ABSTRACT

Bruce’s Sport is a mutant grapevine (Vitis vinifera L.) with green and white variegated fruit derived from the Sultana variety. The white regions of tissue have decreased polyphenol oxidase (PPO) activity resulting in a reduced capacity for browning. Active PPO from Sultana grapes was purified and had an apparent molecular weight of 40,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots indicated that mature Sultana grapes contained a single 40-kilodalton PPO, and young Sultana berries also had small quantities of a 60-kilodalton protein. Bruce’s Sport grapes had much less of the 40-kilodalton PPO and greater amounts of the 60-kilodalton band. Protease digestion of Bruce’s Sport extracts decreased the proportion of the 60-kilodalton protein and increased the 40-kilodalton band. A cDNA clone of grape PPO was used to probe a northern blot of Sultana and Bruce’s Sport RNA and hybridized to a 2.2-kilobase transcript in both grapevines. The level of PPO mRNA was high in the early stages of berry development but then declined. The results suggest that in grapevine the active 40-kilodalton form of PPO is synthesized as a precursor protein of at least 60 kilodaltons, and normal processing is interrupted in Bruce’s Sport resulting in the accumulation of the 60-kilodalton inactive preform of PPO.

PPO (EC 1.10.3.1.), also known as catechol oxidase, is a copper-containing enzyme catalyzing the oxidation of o-diphenols to o-diquinones. These quinones polymerize to form the familiar brown pigments associated with browning in plants. The physiological function of PPO is as yet unknown, although it has been associated with disease resistance (27). Other functions such as mediation of the Mehler reaction or involvement in pseudocyclic phosphorylation (22) have also been suggested.

PPO is encoded in the nucleus and thought to be transported to the chloroplast in an inactive form (25). It is located on thylakoid membranes in healthy green leaves (24) and immature green olive fruit (21). In Vicia faba leaves, PPO is colocalized with PSII proteins on the thylakoid membranes (9). The chloroplastic location of this enzyme ensures that it is normally separated from its phenolic substrates within the vacuole and thus browning only occurs when plant cells are damaged.

The molecular mass of PPO has not yet been clearly established. Gel filtration of the PPO of Mucuna pruriens determined its molecular mass to be 95 kD (28). The protein was found to be a dimer, denaturation revealing two subunits of 42 kD. Partially denaturing gels stained for PPO activity revealed a 40-kD PPO in Vicia faba, the same size as the denatured purified protein (26). The PPOs purified from olive, sago palm, and spinach also consisted of a single subunit of 40 to 42 kD (2, 14, 23). Western blots probed with a polyclonal antibody raised against purified broad bean PPO indicated that a 43- to 45-kD band was present in broad bean, bush bean, lettuce, mung bean, soybean, spinach, and tobacco (8). In vitro translation of leaf mRNA isolated from each of these species produced a protein of approximately 45 kD (4), resulting in the suggestion that in a range of plant tissues PPO is synthesized as a 45-kD protein without a transit sequence.

Although PPO has been extensively studied in grapes (Vitis vinifera L.), much of the work has been concerned with browning during juice or wine production (29). The existence of multiple forms of PPO in grapes has been reported by a number of authors. Wolfe (31) noted at least three forms of PPO in 55 grapevine varieties, whereas as many as eight bands staining for PPO activity were described by Wissemann and Lee (30) and Sánchez-Ferrer et al. (19). There is also little consensus in the literature concerning the mol wt of grape PPO, with values ranging from 15,000 in Noble grapes (7) to 85,000 in DeChaunac (10). Few reports, however, have described PPO purified to homogeneity, and most estimates of mol wt have been determined under nondenaturing conditions. Nakamura et al. (13) purified PPO from Kosher grapes and estimated the mol wt of the single PPO to be 39,000 by gel filtration and 41,000 by SDS-PAGE.

In Australia, the grape variety Sultana (Syn. Thompson Seedless, Kishmish, Sultanina) is used extensively for the production of dried fruit. Bruce’s Sport is a mutant of Sultana, first described by Antcliff and Webster (1). It produces variegated leaves, although this is not constitutively expressed and first appears after one-third of the growing season. The berries, however, are always variegated and have green and white stripes running along the length of the fruit. Bruce’s Sport has an inherently low capacity for browning because it lacks PPO activity in the white regions of berry tissue. The green sections, however, have the same activity as berries of Sultana (16). The aim of this work was to determine the basis...
for this lack of PPO activity and to investigate the PPO enzyme in Sultana and Bruce’s Sport.

