, 18%, 13%, 6%, and 0% of its original activity after 2 min of incubation at 50, 60, 70, 80, 90, and 100 °C, respectively, and more than 90% of activity was lost after incubation at 70 °C for 10 min. *Pa*PPO was completely inactivated following incubation at 80, 90, and 100 °C for 30, 10, and 5 min, respectively (Figure 3c). Our results are close to those of Yemenicioğlu and Cemeroğlu,[26] who reported that apricot PPO (Kabaasi cultivar) lost more than 95% of its activity after 10 min of heating at temperatures ≥ 80 °C, using catechol as substrate. However, they found that the enzyme was quite stable at 70 °C. PPO from plum[30] showed similar thermal stabilities at 55 °C where the enzyme retained only 50% of its activity after 5 min of incubation. Also PPO from blueberry[31] showed similar behavior, the PPO retained only 3% of its activity after 2 min of incubation at 85 °C. By comparison with *Pa*PPO, PPO from strawberry[32] and medlar fruits[33] were relatively thermostable as the enzymes retained more than 60% of their activity after 30 min of incubation at 50 °C. On the other hand, PPO from potato[34] has been found to be sensitive to heat treatment; the enzyme was completely inactivated after heating at 70 °C for 10 min.

Substrate Specificity and Enzyme Kinetics. Substrate specificity for *Pa*PPO was investigated using 9 different substrates (Table 2) at the enzyme's pH optimum for catechol as the substrate (pH 4.5). The purified enzyme (L-*Pa*PPO) was found to be activated in the reaction media in contact with substrates without the addition of an external activator (like SDS). This activation can be attributed to the low pH optimum (4.5) of *Pa*PPO. The latent PPO activation in the reaction media at low pH has been reported before.[15] Activities were compared with the activity observed in the presence of catechol (set as 100%). The highest activity was found using chlorogenic acid, followed by 4-methylcatechol, caffeic acid, pyrogallol, and catechol.

A lower activity toward dopamine and L-dopa (diphenols) was recorded, and no activity was detected with monophenols (phenol and tyramine). The slight monophenolase activity reported for *Pa*PPO after storage using tyramine as substrate suggests that *Pa*PPO has a weak activity on monophenols compared to the strong activity on diphenols. Furthermore, reliable determination of monophenolase activity requires the use of a large amount of enzyme and a substantially extended monitoring time. The results obtained in this study are in agreement with those of Arslan et al.[25] (1998) and Yemenicioğlu and Cemeroğlu[26] (2003), who reported that apricot PPO had no activity toward monophenols using hydroquinone and *p*-cresol as substrate at pH 6.8. PPOs lacking monophenolase activity were reported for other fruits among them mamey,[35] borage,[36] and blueberry.[31] Chlorogenic acid and 4-methylcatechol have been found to be the best substrates for coffee[37] and apple PPO[38] and the here investigated *Pa*PPO. Chlorogenic acid is the main phenolic compound in apricot,[39] which could explain the highest catalytic efficiency of *Pa*PPO on this substrate.

K_M and k_{cat} were calculated by nonlinear regression using the four best substrates at various concentrations under optimal conditions and are shown in Table 3. Substrate specificities were evaluated by using the k_{cat}/K_M ratio (catalytic efficiency). k_{cat}/K_M followed the order of activity of *Pa*PPO on the tested substrates: chlorogenic acid > 4-methylcatechol > pyrogallol >

