Biochemical and Immunochemical Characteristics of Polyphenol Oxidases from Different Fruits of *Prunus*

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Polyphenol oxidases (PPOs) from five *Prunus* species fruits (peach, apricot, almond, plum, and cherry) have been partially purified by temperature-induced phase-partitioning in Triton X-114. The effect of pH on PPO activity with or without SDS shows that optimum pH ranges from 4 to 5.5 and that this enzyme does not occur in a latent form. Multiple forms of PPO (active and inactive) have been detected by SDS–PAGE stained with DL-Dopa and by Western blot revealed with PPO antibodies from apple pulp and broad bean leaves. We demonstrated *in vitro* that the multiple active forms occurred as a result of proteolysis of one major form. SDS–proteinase K digestion leads to a further resistant form in the five species studied. Under totally denaturing conditions, the main active and the proteolyzed resistant forms are, respectively, monomers of 63 and 43 kDa.

Keywords: *Polyphenol oxidases; fruits; Prunus; multiple forms*

INTRODUCTION

Enzymatic browning of fruit is a well-known phenomenon caused by oxidation of phenolic compounds into quinones (Mayer and Harel, 1981; Macheix et al., 1990, 1991; Lee, 1992; Nicolas et al., 1994). Following cell decompartmentation, this reaction is mainly catalyzed by polyphenol oxidases (PPOs; EC 1.10.3.1) in the presence of oxygen (Vamos-Vigyazo, 1981; Mayer and Harel, 1981, 1991; Eskin, 1990; Zawistowski, 1991). The quinones formed are highly reactive and polymerize, giving rise to the brown pigmentation that is characteristic of browning. This brown discoloration leads to organoleptic and nutritional modifications in the plant tissues, thus depreciating the food product (Fleuriet and Macheix, 1990; Macheix et al., 1990, 1991). Browning is currently responsible for serious economic losses in the food industry. Many different techniques have been used to deal with this problem, e.g., breeding for cultivars that are not susceptible to browning and the use of chemical or physical browning inhibitors (PPO, oxygen, substrates, etc.) (Eskin, 1990; Macheix et al., 1990; McEvily et al., 1992; Nicolas et al., 1994). However, no fully satisfactory results have been obtained to date.

Recent studies on leaf PPOs from different plant species revealed the basic characteristics of this enzyme. Cloning and sequencing of leaf PPO genes from broad bean (Cary et al., 1992), tomato (Newman et al., 1993), and potato (Hunt et al., 1993) indicated that all PPO genes encode native proteins with molecular masses ranging from 57 to 62 kDa. It has also been shown that PPO was routed to the thylakoid lumen in two steps by its two domain transit peptide (Sommer et al., 1994). For the first time, antisense gene strategy was used to inhibit PPO activity in potato tubers (Bachem et al., 1994). This approach opened many possibilities of preventing enzymatic browning and helps to pinpoint the physiological function of this enzyme.

Research on fruit PPOs is not yet quite as advanced, even though PPO activity has been detected in many fruits of economic interest (Vamos-Vigyazo, 1981; Mayer and Harel, 1981, 1991; Macheix et al., 1990; Zawistowski et al., 1991). The published results, concerning biochemical studies, suggested the structural heterogeneity of fruit PPOs expression, and recent studies on apple fruit PPO in our laboratory allowed us to clarify the relations between different forms of the enzyme (Marquès et al., 1994, 1995a,b).

Prunus species are of prime economic importance on fresh and processed fruit markets. Investigations on the PPO activity of these fruits, i.e., in peach (Luh and Phithakpo, 1972; Flurkey and Jen, 1978; Lee, 1991), apricot (Dijkstra and Walker, 1991), plum (Siddiq et al., 1992), and cherry (Benjamin and Montgomery, 1973; Pifferi and Cultrera, 1974), have focused mainly on optimum pH, stability, substrate affinity, and isoform number, sometimes with contradictory results. The aim of the present study was to study relationships between multiple forms of PPO from fruits of five *Prunus* species and to compare their immunochemical and biochemical characteristics.

MATERIALS AND METHODS

Plant Material. Fruits of five *Prunus* species (*Prunus persica* L. cv. Aline; *Prunus armeniaca* L. cv. Goldrich; *Prunus amygdalus* L. cv. Lauranne; *Prunus cerasifera* L. cv. P2930; *Prunus avium* L. cv. Durane Neri 1) came from experimental plots of Institut National de la Recherche Agronomique (INRA, Bellegarde) and Centre Technique Interprofessionel des Fruits et Légumes (CTIFL, Balandran). They were manually harvested in May and then lyophilized.