**MATERIALS AND METHODS**

**Plant Material**

Immature Sultana and Bruce’s Sport berries were collected from grapevines (*Vitis vinifera* L.) grown at the Commonwealth Scientific and Industrial Research Organization Division of Horticulture, Merbein, Victoria. Sugar content was measured with a Zeiss hand-held refractometer and berry diameter with spring calipers. When collected, the fruit was <5 mm in diameter and about 4° Brix. The berries were immediately frozen in liquid nitrogen and stored at -80°C.

Berries used for northern blot analysis were obtained from one (Bruce’s Sport) or two (Sultana H5) vines growing at the Commonwealth Scientific and Industrial Research Organization Division of Horticulture, Adelaide, South Australia.

**Partial Purification of PPO**

PPO was partially purified from Bruce’s Sport berries for subsequent purification using an antibody-bound affinity column. All procedures were carried out at 4°C. Immature, frozen berries (300 g) were homogenized in a Polytron blender in 1500 mL of ice-cold extraction medium containing 0.1 m NaH₂PO₄, 0.4 m sucrose, 1 mM MgCl₂ (pH 7.2), and 5 mM DTT. The homogenate was centrifuged at 10,000g for 10 min and the pellet resuspended in 300 mL of extraction medium containing 1% (v/v) Triton X-100. This was stirred on ice for 10 min and centrifuged at 40,000g for 10 min, and the supernatant was strained through Miracloth. Ammonium sulfate was added to 95% (w/v) over 2 h, and the mixture was stirred for a further 20 min before centrifugation at 40,000g for 20 min. The pellet was resuspended in BTP buffer (20 mM BTP [pH 7.5], 5 mM DTT) before further centrifugation at 40,000g for 30 min. The supernatant was loaded onto a Sephadex G-25 column and eluted with BTP buffer. Fractions (30 × 10 mL) were collected, and six of these were pooled and are referred to as the “partially purified extract” used for subsequent purification with an affinity column. PPO activity was measured polarographically at 25°C in an oxygen electrode (Hansatech) containing 0.6 mM SDS, 50 mM NaH₂PO₄ (pH 5.0), and extract in a final volume of 1 mL. Addition of SDS was undertaken to ensure complete activation of grape PPO (16). Oxygen uptake was initiated with the addition of 2 mM 4-methyl catechol. Protein concentrations were determined using the Bio-Rad protein assay, based on the method of Bradford (3) using bovine γ-globulin as a standard.

**Antibody Preparation**

PPO was purified to homogeneity from mature Sultana berries (S.P. Robinson, unpublished data) and a sample used to raise polyclonal antibodies in New Zealand white rabbits. γ-Immunoglobulin was purified on a protein A column. The γ-Immunoglobulin fraction (23.3 mg mL⁻¹) was diluted 1:2000 or 1:1000 for western blotting.

**Affinity Column**

A CNBr-activated Sepharose 4B column (Pharmacia) was prepared according to instructions of the manufacturer. Approximately 5 mg of anti-grape PPO was bound to 2 g of CNBr-activated Sepharose 4B.

Before use, the column was brought to room temperature and equilibrated with 25 mL of sample buffer (20 mM BTP pH 7.5, 0.1 mM NaCl, 5 mM DTT, 0.1% [w/v] Triton X-100). Partially purified Bruce’s Sport PPO (10 mL) was added and allowed to pass through the column. The column was then washed with 25 mL of sample buffer and the sample eluted with 0.1 M glycine-HCl (pH 2.8), 1 mM DTT. Fractions (10 × 1 mL) were collected and the pH of each neutralized with 50 µL of 1 M Tris (pH 9.5). PPO activity was measured using an oxygen electrode as described above. Fractions containing active PPO were pooled and concentrated with a Centricon-10 micro concentrator (Amicon) with the addition of 0.05% (v/v) Tween-20. The concentrated PPO was transferred into 50 mM Tris (pH 6.8), 0.05% (v/v) Tween-20 using the Centricon-10 micro concentrator.