DOI: 10.1021/acs.jafc.7b03210
J. Agric. Food Chem. 2017, 65, 8203−8212

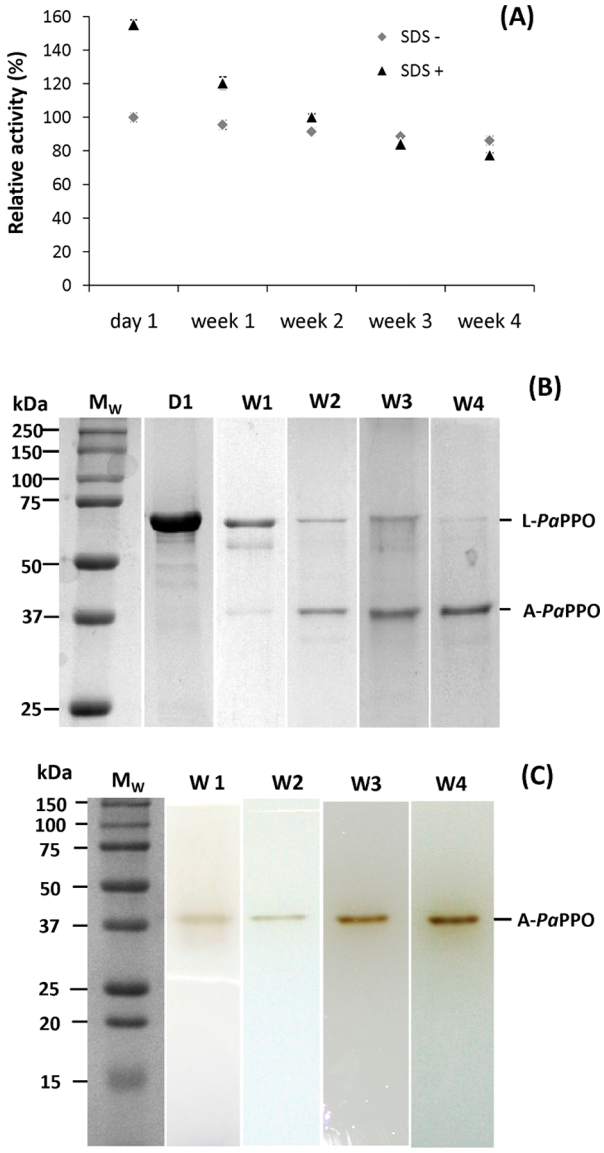

Figure 5. Effect of storage at 4 °C on *Pa*PPO. (A) activity of *Pa*PPO on catechol in the absence (SDS −) and presence (SDS +) of 0.5 mM SDS, (B) denaturing SDS−PAGE gels (reduced sample) of purified *Pa*PPO, (C) activity gels of purified *Pa*PPO. (M_w) molecular weight marker (values given in kDa), (D1) freshly purified protein, (W1) 1 week of storage, (W2) 2 weeks of storage, (W3) 3 weeks of storage, (W4) 4 weeks of storage. The enzyme was stored at 4 °C in Tris-HCl buffer (pH 8).

catechol. The lowest K_M was observed for 4-methylcatechol (2 mM), followed by chlorogenic acid (2.7 mM), catechol (5.3 mM), and pyrogallol (11 mM). The K_M values reported for PPOs from apricot are 6.6 mM (cultivar Malatya)[25] and 1 mM (cultivar Moorpark)[40] using catechol and chlorogenic acid, respectively. Close K_M values were reported for mango (3.1 mM)[41] using 4-methylcatechol and Yali Pear (1.5 mM)[42] using chlorogenic acid as substrate.

Effect of Inhibitors, Metal Ions, and SDS on *Pa*PPO Activity. The effects of ten various inhibitors, among them the most commonly used inhibitors of enzymatic browning, on *Pa*PPO activity were investigated at four different inhibitor concentrations (0.1, 1, 5, and 10 mM). The results are presented in Table 4. At 0.1 mM, 4-hexylresorcinol was the most effective inhibitor with only 34% activity retained. Increasing the concentration of inhibitors to ≥ 1 mM, sodium metabisulfite, ascorbic acid, and L-cysteine were the most effective inhibitors for *Pa*PPO activity, followed by reduced glutathione, 4-hexylresorcinol, kojic acid, and benzoic acid. Complete inhibition was observed with sodium metabisulfite and ascorbic acid at 1 mM, with L-cysteine and glutathione at 5 mM, and with 4-hexylresorcinol at 10 mM. Citric acid, EDTA, and succinic acid were the weakest inhibitors with 81%, 87% and 89% of residual activity, respectively, even at 10 mM concentration. All of the inhibitors hinder the formation of melanin by preventing the accumulation of *o*-quinones or by forming stable colorless products.[13] Our results are in agreement with those of Ünal and Şener[16] who detected a complete inhibition of apricot PPO with 1 mM of sodium metabisulfite and ascorbic acid. Metabisulfite and ascorbic acid are reducing agents, which inhibit enzymatic browning reactions by reacting with quinones formed by PPO catalyzed oxidation of *o*-dihydroxy phenols, forming a stable, colorless product.[7] In addition metabisulfite can act on the enzyme itself by irreversibly binding to the "met" and "oxy" forms of binuclear copper at the active site.[7]