Reagents. Biochemicals were purchased from Sigma and used without further purification, except Triton X-114 (TX-114), which was concentrated three times as described by Bordier (1981), but using 100 mM Na–P_i buffer (pH 7.3) instead of 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl.

Enzyme Extraction. All extraction procedures were carried out at 4 °C. Pieces of lyophilized fruits (250 mg) were first cooled by liquid nitrogen and then reduced to powder with a crusher (Dangoumau). This powder was homogenized for 15 s in an Ultra-Turax in 10 mL of sodium phosphate buffer (0.1 M; pH 7.3) containing 40 mM ascorbic acid, 1.5% (v/v) TX-114, and 20 mg mL^{-1} α_2-macroglobulin (protease inhibitor) or 50 mg mL^{-1} phenylmethanesulfonyl fluoride (PMSF: serine–protease inhibitor). This homogenate was stirred for 30 min

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and then centrifuged for 20 min at 40000*g*. The supernatant constituted the TX-114 extract.

Enzyme Purification. The supernatant was subjected to temperature-induced phase partitioning as described by Sanchez-Ferrer et al. (1989) by adding TX-114 at 4 °C, so that the final detergent concentration was 4% (w/v). The mixture was kept at 4 °C for 15 min and then warmed to 25 °C until the solution became turbid, due to the formation, aggregation, and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins, phenolic compounds, and chlorophylls. This solution was centrifuged at 5000*g* for 10 min at room temperature. The dark green detergent-rich phase with no PPO activity was discarded, and the clear supernatant was used as enzyme source. The latter was named the native extract.

Enzyme Assay. Catecholase activity was determined spectrophotometrically at 400 nm and 30 °C with or without 3.5 mM SDS (0.1% w/v). One unit of enzyme was defined as the amount of the enzyme that produced 1 μmol of *o*-benzoquinone/min. The reaction medium (2.5 mL) contained 20 mM 4-methylcatechol, 5–20 μL of PPO extract, and 0.05 mM citrate phosphate buffer (pH 3–5.5) or 0.1 M sodium phosphate (pH 6–7.5). The total activity was expressed in ΔDO min^{-1} g^{-1} of dry weight (DW). The coefficient of variation due to the protocol is below 6%.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out according the method of Laemmli (1970), using a minigel Bio-Rad system (6.5 cm × 10 cm). Samples were mixed with 0.1% bromophenol blue and a few grains of sucrose before being applied to 12% polyacrylamide gels. Electrophoresis was carried out for about 1.5 h at constant current (18 mA/gel) in a buffer (pH 8.3) containing 1.5% (w/v) Tris base, 7.2% (w/v) glycine, and 0.5% (w/v) SDS.

Gels were stained for PPO activity in 0.1 M sodium phosphate (pH 7.3), containing 5 mM 3,4-dihydroxyphenylalanine (DL-Dopa) and catalase (100 units mL^{-1}). The reaction was stopped with 0.02 mM diethyldithiocarbamic acid. Catechin (10 mM), chlorogenic acid (3 mM), and 4-methylcatechol (20 mM) were also used to stain gels. Peroxidase activity was stained with 50 mM guaiacol and a few drops of 1% H_2O_2.

The molecular masses were determined using SDS–PAGE with denatured enzymatic extracts. Samples were diluted with 50 mM Tris-HCl buffer (pH 6.8), containing 8% (v/v) glycerol, 0.5 mM β-mercaptoethanol, 1.6% (w/v) SDS, and 0.1% bromophenol blue and then heated for 3 min at 100 °C. Gels were stained for protein using silver staining (Oakley et al., 1980). Seven SDS molecular mass markers were used: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