**Electrophoresis**

Crude protein extracts were made from immature, frozen Sultana and Bruce’s Sport berries for protease digestion, electrophoresis, and western blotting. Two grams of berries were ground in a Polytron blender in 6 mL of grinding buffer (0.1 mM NaH₂PO₄, 0.4 mM sucrose, 1 mM MgCl₂ [pH 7.2]). The homogenate was centrifuged at 10,000g for 5 min and the resulting pellet resuspended in 2 mL of grinding buffer containing 1% (v/v) Triton X-100. This suspension was incubated on ice for 10 min and centrifuged at 10,000g for a further 5 min, and the supernatant was collected for subsequent analysis. This is referred to as the “crude protein extract.”

Electrophoresis was conducted using a minigel system (Hoeffer Mighty Small II) and Tricine polyacrylamide slab gels (20), consisting of a 10% (w/v) resolving gel and a 4% (w/v) stacking gel in a discontinuous buffer system. Protein samples loaded onto activity-stained (partially denaturing) gels were diluted into 50 mM Tris (pH 6.8), 0.1% (w/v) SDS, 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue but were left at room temperature. These gels were washed for 10 min in two changes of wash buffer (20 mM NaH₂PO₄ [pH 6.0], 0.1% [w/v] SDS) immediately after electrophoresis. They were then shaken in 50 mL of wash buffer containing 2 mM l-dihydroxyphenylalanine, 1 mM catechol, 0.5 mM p-phenylenediamine, and 100 units mL⁻¹ catalase (Sigma) for 1 to 2 h, although most bands were visible after 15 to 30 min. After staining, the gels were rinsed in wash buffer and dried on a slab dryer.

For all other forms of electrophoresis, protein samples were denatured by dilution in 62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM DTT, and 0.01% (w/v) bromophenol blue and heated to 100°C for 3 min. Gels to be stained for total protein were stained in 0.15% (w/v) Coomassie blue, 50% (v/v) methanol, and 10% (v/v) acetic acid and then destained in 50% (v/v) methanol, 10% (v/v) acetic acid for approximately 1 h. Remaining background
color in the gels was removed by washing in 5% (v/v) methanol, 7% (v/v) acetic acid before drying on a slab dryer. Molecular mass standards from Sigma were BSA (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), and lactalbumin (14 kD).

For western blotting, proteins were transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH approximately 8.3) using a Bio-Rad Mini Trans-Blot apparatus. The primary antibody (anti-grape PPO) was prepared as described above. The secondary antibody was Bio-Rad goat anti-rabbit conjugated with alkaline phosphatase. Prestained molecular mass standards from Bio-Rad were phosphorylase (89 kD), BSA (71 kD), ovalbumin (49 kD), carbonic anhydrase (35 kD), trypsin inhibitor (24 kD), and lysozyme (20 kD). Prestained standards were calibrated in the Tricine gel system by comparison with unstained standards of known molecular mass.

Protease Digestion

An extract of predominantly white Bruce’s Sport berries was made as described for electrophoresis and 50 μL of the nondenatured extract incubated with 1:20 (w/w) trypsin or thermolysin at 25°C. Digestion was stopped with the addition of denaturing buffer. Extracts were further denatured by heating to 100°C for 3 min before electrophoresis and western blotting.

RNA Extraction

RNA was extracted from berries using a modification of the method of Rezaian and Krake (17). Four grams of frozen berries were ground in liquid nitrogen, and the resulting powder was stirred with 16 mL of extraction buffer (0.2 M Tris [pH 8.3], 5 M sodium perchlorate, 5% [w/v] SDS, 8.5% [w/v]) polyvinylpolypyrrolidone, 0.1% [v/v] β-mercaptoethanol) at room temperature for 30 min. The homogenate was loaded onto an Amicon Centriflone cone plugged with approximately 1.5 cm of glass wool, inserted in a centrifuge tube, and centrifuged at 200g for 10 min in a swing-out rotor. An equal volume of cold (~20°C) ethanol was mixed with the eluate and immediately centrifuged at 4000g for 10 min in a swing-out rotor. The pellet was briefly air dried before resuspension in 1.2 mL of buffer (10 mM Tris, 0.1 mM EDTA [pH 7.6]). The suspension was extracted three times with phenol-chloroform (1:1) and once with chloroform alone. A one-tenth volume of 3 M sodium acetate (pH 5.2) and an equal volume of cold ethanol were added and the nucleic acids collected by immediately centrifuging at 12,000g for 15 min. The pellet was washed with 70% (v/v) ethanol, centrifuged at 12,000g for a further 15 min, air dried, and resuspended in 50 μL of sterile water. Typically, 5 to 50 μg of RNA per gram of tissue was extracted from berries, depending on the age of the tissue.

