Partial Characterization of Polyphenoloxidase Extracted from 'Anna' Apple

Augusto Trejo-Gonzalez¹ and Herlinda Soto-Valdez

Research Center in Food and Development, Apartado Postal 1735, Hermosillo, Sonora 83000
Mexico

Additional index words. enzyme thermostability, hydrophobic chromatography, *Malus domestica*

Abstract. Polyphenoloxidase (PPO) was studied in extracts from 'Anna' apples (*Malus domestica* Borkh.) produced under the desert conditions of the state of Sonora, Mexico. A crude extract of PPO was prepared in phosphate buffer from an acetone powder of pulp from ripe apples. Optimum conditions for enzyme activity were pH 5.36 at 35°C. Specificity toward substrates, in order of decreasing activity, was 4-methylcatechol, chlorogenic acid, catechol, caffeic acid, and 3,4-dihydroxyphenylalanine. Activation energy for the reaction with 4-methylcatechol was 6543 J·mol⁻¹ (1563 cal·mol⁻¹). The PPO from 'Anna' was similar to that described from other apple cultivars, except that it was more thermostable between 35 and 60°C. A single peak of PPO activity was achieved by hydrophobic chromatography of the crude extract, resulting in a 300-fold purification. 'Anna' apples contained high levels of phenolic substances, 1.16% on a fresh-weight basis. The high phenolic content and PPO thermostability help explain the browning susceptibility of 'Anna' apples during postharvest handling.

'Anna' apple is among the fruit species under investigation for adaptation to the Sonoran Desert region (Diaz et al., 1986). This cultivar was developed in 1967 in Israel from a cross between 'Red Delicious' and the local 'Hashab' (Sherman et al., 1971). 'Anna' has a low chilling requirement and is one of the few cultivars that are productive with irrigation under hot desert conditions (Miller and Baker, 1982; Rushing and Sherman, 1981). There are ≈1600 ha of 'Anna' in Sonora, probably the largest planted area, of this cultivar in the world (J.J. Martinez, 1988, personal communication). Fruit development requires ≈120 days, with the fruit ripening during a period of extremely high day temperatures (> 40°C). The limited shelf-life of the fruit is related to rapid softening and also to the tendency to bruise and brown easily (Rushing and Sherman, 1982).

Browning in apple pulp is principally initiated by the activity of polyphenoloxidase (EC 1.10.3.2; PPO) on simple phenols, converting them, in the presence of oxygen, to o-quinones. These subsequently polymerize by nonenzymatic reactions to form brown melanin pigments that are partially stabilized by linkage with proteins (Martins-Galeazzi, 1984). PPO has been characterized in many fruits, including apple, but has not been studied in 'Anna' (Satjawatcharaphong et al., 1983; Walker, 1964). Such information is important for the postharvest handling of 'Anna' fruit for fresh market as well as processing.

The objective of this study was to characterize and purify PPO from the pulp tissue of 'Anna' apple.

Materials and Methods

Fruit material. 'Anna' apple fruits were harvested in the coastal region of Hermosillo, Sonora, from a commercial orchard of 3-year-old trees between June and August in 1985 and 1986. From an orchard of 7200 trees, nine fruits each from 85 trees were harvested at random (Dixon and Massey, 1980) from the east side of the trees. The same criterion used for commercial harvest, 40% red coloration, was used to select the apples. From

the freshly harvested fruits, nine subsamples were composed at random, and each was analyzed in triplicate.

Fruit composition and degree of browning. Water content, protein, lipid, crude fiber, and ash content were determined by standard procedures (AOAC, 1984; Procedures 3.003, 22.052, 7.062, 3.123, and 22.027, respectively), and total carbohydrate content was calculated by difference. Phenolic substances were analyzed colorimetrically by the Folin-Ciocalteu method from aliquots of consecutive extractions with 100%, 50%, and 0% methanol in water (by volume) using 10 g tissue and 20 ml solvent (Singleton and Rossi, 1965). Reducing sugars were determined on a water extract of the fruit pulp by the colorimetric Nelson-Somogyi method (Somogyi, 1952).

The degree of browning of the apple tissue was determined from juice prepared from homogenization of 100 g of diced apple in a juice extractor (Moulinex, Bagnolet, France). A 5-ml aliquot of apple juice was added to a test tube containing 5 ml of 95% ethanol and immediately shaken and centrifuged at 800 × g for 3 min. The degree of browning of the supernatant was measured at 440 nm (Coseteng and Lee, 1987).