Immunoblotting. Proteins from SDS–PAGE were directly transferred to an Immobilon P membrane using the Milliblot-SDE transfer system (Millipore) at room temperature, 2.5 mA cm^{-2}, for 30 min with a single transfer buffer containing 48 mM Tris base, 39 mM glycine, 0.037% (v/v) SDS, and 20% MeOH. After two washings with phosphate-buffered saline (PBS), the membrane was incubated for 1.5 h with agitation in PBS, containing 5% (w/v) nonfat dry milk, 0.2% Tween 20, and 0.02% NaN_3. The membrane was rinsed 2 × 5 min with PBS and incubated at room temperature for 1 h in PBS containing 3% SAB and serum/anti-apple PPO diluted 1:1000 (Marquès et al., 1994a) or serum/anti-broad bean PPO diluted 1:1500 (a gift from Bob Buchanan, Berkeley, CA). After 4 × 5 min washing with Tris-buffered saline (TBS), the membrane was treated for 1 h in 50 mM Tris and 150 mM NaCl containing alkaline phosphatase-conjugated goat anti-rabbit Ig G at a dilution of 1:30000 (Sigma). The membrane was rinsed 4 × 5 min in TBS and then incubated with 10 mL of alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$) containing 0.33% nitro blue tetrazolium (NBT) and 0.16% 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The reaction was stopped by rinsing the membrane with PBS containing 20 mM EDTA.

Proteolysis. Proteolysis were carried out in liquid medium or directly during SDS–PAGE, according to the method established for apple (Marquès et al., 1994). Here, after the application of PPO extract in wells of gels, proteinase K, diluted in 0.125 mM Tris buffer containing 20% (v/v) glycerol and 0.1% (w/v) SDS, was added to the wells. Electrophoresis was started. It was stopped when the proteins reached the middle of the stacking gel. After digestion for 30 min at room temperature, electrophoresis was started again. Gels were stained for PPO activity with 5 mM DL-Dopa.

For the other method of proteolysis, 15 mL of the PPO extract was incubated with 37.5 μL of proteinase K (43 U mL^{-1}) at 25 °C. The reaction was stopped by cooling or with PMSF. One unit of proteinase K hydrolyzes casein to produce color equivalent to 1 mmol (181 mg) of tyrosine/min at pH 7.5 at 37 °C.

RESULTS

Effect of pH and SDS. Optimum pH of the PPO from phase-partitioning extracts has been studied for the five species (Figure 1). Optima exist for peach (pH 5), almond (pH 5), and cherry (pH 4.5), with wider ranges for apricot (pH 5–5.5) and plum (pH 4–5.5). In cherry, unlike the four other species, there was no activity at pH 3 or above pH 6. In the five species studied, treatment with SDS, an anionic detergent that is widely used to obtain high PPO activity, resulted in a shift in the optimum pH. In some cases, this was accompanied by changes in activity. In cherry, this shift was very marked (from pH 4.5 to pH 5.5) and the activity was 8 times as high as that at the same pH without SDS. However, maximum activity with SDS was only 1.5 times that without SDS. Maximum activity in plum in the presence of SDS (pH 5.5–6) was 2 times as high that without the SDS treatment. The increase in activity due to SDS was not as marked in almond and apricot, and the optimum pH shift was not as clear as it was in plum and cherry. In peach, a slight inhibition by SDS was noted at pH 4.5–5.5, with a second maximum occurring at pH 6.5 after SDS treatment.

PPO Profile by SDS–PAGE. In partially denaturing conditions and at similar specific activities, the number of forms stained on polyacrylamide gel with Dopa varied according to species (Figure 2). Three bands were visible for peach, cherry, and apricot, two for plum, and only one for almond. In all cases, a major form with an electrophoretic mobility of 43 kDa was dominant, except for cherry, which gave three major bands at 43, 40, and 36 kDa (Figure 2). The dominant 43 kDa form was identical to that detected in apple (Figure 2), another Rosaceae fruit which has been the topic of extensive laboratory studies (Marquès et al., 1994). The same enzymatic profile was obtained with other *Prunus* cultivars with relatively clear intermediate forms with electrophoretic mobilities in the range 27–43 kDa. PPO activity was also revealed with two other substrates, catechin and chlorogenic acid (data not shown), two *o*-diphenols that are known to be very good PPO substrates. Profiles obtained with these substrates were quite close to those obtained with Dopa, with a very clear 43 kDa band for each *Prunus* species. All SDS–PAGE separations were performed in the presence of catalase to reduce possible interference from peroxidase activity. However, for the five *Prunus* species, peroxidases had isoforms with lower electrophoretic mobility than those of PPO (data not shown).