Total RNA (5 μg) was loaded onto a 1.2% (w/v) formaldehyde gel for northern blotting and transferred onto a Bio-Rad Zeta-Probe membrane by alkaline capillary transfer. RNA markers obtained from BRL (Gaithersburg, MD) were also loaded to allow sizing of the transcript. The filter was probed with a fragment of a grape PPO clone GPO1 isolated from Sultana berries, verified from the amino acid sequence of the active, 40-kD PPO (I.B. Dry and S.P. Robinson, unpublished data), and labeled using a hexamer-primer DNA-labeling kit (Bresatec Pty Ltd, Adelaide, South Australia). After hybridization, the filter was washed in 2 × standard sodium citrate (150 mM sodium chloride, 150 mM sodium citrate [pH 7.0]), 0.1% (w/v) SDS for twice for 15 min at room temperature followed by two washes of 15 min in 0.1 × standard sodium citrate, 0.1% (w/v) SDS at 65°C.

Sequencing

A total of 240 μg of Bruce’s Sport PPO purified using the affinity column was loaded into seven tracks of a 10% (w/v) Tricine gel. Separated proteins were transferred onto a Bio-Rad Transblot Protein Sequencing PVDF membrane in 3-(cy clohexylamino)propanesulfonic acid buffer as described by Ploug et al. (15).

RESULTS

Active PPO can be detected by separating extracts on a partially denaturing SDS-PAGE gel and staining with a phenolic substrate. Crude extracts of Sultana and Bruce’s Sport berries were separated on a partially denaturing gel and washed in a buffer containing the PPO substrate, catechol. A single band of approximately 40 kD was evident in Sultana extracts (Fig. 1). Bruce’s Sport berries also had a single band with an apparent molecular mass of 40 kD but had <10% of the activity of Sultana berries (Fig. 1). This was consistent with our earlier polarographic measurements of PPO activity in Sultana and Bruce’s Sport berries (16).

Purification of PPO from Sultana berries yielded a single protein that comigrated with the band of PPO activity in crude extracts and had an apparent molecular mass of 40 kD

Figure 1. Crude grape berry extracts electrophoresed on a partially denaturing SDS-PAGE gel and stained for PPO activity. Lanes 1 to 4 represent Sultana extracts of 1, 2, 5, and 10 μL, respectively; lanes 5 to 8 show Bruce’s Sport extracts also of 1, 2, 5, and 10 μL, respectively. The positions of fully denatured molecular mass markers (kD) are indicated in the left margin.
in denaturing SDS-PAGE (S.P. Robinson, unpublished data). This was similar to PPO purified from Koshu grapes, which had a mol wt of 39,000 to 41,000 (13). An antibody raised against the purified 40-kD Sultana PPO was used to detect PPO proteins in western blots of crude extracts of Sultana and Bruce’s Sport. The specificity of this antibody was ascertained by probing a western blot with preimmune and postimmune serum. Preimmune serum did not react with crude extracts of either Sultana or Bruce’s Sport berries, whereas postimmune serum gave the expected single band at 40 kD with extracts of mature Sultana berries (data not shown).

Extracts of immature Sultana berries probed with postimmune serum also exhibited a prominent band of 40 kD, but in addition a small amount of an antigenic protein of 60 kD could be observed (Fig. 2, lane 2). In extracts of Bruce’s Sport, there was much less of the 40-kD protein, in agreement with the measurements of PPO activity, but relatively more of the 60-kD protein (Fig. 2, lane 1). The mainly white Bruce’s Sport berries (Fig. 2, lane 3) contained relatively less active 40-kD PPO and relatively more inactive 60-kD protein than the predominantly green berries (Fig. 2, lane 4), which had only small amounts of the 60-kD protein. This suggested that the inactive 60-kD protein was derived from the white tissue, which lacks PPO activity (16), and that there was a direct correlation between the amount of the 40-kD protein and measured PPO activity. The presence of the 60-kD protein in immature, but not mature Sultana berries, and its elevated level in the white regions of Bruce’s Sport berries, suggested that this may be an immature protein whose normal processing is interrupted in the mutant.