Ascorbic acid and L-cysteine were found to provide effective inhibition of PPO in blueberry.[31] Sodium metabisulfite was reported as the best inhibitor for PPOs from a wide variety of species, among them pears,[43] potato,[34] grape,[44] mamey,[35] mango,[41] and blueberry.[31] Citric acid, succinic acid, and EDTA have also been found not to be effective as inhibitors for PPO from mango, even at 10 mM concentration, with 67%, 69% and 79% retained activity, respectively.[41]

The effect of metal ions on *Pa*PPO is shown in Table 5. The presence of most of the tested metal ions did affect the enzymatic activity negatively. Metal ions such as Al^{3+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, and Fe^{2+} inhibited PPO activity markedly; less than 35% of PPO activity was retained at a concentration of 10 mM. The exceptions were $MgSO_4$, $ZnSO_4$, and $CuSO_4$; their presence stimulated the *Pa*PPO activity. Similar effects were observed in apple[29] where 50 mM of $CuSO_4$ enhanced PPO activity to 145% and 3.69 mM of $AlCl_3$ decreased it to 50%. Conversely PPO activity in litchi was reduced in the presence of $FeSO_4$ and NaCl and stimulated in the presence of $MnSO_4$ and $CuSO_4$.[45] PPO of longan fruit was found to be activated in the presence of $MnSO_4$ and $CuSO_4$.[46] The activities of PPO from mango[41] and Japanese honeysuckle[47] were found to be stimulated in the presence of Mg^{2+}; however in contrast to our results, they noted that the activity was decreased in the presence of $CuSO_4$ and increased in the presence of $ZnSO_4$. According to Sun et al.,[48] metal ions like Ca^{2+} can cause the repulsion of oxygen and therefore the inhibition of PPO activity. However, some metal ions can promote the binding of the substrate to the active site and enhance enzymatic activity by combining with substrate or PPO.[35]

PPO exists in plant tissues in its active form and its latent form where full activity can only be observed by adding detergents or fatty acids or by treatment with proteases.[49] For this reason the enzymatic activity of the purified *Pa*PPO was determined under normal assaying conditions using catechol as substrate with various concentrations of SDS. The PPO activity without SDS was taken as 100%. As displayed in Figure 4, SDS at lower concentrations (0.1, 0.5, 1, and 2 mM) enhanced the enzymatic activity, with the maximum activity (155%) at 0.5 mM, whereas at higher concentrations of SDS (5 and 10 mM) *Pa*PPO activity diminished markedly to 80% and 55%, respectively. The activation of the enzyme with SDS suggests

DOI: 10.1021/acs.jafc.7b03210
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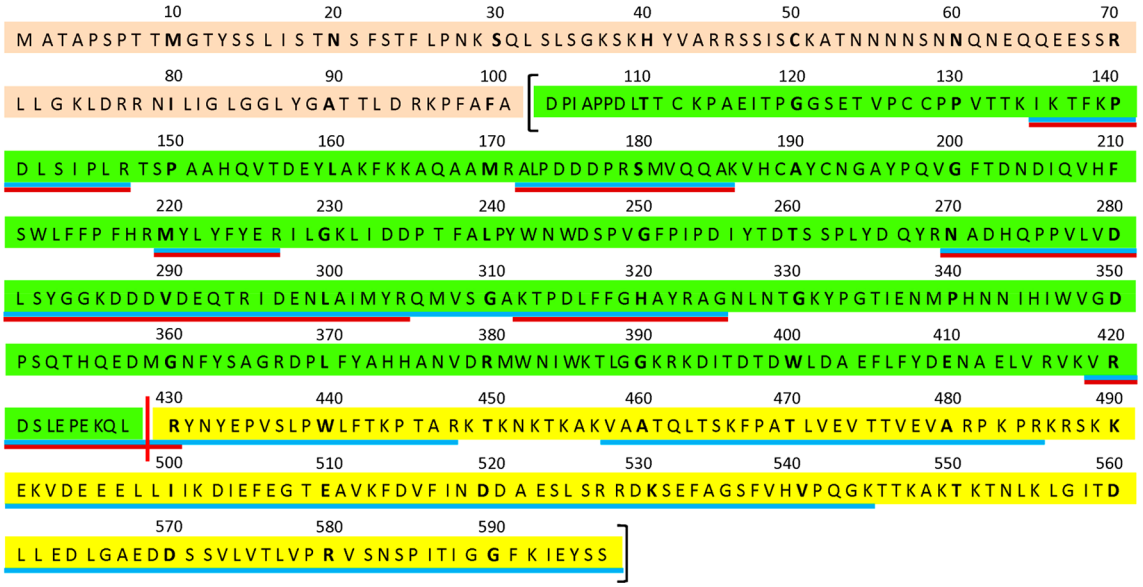