Immunological Characteristics of PPO. After separation under partially denaturing conditions, proteins were blotted to a membrane and two types of PPO antibodies were assessed. One was obtained in our

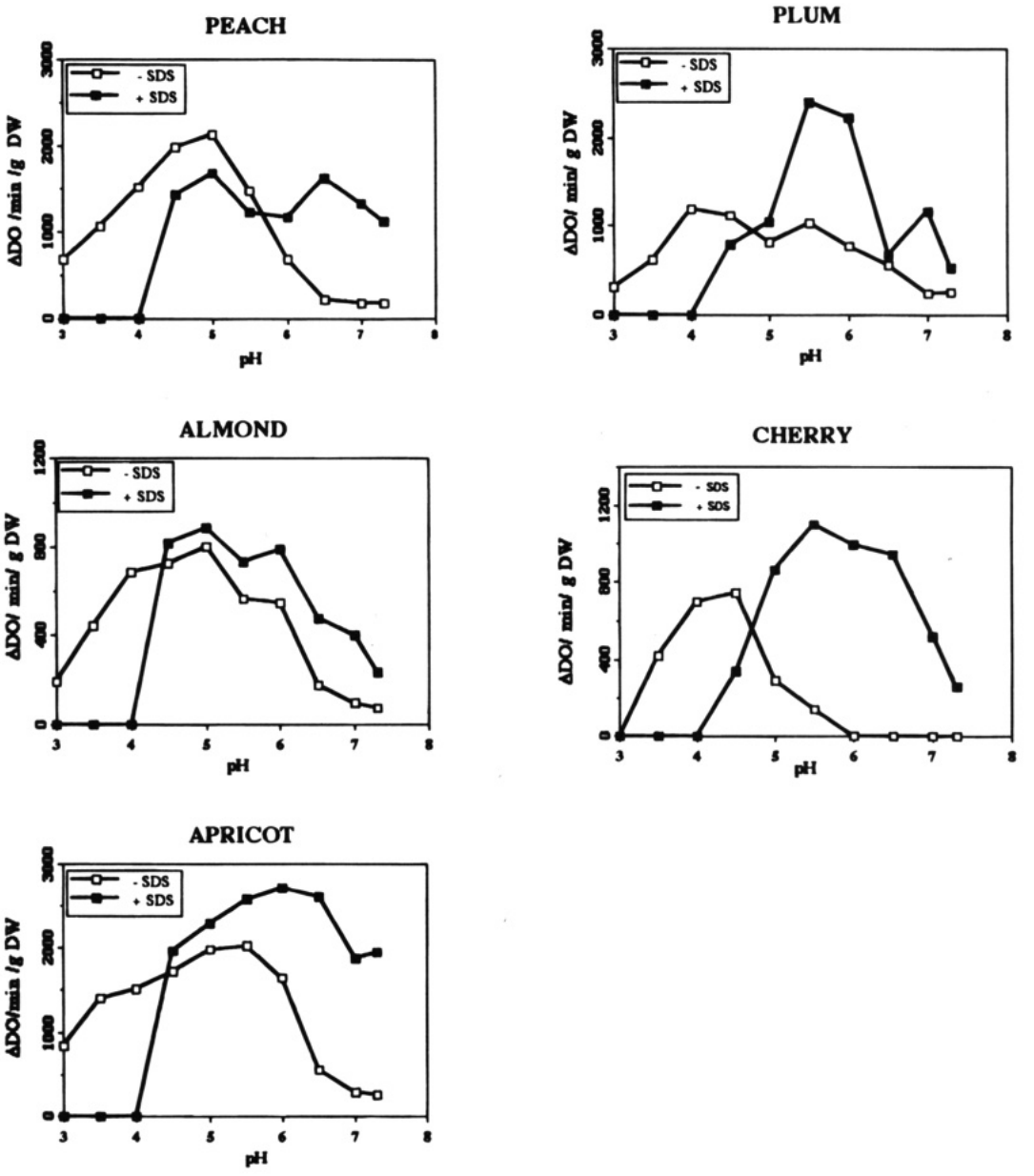

Figure 1. Effect of pH and SDS on PPO activity of the native extracts from five *Prunus* fruits.