PPO preparation and characterization. PPO was prepared from apple fruits containing ≥12% soluble solids by an acetone extraction procedure according to Flurkey and Jen (1978), as modified by Sciancalepore and Longone (1984). The acetone powder was stored in a desiccator at -5°C for subsequent work; under these conditions it maintained activity for >1 year. The enzymatic extract was prepared by suspending 2 g of the acetone powder in 100 ml phosphate buffer (0.05 M, pH 7) with 1 M KCl. After agitation at 4°C for 30 min, centrifugation at 34,000 × g for 45 min at 4°C, the supernatant was assayed for enzyme activity.

Specific activity of the enzyme was determined spectrophotometrically according to Sciancalepore and Longone (1984). The reaction mixture consisted of 1.9 ml citrate buffer (0.1 M, pH 5.3), 1.0 ml substrate (0.02 M) in citrate buffer (pH 5.3), and 0.1 ml of the enzyme extract. The reaction was carried out for 3 min at 30°C and the change in absorbance with time was recorded at different wavelengths, depending on the substrate used. The initial velocity of the reaction was calculated from the slope of the linear part of the curve obtained. Protein content

Received for publication 28 Dec. 1990. We appreciate the assistance of Marita Cantwell (Dept. Vegetable Crops, Univ. of California, Davis) in the translation and revision of this paper. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

¹Present address: CIIDIR-IPN, Justo Sierra No. 28, Jiquilpan, Michoacan 59510, Mexico.

Abbreviations: PPO, polyphenoloxidase.

of the enzymatic extract was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Five substrates were tested with PPO extracted from the apple tissue: chlorogenic acid, catechol, 4-methylcatechol, caffeic acid, and 3,4-dihydroxyphenylalanine (DOPA). The substrates were dissolved in 0.1 M citrate buffer pH 5.0 at a 0.01 M concentration.

To determine the optimum pH of the PPO, the method of Satjawatcharaphong et al. (1983) was used, testing the equilibrium activity from pH 5 to 7 with 0.1-pH increments. The thermal stability of the enzyme was tested by the method described by Whitaker (1984), where the enzyme extract was held for 10 min at 15 to 90°C, before assaying activity at 30°C under optimum pH and substrate conditions. The energy of activation was estimated from an Arrhenius plot.

The enzymatic extract was partially purified by hydrophobic chromatography (Flurkey and Jen, 1980; Jen and Flurkey, 1979). A glass column (0.7 × 20 cm) was packed with Phenyl-Sepharose Cl-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) that previously had been vacuum deaerated. The column was equilibrated with a phosphate buffer 0.05 M with 1 M KCl and 1 M (NH₄)₂SO₄ at pH 6.8. The enzymatic extract was applied and eluted with decreasing molar concentrations of the equilibration buffer (0.05, 0.04, 0.03, 0.02, 0.01, and 0.005 M). Finally, the column was washed with 50% ethylene glycol in water. The total volume of each eluent was 20 ml and 2.0-ml fractions were collected. The protein was assayed by absorbance at 280 nm, and the enzymatic activity was assayed as described above.

Results and Discussion

The general composition of the pulp of 'Anna' apple (Table 1) is very similar to that of other apple cultivars. However, the content of phenolic substances was higher than the range of 0.1 to 1 g/100 g of fresh tissue reported for other apple cultivars (Hulme, 1970). This high phenolic content could be important in determining the susceptibility of this cultivar to discoloration (Matheis, 1983).

The degree of browning of juice prepared from freshly harvested ripe 'Anna' apples over time is shown in Fig. 1. The absorbance values attained after 45 min were in the range of those reported by Coseteng and Lee (1987) for other apple cultivars.

The affinity of PPO for various substrates showed that greatest activity was achieved with 4-methylcatechol (Fig. 2). This result is in agreement with the studies of Vamos-Vigyazo and Gajzago (1978) on 'Jonathan' and Stelzig et al. (1972) on 'Cox's Orange Pippin'. Therefore, the 4-methylcatechol substrate was used in all subsequent work.

Table 1. Chemical composition of 'Anna' apple fruits produced in the coastal region near Hermosillo, Sonora, Mexico.