Figure 6. Sequence of *Pa*PPO (UniProt O81103). (Peach shading: signal peptide domain. Green shading: main domain (A-*Pa*PPO). Yellow shading: C-terminal domain. Square brackets indicate the start and the end of L-*Pa*PPO. Pink vertical line indicates the cleavage position (the end of A-*Pa*PPO and the start of C-terminal domain) as deduced from matching the amino acid sequence with the molecular mass determined for A-*Pa*PPO by ESI LTQ Orbitrap Velos MS. The peptides identified by UHPLC-ESI-MS/MS for L-*Pa*PPO and A-*Pa*PPO are underlined in blue and crimson, respectively.

that purified *Pa*PPO is present in its latent form. Activation of latent PPO by SDS was reported in many organisms, among them banana,[50] coffee,[37] peach,[15] mushroom,[51] and petals of *C. grandiflora*.[52] The effect of SDS (3.5 mM) on PPO activity of five *Prunus* species (peach, almond, cherry, plum, and apricot) was investigated by Fraignier et al.[24] They noted that the activity of PPO in the presence of SDS was found to be 1.5 and 2 times higher than the activity without SDS in plum and cherry, respectively, and a slight increase in activity was noticed in almond and apricot. In peach, however, they found a little decrease in activity due to SDS. The increase of PPO activity in the presence of SDS might be due to the activation of latent PPO through conformational changes.[53] Thus, the activation of PPOs is observed at low concentrations of SDS, and higher concentrations can cause the opposite effect and decrease the enzyme activity instead of increasing it,[50] which is also what we observed for *Pa*PPO.

Storage Stability of the Purified *Pa*PPO. In order to determine the effect of storage at 4 °C (in 10 mM Tris-HCl buffer at pH 8) on stability of the purified *Pa*PPO, we monitored the enzymatic activity with and without SDS over 4 weeks, where *Pa*PPO activity without SDS at day one was taken as 100%. The results are shown in Figure 5A; *Pa*PPO activity assayed in the presence of SDS diminished by 50% after one month of storage from 155% activity at day one to 78% activity at week 4. On the other hand just a small decrease (13%) in *Pa*PPO activity was observed in the tests without SDS from 100% to 87% activity. Furthermore, starting from the third week, SDS switched from being an activator to acting as an inhibitor of the enzymatic activity, where we noted a decrease of 10% of activity at week 4 from 87% in the absence of SDS to 77% in the presence of SDS. Loss of PPO activity during storage at 4 °C is a common observation in studies on PPOs.[28] However, the peculiar behavior of *Pa*PPO during storage noted in this study, in the presence and absence of SDS was not reported in earlier works. For that and to better understand this behavior, denaturing SDS-PAGE (reduced samples) and partially denaturing SDS-PAGE were performed over the storage period (Figure 5B,C).

Gel Electrophoresis and Mass Determination. Reported molecular weights of PPO, range from very low masses like 25 kDa for pineapple PPO[54] to relatively high molecular masses, for example, 120 kDa for field bean PPO.[55] In our study, the purified L-*Pa*PPO collected from cation exchange chromatography appeared as a single protein band on an SDS-PAGE gel with an apparent molecular mass of 63 kDa (Figure 2). This mass is identical to the molecular weight of the latent PPO detected in apricot and four other species of *Prunus*.[24] Chevalier et al.[10] also reported a similar mass of 60 kDa. Our apparent mass is higher than the molecular weight of the mature protein (56.2 kDa) calculated by Chevalier et al.[10] and the mass of the purified apricot PPO (57 kDa) detected by Ünal and Şener.[16]