**Purification of PPO by Affinity Chromatography**

Anti-grape PPO antibodies were bound to a CNBr-Sepharose matrix and used to purify antigenic proteins from a crude extract of Bruce’s Sport berries. Three proteins, with apparent molecular masses of 60, 40, and 20 kD by SDS-PAGE, were eluted after this extract was passed through the column (Fig. 3). The 40-kD protein was the most abundant, and approximately 20% of the mixture consisted of the 20-kD protein. The 60-kD protein made up only about 10% of this purified PPO extract. A western blot of the partially purified extract, before passing through the column, indicated that passage through the anti-grape PPO affinity column decreased the relative amount of the 60 kD and increased the proportions of the 40- and 20-kD proteins present in the extract (Fig. 4). The 20-kD peptide was only mildly antigenic (cf. Figs. 3 and 4) and was difficult to detect in the partially purified extract by western blotting (data not shown). Therefore, it seemed to be a breakdown product of either the column or of the 60- or 40-kD protein rather than being recognized and bound by the anti-grape PPO antibody. Experiments with two different column types (Pharmacia CNBr-activated Sepharose 4B and Pierce AminoLink) indicated that
the appearance of the 20-kD peptide was not connected with column type or age and thus was unlikely to be a result of column breakdown.

Amino Acid Sequencing

The three proteins purified by the anti-grape PPO affinity column (Fig. 3) were blotted onto a PVDF membrane and sequenced. The amino-terminal sequence of the 40-kD PPO protein was found to be identical with that obtained from the active 40-kD PPO purified from Sultana berries (S.P. Robinson, unpublished data). The 60-kD protein was blocked at the amino terminus and was unable to be sequenced. The amino-terminal sequence of the 20-kD protein was identical with a region in the carboxyl terminus of a cDNA clone of grape PPO isolated from Sultana (I.B. Dry and S.P. Robinson, unpublished data).

Protease Digestion of PPO

To determine the relationship between the 60- and the 40-kD proteins, extracts of predominantly white Bruce’s Sport berries (cf. Fig. 2, lane 3) were digested with trypsin and thermolysin and analyzed by western blotting (Fig. 5). When the extract was denatured, it remained largely undigested even after 24 h under the conditions of proteolysis. When undenatured, however, after 1 h only faint traces of the 60-kD band remained, whereas there were increased levels of the 40-kD protein. Although not visible in Figure 5, a small amount of the 20-kD peptide also became apparent after digestion by trypsin. The 40-kD protein produced by digestion of the 60-kD band was not significantly cleaved even after 3 h (data not shown). Analysis by activity-stained gels was unsuccessful in detecting any increase in PPO activity resulting from cleavage of the 60-kD protein (data not shown). Extracts of Sultana berries and white Bruce’s Sport grapes were also mixed and incubated at room temperature, but polarographic measurements over a period of 3 h failed to detect an increase in PPO activity (data not shown).

Northern Blot Analysis

The lack of active 40-kD PPO in Bruce’s Sport and its apparent replacement by a much larger, 60-kD, inactive protein could be due to incorrect protein processing, abnormal RNA splicing, or a mutation in the PPO gene itself. The latter possibilities would lead to the production of an oversized mRNA encoding PPO; whereas if the fault were in the processing of the protein, similar-sized transcripts would be expected. RNA isolated from Bruce’s Sport and Sultana berries at differing stages of development was probed with a cDNA clone of Sultana PPO, revealing that both varieties produced similar-sized transcripts of PPO, 2.2 kb in length (Fig. 6). This would encode a protein of at least 60 kD. High levels of PPO mRNA were extracted from Sultana berries early in development, but these were reduced by the time the berries reached veraison (11 mm diameter). Only a small percentage of the initial levels of mRNA was evident in 12 mm berries, with this being reduced even further as the berries matured. At these later stages, expression was at a constant, although very low, level. RNA could not be successfully extracted from berries 9 and 10 mm in diameter. A similar pattern of expression was evident in Bruce’s Sport, although expression was sharply curtailed earlier in development when the berries were approximately 7 mm in diameter. Again, after the initial abrupt reduction, the amount of PPO mRNA extracted from the berries tended to decrease, with expression in the more mature berries (>12 mm) being at a consistently low level.