Component	Content, percent fresh wt ²
Water	84.11 ± 1.11
Protein (N × 6.25)	0.32 ± 0.02
Lipids	0.19 ± 0.04
Fiber	0.79 ± 0.06

²Data are based on five samples analyzed in triplicate.

Fig. 1. Change with time in degree of browning of 'Anna' apple juice. Means of eight measurements from four juice samples. SD are indicated.

Fig. 2. Effect of substrate on specific activity of PPO from 'Anna' apple. Means of six measurements from three enzyme preparations. SD are indicated.

Fig. 3. Determination of pH optimum of PPO from 'Anna' apple. Means of six measurements from three enzyme preparations. SD are indicated.

The effect of pH on enzyme activity resulted in the typical bell-shaped curve with an optimum at pH 5.4 (Fig 3). This value is slightly higher than the 5.2 reported for 'Rome Beauty', 'Winesap', and 'Cortland' (Shannon and Pratt, 1967) and the 5.0 reported for 'Pippin' apple (Walker, 1966).

The PPO-specific activities of the nine apple subsamples were highly distinct. These could be grouped into three ranges of activity, which we designated as low, medium, and high (Table

2). Other researchers have also found high variability in apple PPO activity. Harel and Mayer (1968) demonstrated that different forms of PPO exist in the chloroplast and may vary according to the developmental stage of the chloroplast and the stage of maturity of the apple. Although we used ripe apples in this study, the development of red pigmentation is highly variable under desert conditions and could result in these differences. These differences could also be explained by genetic variation in degree of enzymatic browning as has been demonstrated for peach (Hanschke and Boynton, 1986). If this is the case, it may be possible to select specimens with lowered PPO activities from an apple population.

Purification of the crude extract by hydrophobic chromatography showed that the protein eluting at 0.01 M phosphate had the highest PPO activity, resulting in a 307-fold purification (Fig. 4). The elution of PPO activity at this buffer strength coincides with results reported for hydrophobic chromatography of PPO from peach and grape (Jen and Flurkey, 1979; Wisse- mann and Lee, 1980).

The optimum temperature for enzyme activity was 35C (Fig. 5), and the Arrhenius plot demonstrated that the enzyme was very thermostable between 15 and 60C (Fig. 6). It was not until 60 to 80C that the enzyme was inactivated or the protein denatured. Walker (1966) also found a high inactivation temperature for PPO extracted from ‘Sturmer Pippin’ apples. The energy of activation (E_a) calculated from the slope between 15 and 35C was estimated at 6543 J·mol⁻¹ (1563 cal·mol⁻¹). The E_a is low in comparison to that generally required for enzymatic reactions (Whitaker, 1972) in which the E_a usually falls in the range of 25,125-50,250 J·mol⁻¹. Our result is probably related to the small change in PPO activity in the range of 20 to 30C, which contrasts with the large increase in activity of other enzymes over this temperature range (Whitaker, 1972). The activity of

Table 2. Classification of specific activities of PPO from ‘Anna’ apple.

Activity class	Subsamples (no.)	Activity (units)
Low	4	46.8 ± 2.6
Medium	2	61.4 ± 1.5
High	3	96.2 ± 4.4

^aPPO activities were based on triplicate analyses of extracts from acetone powders prepared from a composite of 85 apples per subsample.

Fig. 4. Purification of PPO from ‘Anna’ apple by hydrophobic chromatography. Proteins were eluted with a stepwise gradient from 1.0 to 0.1 × equilibration buffer (EB) (0.0 5-M phosphate buffer with 1 M KCl and 1 M (NH₄)₂SO₄, pH 6.8). The column was washed finally with 50% ethylene glycol (EG 50%) in water.

Fig. 5. Effect of temperature on the activity of PPO from ‘Anna’ apple. Means of three measurements from three enzyme preparations. SD are indicated.

Fig. 6. Arrhenius plot of the thermostability of PPO from ‘Anna’ apple.

the PPO enzyme extract was reduced to an undetectable level at 80C for 10 min.

The PPO activity of ‘Anna’ apple is similar to that of other apple cultivars in relation to substrate specificity and pH optimum, with the major difference being the greater thermostability of the PPO from ‘Anna’. The concentration of endogenous phenolic substrates was very high, reaching 1.2% on a fresh-weight basis. These results help explain this cultivars susceptibility to browning when handled under the growing conditions of Sonora, where ambient day temperatures are often > 40C.