Denaturing SDS-PAGE (reduced samples) stained with Coomassie brilliant blue G-250 was performed to monitor storage behavior of L-*Pa*PPO, and the respective gels are presented in Figure 5B. The results show the formation of a new band at 38 kDa (A-*Pa*PPO) during storage, which is apparent starting from the first week of storage and becomes thicker over the weeks, and in parallel the original band at 63 kDa (L-*Pa*PPO) becoming thinner until it almost totally disappears after the fourth week (Figure 5B). Under partially denaturing SDS-PAGE, a single band was detected by in-gel enzymatic activity staining with catechol as substrate (Figure 5C). The band appeared at 38 kDa in the same position as the band observed when stained with Coomassie blue (A-*Pa*PPO of Figure 5B). From these results, it is concluded that the band at 38 kDa represents the active form of apricot *Pa*PPO, a mass that is close to the molecular weight (43 kDa) detected by activity staining previously reported by Fraignier et al.[24]

The mass spectrum (ESI-LTQ) of the purified enzyme (after storage) is given in Figure S3 along with a zoomed-in section of this spectrum, indicating the presence of one major protein species. Twelve distinct peaks were used, and assuming that the

DOI: 10.1021/acs.jafc.7b03210
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positive charge states are solely caused by the attachment of protons, the average molecular mass can be assessed as 37 455.6 ± 0.9 Da. Those results confirm the results obtained by SDS-PAGE and indicate that the latent apricot PPO (L-*Pa*PPO) was practically quantitatively converted to its active form after one month of storage at 4 °C in Tris-HCl buffer (pH 8).

PPOs are believed to be stored in latent form in plants.[13] Latent PPO could exist in a completely latent or a partially active form,[56] as in the case of L-*Pa*PPO. The C-terminal domain is thought to shield access of phenolic substrates to the active site.[57] From that, it can be concluded that following harvest *Pa*PPO is present in the fruit in the latent form. Our results give a strong indication that latent *Pa*PPO can also be activated spontaneously during storage at 4 °C.

Protein Identification and Sequence Confirmation. The mature protein of apricot PPO has 496 amino acids (Asp102 → Ser597) with a calculated molecular weight of 56.2 kDa.[10] The mass for A-*Pa*PPO (37 455.6 Da) determined by mass spectrometry (ESI-LTQ) matches perfectly to the mass of the polypeptide Asp102 → Leu429 with one thioether bridge[58] and two closed disulfide bridges containing all five cysteines present in the peptide chain (Figure 6). This is additionally supported by the results of enzymatic digestion. UHPLC-ESI-MS/MS identified the purified latent PPO (L-*Pa*PPO) at 63 kDa as apricot PPO (UniProt O81103) yielding a sequence coverage of 48.87% (*Pa*PPO (Asp102 → Ser597)) and a total of 36 identified peptides (Table S1), 16 peptides from the main domain and 20 peptides from the C-terminal part defining the enzyme latent form, including peptides that cover the C-terminus of the protein. The peptides are underlined in blue in Figure 6 and listed in Table S1. For A-*Pa*PPO, we had a sequence coverage of 41.4% (*Pa*PPO (Asp102 → Leu429)), and all the peptides that have been identified (12 peptides) are in the main domain region. No tryptic peptides were detected and identified in the C-terminal part beyond Arg430. The presence of Arg430 in the last peptide identified can provide an indication that the removal of the enzyme's C-terminal domain proceeds via more than one cleavage site, as it was also reported in recent research.[59] The identified peptides are underlined in crimson in Figure 6 and listed in Table S2. *Pa*PPO (Uniprot O81103) shows 96.65% and 94.14% sequence identity to PPO from japanese apricot (*Prunus mume*; GenBank 645236994) and peach (*Prunus persica*; UniProt I1U4K7), respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03210.

List of identified peptide sequences of L-*Pa*PPO (Asp^{102} → Ser^{597}) and of A-*Pa*PPO (Asp^{102} → Arg^{430}), Michaelis−Menten diagrams for the four tested di- and triphenols, photograph showing the tyramine test activity for *Pa*PPO after storage (A-*Pa*PPO), and mass spectra of A-*Pa*PPO (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

The research was funded by the Austrian Science Fund (FWF) P25217 and P29144.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully recognize the opportunity given by the University of Vienna for the Ph.D. student visiting program. The authors are grateful to INATAA, University Frères Mentouri Constantine 1, for financial support of a fellowship to A.D. We thank Mag. Alexander Ronacher and Anna Fabisikova, M.Sc., for the support during the ESI-LTQ-MS experiments.

■ ABBREVIATIONS

*Pa*PPO, polyphenol oxidase from *Prunus armeniaca*; SDS, sodium lauryl sulfate; FPLC, fast protein liquid chromatography; AEX, anion exchange; CEX, cation exchange

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