This pattern of PPO gene expression correlates well with previous measurements of PPO activity in developing berries of Sultana and Bruce’s Sport (16). As Sultana berries developed, PPO activity per berry, which provides an indication of PPO synthesis, increased markedly until the berries reached approximately 11 mm in diameter. Thereafter, the PPO activity remained at a constant level. Bruce’s Sport berries, however, reached a peak in PPO activity per berry.

Figure 5. Western blot of proteolysis of PPO in Bruce’s Sport berries. Crude extracts of predominantly white berries (refer to Fig. 2, lane 3) were digested with trypsin and thermolysin for 1 h. Lanes: 1, undigested sample; 2, extract treated with trypsin; 3, extract treated with thermolysin. All lanes contained 5 \( \mu L \) of crude extract. The positions of molecular mass markers (kD) are indicated in the left margin.

Figure 6. Northern blot analysis of total RNA extracted from developing grape berries. The blot was hybridized with a grape PPO clone isolated from Sultana H5. Lanes 1 to 7 show RNA extracted from Sultana berries of 6, 7, 8, 11, 12, 13, and 14 mm diameter, respectively. Lanes 8 to 14 contain RNA extracted from berries of Bruce’s Sport of 6, 7, 8, 10, 12, 13, and 14 mm diameter, respectively. Each lane was loaded with 5 \( \mu g \) of total RNA. The positions of RNA markers (kb) are indicated in the left margin.
when the berries were 7 to 8 mm in diameter, after which the PPO activity remained constant.

**DISCUSSION**

Bruce’s Sport is a variegated mutant of Sultana with an inherently low capacity for browning. This was found to be due to its low PPO activity, which was 20 to 30% of that in Sultana (16). The two grapevines had similar levels of phenolic compounds, substrates of PPO, and no evidence of PPO inhibitors in Bruce’s Sport or activators in Sultana was found. Measurements of PPO activity in Sultana throughout development indicated that PPO activity per berry increased from fruit set until veraison and then remained at a constant level. A similar pattern was observed in Bruce’s Sport, although PPO activity was significantly lower than in Sultana at all stages of development. The green regions of skin in Bruce’s Sport grapes had a similar PPO activity to that of Sultana skin, whereas the white regions were shown to have negligible PPO activity (16).

Purification of Sultana PPO yielded a single, 40-kD protein (S.P. Robinson, unpublished data), and a single, 40-kD protein was detected in extracts of mature Sultana grapes probed with the anti-grape PPO antibody. The additional recognition of small amounts of a 60-kD protein in immature Sultana berries, in which synthesis of PPO appears to be greatest (16), suggested the existence of an immature form of PPO. This 60-kD protein was found in much larger amounts in the white tissue regions of Bruce’s Sport where PPO activity is known to be low. The relatively high levels of the 40-kD, active enzyme in green berries of Bruce’s Sport, with the concomitant small amounts of the 60-kD protein, suggested that the presence of the 40-kD PPO was dependent upon the existence of normal plastids. Equally, the amount of the 60-kD form was proportional to the amount of white tissue, with more occurring in those regions where the plastids lacked normal internal membrane structure.

When subjected to proteases, the undenatured 60-kD protein readily cleaved to a 40-kD peptide. When denatured, however, it remained largely intact after 24 h under the same conditions (data not shown). This suggested that the susceptibility of the 60-kD protein to cleavage was structurally conferred, perhaps being in the form of a proteolytically sensitive protruding loop. The 40-kD protein produced by digestion with trypsin and thermolysin, however, did not have any detectable PPO activity, as might be predicted from the activity of the 40-kD protein in extracts of Sultana (Fig. 1). There may be two reasons for this. First, PPO is a copper-containing enzyme, and it is known whether its immature, inactive form has an incorporated copper atom. Second, trypsin and thermolysin may not cleave at exactly the same site as an in vivo protease involved in processing, and the precise location of cleavage may be required for activation. Further investigation is needed to ensure that activity indeed cannot be induced in vitro.