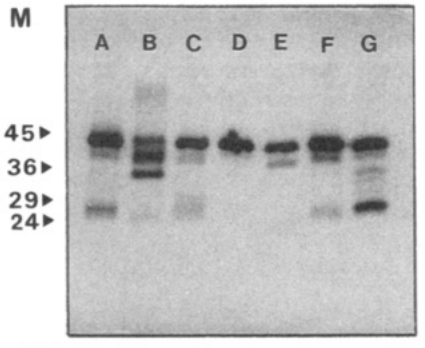

Figure 2. SDS–PAGE profile of apricot cv. Bergeron (A), cherry cv. Durane Neri 1 (B), plum cv. P2930 (C), almond cv. Lauranne (D), apple cv. Granny Smith (E), apricot cv. Goldrich (F), and peach cv. Aline (G) native extracts (6 μg of protein/well). Gels were stained with DL-Dopa. M, molecular mass markers.

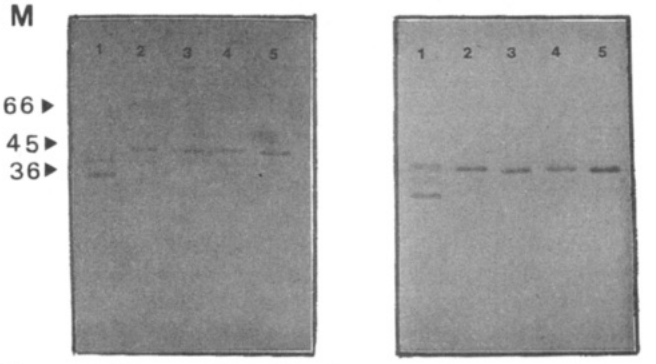

Figure 3. Western blot of five *Prunus* native extracts (7.5 μg of protein/well) revealed with anti-apple PPO (A) and anti-broad bean PPO (B) antibodies. 1, Cherry; 2, plum; 3, almond; 4, apricot; 5, peach. M, molecular mass markers.

laboratory from apple pulp PPO (Marquès et al., 1994), and the other was derived from bean leaf PPO. With apple anti-PPO antibodies (Figure 3) and at similar specific activities, the 43 kDa form was detected in extracts from all five *Prunus* species along with a weaker mobile form at around 63 kDa. This latter form was inactive since it was not revealed with Dopa. In cherry, only two of the three bands revealed with Dopa were detected by these antibodies (43 and 36 kDa

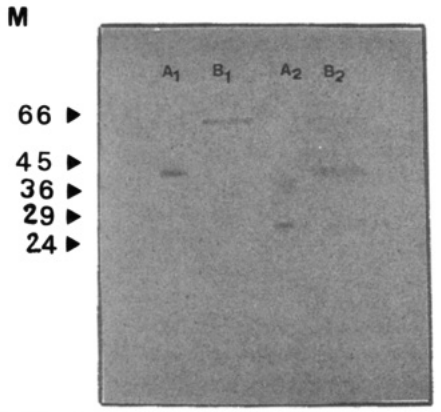

Figure 4. Western blot of native (1) and proteolyzed (2) extracts (5 μg of protein/well) from peach after separation by SDS–PAGE under partially (A) and totally (B) denaturing conditions. M, molecular mass markers.

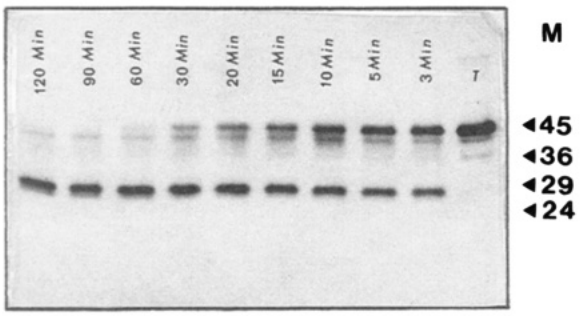

Figure 5. SDS–PAGE stained for PPO activity of proteolysis kinetics of peach extract by 37.5 μL of proteinase K (43 units mL^{-1}).

forms). With broad bean leaf anti-PPO antibodies (Figure 3) similar results were obtained; i.e., the major 43 kDa form was recognized in all fruits except cherry. However, the 40 and 60 kDa forms were more clearly revealed for the cherry with this serum than with that derived from apple.

Anti-PPO polyclonal serum was also used to study molecular masses of the PPO proteins. Extracts were completely denatured before being separated by SDS–PAGE, blotted, and then immunodetected (Figure 4). The electrophoretic mobility of the major form was reduced, and its molecular mass was found to be 63 kDa for all *Prunus* species studied.