Literature Cited

AOAC. 1984. Official methods of analysis, 14th ed. Assn. Offic. Agr. Chemists, Washington, D.C.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Coseteng, M.Y. and C.Y. Lee. 1987. Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. *J. Food Sci.* 52:985-989.

Diaz, D. H., J.J. Martinez, and W.B. Sherman. 1986. Apple and peach production in warm climates of northwest Mexico. *Fruit Var. J.* 40:121-125.

Dixon, W.J. and F.J. Massey. 1980. *Introducción al análisis estadístico*. 2nd ed. McGraw-Hill, Mexico.

Flurkey, W.H. and J.J. Jen. 1978. Peroxidase and polyphenoloxidase activities in developing peaches. *J. Food Sci.* 43: 1826-1828, 1831.

Flurkey, W.H. and J.J. Jen. 1980. Hydrophobic Adsorption Chromatography of peach polyphenoloxidase. *J. Food Sci.* 45:1622-1624.

- Hansch, P.E. and B. Boynton. 1986. Heritability of enzymatic browning in peaches. *HortScience* 21: 1195-1197.
- Harel, E. and A.M. Mayer. 1968. Interconversion of sub-units of catechol oxidase from apple chloroplasts. *Photochemistry* 7:199-204.
- Hulme, A.C. (ed.) 1970. The biochemistry of fruits and their products. vol. 1. Academic, London. p. 4, 270.
- Jen, J.J. and W.H. Flurkey. 1979. Hydrophobic chromatography of peach fruit polyphenol oxidase. *HortScience* 14:516-518.
- Martins-Galeazzi, M.A. 1984. Comportamento das polifenol-oxidases em alimentos. *Arch. Lat. Nutr.* 34:269-289.
- Matheis, G. 1983. Enzymatic browning of foods. *Z. Lebensm. Unters. Forsch.* 176:454-462.
- Miller, E.P. and L.H. Baker. 1982. An evaluation of apple cultivars for Central and North Florida. *Proc. Fla. State Hort. Soc.* 95:88-90.
- Rushing, J.W. and W.B. Sherman. 1981. Storage and marketing potential of Florida apples and pears. *Proc. Fla. State Hort. Soc.* 94:358-359.
- Satjawatcharaphong, C., K.S. Rymal, W.A. Dozier, Jr., and R.C. Smith. 1983. Polyphenol oxidase system in Red Delicious apples. *J. Food Sci.* 48:1879-1880.
- Sciancalepore, V. and V. Longone. 1984. Polyphenol oxidase activity and browning in green olives. *J. Agr. Food Chem.* 32:320-321.
- Shannon, C.T. and D.E. Pratt. 1967. Apple polyphenol oxidase activity in relation to various phenolic compounds. *J. Food Sci.* 32:479-483.
- Sherman, W. B., R.H. Sharpe, and J.B. Aitken. 1971. Subtropical apples. *Proc. Fla. State Hort. Soc.* 84:9-11.
- Singleton, V.L. and J.H. Rossi, Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *J. Enol. Vitic.* 16:144-158.
- Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* 195:19-23.
- Stelzig, D.A., S. Ashtar, and S. Ribiero. 1972. Catechol oxidase of Red Delicious apple peel. *Photochemistry* 11:535-539.
- Vamos-Vigyazo, L. and I. Gajzago. 1978. Substrate specificity of the enzymatic browning of apple. *Acta Alimentaria* 7:79-90.
- Walker, J.R.L. 1964. Studies on the enzymic browning of apples. *Austral. J. Biol. Sci.* 17:360-371.
- Walker, J.R.L. 1966. Studies on the enzymic browning of apple. III. *Phytochemistry* 5:259-262.
- Whitaker, J.R. 1972. Principles of enzymology for the food sciences. Marcel Dekker, New York.
- Whitaker, J.R. 1984. Analytical uses of enzymes, p. 297-377. In: D.W. Gruenwedel and J.R. Whitaker (eds.). Food analysis, vol. 3. Biological techniques. Marcel Dekker, New York.
- Wissemann, K.W. and C.Y. Lee. 1980. Characterization of polyphenoloxidase from Ravat 51 and Niagara grapes. *J. Food Sci.* 46:506-508, 514.