The pattern of expression of PPO indicated by the northern blot of Sultana and Bruce’s Sport RNA is similar to that predicted from measurements of PPO activity throughout berry development (16). The high levels of PPO early in fruit development, found also in peaches, apples, and pears (5, 12) may indicate a role, at least in part, in protecting the berry against predation. This would support the suggestion that PPO is associated with disease resistance (27). The 2.2-kb transcript of grape PPO mRNA would appear to encode a protein of approximately 70 kD. A full-length cDNA clone has recently been isolated for grape PPO, encoding a mature protein of 57 kD (I.B. Dry and S.P. Robinson, unpublished data). This clone also has 11 kD upstream of the N terminus of the mature PPO protein, which exhibits the characteristics of a chloroplast transit peptide. Upon reaching its target site in the chloroplast, the transit peptide would be removed, leaving an inactive immature protein of close to 60 kD. This inactive protein appears to be subsequently cleaved at the carboxyl terminus to yield the 40-kD active enzyme and a 20-kD inactive peptide. This may, in fact, explain the much sharper cessation of PPO synthesis noted in Bruce’s Sport as being due to a feedback mechanism, the lack of processing of the enzyme preventing the synthesis of any immature protein.

The 20-kD protein eluted from the affinity column was found to be only mildly antigenic on a western blot and hence was likely to have been the result of cleavage of the 60-kD protein to the 40-kD PPO. Strong evidence for the origin of the 20-kD protein as a PPO breakdown product was provided by a western blot of the partially purified extract, which revealed that relatively much larger amounts of the 60-kD protein were present. After elution of the bond protein with highly acidic buffer, the amount of 60-kD protein was decreased, the 40-kD PPO was increased, and the 20-kD peptide became evident. A tenfold activation of grape PPO after a 5-min exposure to pH 5.0 was reported by Lerner et al. (11). This activation was attributed to a conformational change in the enzyme and was reversible for acid shocks of short periods. We did not detect any increase in PPO activity after exposure to the elution buffer of pH 2.8, which may have been due to factors similar to those preventing activation after proteolysis.

The sequencing of the 20-kD peptide resulted in the final evidence that it was, indeed, a breakdown product of the PPO protein, because it was found to have an identical sequence with a region at the carboxyl terminus of the grape PPO clone. C-terminal cleavage of an immature protein is somewhat unusual. However, it has also been found to occur in tyrosinase, a close relative of PPO, in Neurospora crassa, where protyrosinase, with a mol wt of 75,000, was cleaved to produce the 46-kD mature tyrosinase (6). Cloning of the gene encoding this protyrosinase revealed that this cleavage involved the removal of 213 amino acids from the C terminus. By analogy with hemocyanin, the authors suggested that this C-terminal extension was involved in shielding the enzyme’s active site; thus, its cleavage resulted in activation of the enzyme. It is possible that the C-terminal 20-kD extension cleaved from the immature form of grape PPO serves a similar purpose in preventing enzyme activation, although further investigation will be required for confirmation.

PPO is encoded in the nucleus, translated in the cytoplasm, and transported to the chloroplast where it is thought to be located on thylakoid membranes in healthy, green leaves (9, 27). It is known to be inactive until correctly incorporated into the chloroplast (25) but was previously thought to be

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synthesized as a 40- to 45-kD protein without a transit peptide to target it to the plastid (4, 27). A western blot of broad bean, bush bean, lettuce, mung bean, soybean, spinach, and tobacco, probed with an anti-broad bean PPO antibody, revealed bands of 60 to 63 kD and 40 to 45 kD in each species (8). In vitro translation of RNA from each species produced a single, dominant band of 43 to 45 kD, and it was concluded that PPO was synthesized without a transit peptide (4). The reason for the occurrence of this 45-kD, single band is unclear, because more recently the gene encoding broad bean PPO has been isolated, showing conclusively that like the grape enzyme it is synthesized as a protein of >60 kD (18). It is possible that cleavage of the PPO precursor had already occurred in the in vitro translation system. We propose that grape berry PPO is synthesized as a protein of approximately 70 kD with a transit peptide of 10 kD targeting the protein to the chloroplast. This is removed to produce an immature PPO of 60 kD, further cleavage of 20 kD at the carboxyl terminus being required before the enzyme becomes active. In the white regions of Bruce’s Sport, it seems that the specific protease necessary for this cleavage is absent or dysfunctional, allowing the accumulation and subsequent detection of the inactive, immature form of PPO.

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LITERATURE CITED