Resistance to Proteinase K Proteolysis. SDS–proteinase K digestion of PPO extracts was carried out to test the resistance of *Prunus* PPOs to proteolysis. Proteolysis kinetics were followed by partially denaturing SDS–PAGE stained for activity by Dopa. During the first few minutes a very electrophoretic mobile band (27 kDa) appeared (Figure 5). It was derived from the 43 kDa form, whose intensity faded progressively with complete disappearance within 1 h. Moreover, the activity measurements during digestion showed almost no loss of activity as long as the 27 kDa form remained intact (3 h). Protein detection with silver nitrate before and after proteolysis of the enzyme extract demonstrated that PPO is one of the most resistant proteins to this type of proteolysis (data not shown). This resistant form has a molecular mass of 43 kDa after denaturing conditions (Figure 4).

Mild proteolysis of the extract in the gel revealed intermediate forms between the main active 43 kDa form and the resistant 27 kDa (Figure 6). We noted that all of these intermediate forms were active. This

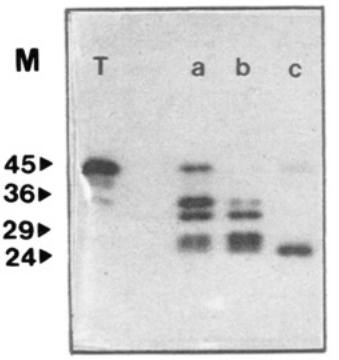

Figure 6. SDS–PAGE stained for PPO activity of mild SDS–proteinase K digestion of the peach native extract (6.7 μg of protein/well): (a) 6.45×10^{-6} units; (b) 6.45×10^{-4} units; (c) 6.45×10^{-2} units; (T) control not proteolyzed. M, molecular mass markers.

in vitro proteolysis enabled identification of electrophoretically very mobile forms that occurred in the native extract despite the presence of anti-proteases (Figure 2).

DISCUSSION

The different biochemical characteristics of numerous fruit PPOs (optimum pH, latency, numerous multiple forms) could be explained by genetic diversity and also by the probable presence of extraction and purification artifacts. Nevertheless, our study of native and proteolyzed PPOs from different *Prunus* fruit species allows several points to be clarified.

The optimum pH for PPO activity in the five *Prunus* species studied ranged from 4 to 5.5, close to the optimum levels reported for other fruit species (Vamos-Vigyazo, 1981; Zawistowski et al., 1991; Mayer and Harel, 1991; Marquès et al., 1995b). This is of particular interest, since this optimum pH corresponds to the pH in the thylakoid lumen from which PPO is derived (Vaughn and Duke, 1984; Newman et al., 1993). However, there were some differences with previous results. Pifferi and Cultrera (1974), for instance, reported two optimal pH levels (pH 4.2 and 6.5) for PPO activity in cherry, whereas we found only one peak at pH 4.5 for this species.

SDS is known to activate most leaf PPOs (Flurkey, 1986), and a detailed study focusing on broad bean leaf PPOs has already been carried out (Moore and Flurkey, 1990). SDS activation of PPOs is relatively unique, since this detergent inhibits most other enzymes. One particularly interesting aspect of this SDS effect on PPOs is that it allows detection of PPO activity in gels containing SDS. In fruit, the latency of PPO has only been reported in mango (Robinson et al., 1993), avocado (Kahn, 1977), and grape (Sanchez-Ferrer et al., 1989). Otherwise, PPO activity has been detected without SDS at pH 4.8 in grape (Valero et al., 1988). We believe that this phenomenon might not actually be latency *per se*, but rather a joint effect of pH and SDS. In fact, the effect of SDS in relation to pH revealed that in most cases this detergent reinforced the inhibiting effect of acidic pH on PPO activity, while activating the enzyme at levels higher than pH 5 (Figure 1). This was especially clear for PPO activities of plum and cherry. However, when we compare maximal PPO activities with or without SDS treatment, no clear SDS activation appeared, at least in the fruit species investigated here. Indeed, in cherry, maximum PPO activity (at pH 5.5)

with SDS was only 1.5-fold higher than that obtained without this detergent (at pH 4.5). The PPOs extracted from our *Prunus* species were therefore not latent. This has also been noted with apple PPOs by Marquès et al. (1995b), who showed that this SDS effect was not modified by the substrate used for PPO activity measurements. This may be a specific characteristic of fruit PPOs, since broad bean leaf PPO has been described as completely latent (Flurkey, 1986).

SDS is known to modify the tertiary and quaternary structures of proteins, which explains the inactivity of most enzymes in the presence of this detergent. The resistance of PPO to SDS thus suggests that the tertiary structure of this protein is stronger. One hypothesis put forward to explain this phenomenon vis-à-vis broad bean PPOs (Robinson and Dry, 1993), and confirmed for apple PPOs (Marquès et al., 1995b), is the presence of disulfide bonds. This would account for the unique resistance of this protein to proteinase K proteolysis. This particular property has been fully investigated with mouse tyrosinase (Yurkow and Laskin, 1989) and demonstrated for the first time in a plant species (Marquès et al., 1994). Only partial resistance was obtained in the fruit species studied, as 33% of the *Prunus* PPOs were degraded, the remaining 67% continuing to contain active sites. In fact, the PPO electrophoretic mobility dropped from 43 to 27 kDa after proteolysis of *Prunus* extracts (Figure 4). This form and all of the intermediate forms that appeared after mild proteolysis were active (Figure 6). Our criterion for considering that PPO is resistant to this type of proteolysis is based on the fact that most of the other proteins present in the extract disappeared during treatment. The presence of this resistant PPO form in apple has enabled development of a quick purification method to obtain polyclonal antibodies (Marquès et al., 1994). It was also found that the proteolyzed form in apple is active over a broader pH range than the major native form (Marquès et al., 1995b). This *in vitro* proteolysis of PPOs also provided interesting details on various aspects of the multiple forms present in the extracts. In native extracts from peach and apricot, we noted the presence of forms with higher electrophoretic mobility and ranging from 27 to 40 kDa in addition to the major 43 kDa band (Figure 2). We consider that these intermediate forms occurred as a result of *in vivo* proteolysis of a main form by native proteases, which react in different ways according to the physiological state of the fruit. This proteolysis could also take place during extraction if there is no addition of anti-proteases such as PMSF, a serine–protease inhibitor, or α_2-macroglobulin.

There were few immunochemical differences between these forms. In partially denaturing conditions, the active (27 and 43 kDa) and inactive (63 kDa) forms were recognized using apple anti-PPO antibodies. In totally denaturing conditions, the molecular masses of the main form and the proteolyzed form were found to be 63 and 43 kDa, respectively. Recent studies carried out under the same conditions on leaves (Robinson and Dry, 1992) and fruits (Murata et al., 1993; Rathjen and Robinson, 1992; Marquès et al., 1995b) also demonstrated that the major PPO form is a protein of about 63 kDa molecular mass. These results were confirmed by mRNA analysis in grape and broad bean leaves, where a 2.2 kb transcript was identified, which would certainly be large enough to encode a 63 kDa protein.

The main forms were also recognized with bean anti-PPO antibodies. The fact that common forms were recognized by anti-PPO antibodies raised against PPO from very different species indicates that some parts are highly conserved, especially around the two active copper sites. This has been confirmed by comparisons of PPO amino acid sequences from tomato (Newman et al., 1993), broad bean (Cary et al., 1992), and potato leaves (Hunt et al., 1993), the degree of homology shown being high.

In conclusion, the present study revealed there are many common PPO biochemical characteristics among different *Prunus* species and more generally among different Rosaceae species. This group of enzymes has the unique property of being resistant to mild proteolysis reactions and to treatment by anionic detergents such as SDS. The multiple active forms depend on cellular proteolytic activity in relation to cultivar, physiological state, or shelf storage. The presence of an inactive PPO form is very interesting with regard to regulation of PPO expression. This form could be a precursor of the enzyme with a high molecular weight, as was suggested in the grape (Rathjen and Robinson, 1992), or the unfolded form of the native form which is probably the translocation-competent form entering thylakoid membranes, as was suggested in the apple (Marquès et al., 1995a,b).

ABBREVIATIONS USED

PPO, polyphenol oxidase; TX-114, Triton X-114; DL-Dopa, D-β-3,4-dihydroxyphenylalanine; SDS, sodium dodecyl sulfate; PMSF, phenylmethyanesulfonyl fluoride; DIECA, diethyldithiocarbamic acid.

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