Characterisation of Apricot Polyphenoloxidase
During Fruit Development

Robert B. Barrett, 2002

Thesis submitted for the degree of Master of Applied Science
(Agriculture)
in
The University of Adelaide

(Faculty of Sciences)

“It seems to me that all sciences are vain and full of error that are not born of experience, mother of all certainty, and are not tested by experience, that is to say, that do not at the origin, middle or end pass through any of the five senses.”
Leonardo da Vinci 1452-1519
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Abstract

This study was aimed at determining the expression and activity of polyphenoloxidase (PPO) during apricot fruit development together with the biochemical characteristics of the enzyme extract at different development stages. Biochemical factors considered include substrate, pH, NaCl level, inhibitor type, high temperature inactivation and sulphur dioxide level.

Changes in apricot (Prunus armeniaca L., cv. 'Moorpark') polyphenoloxidase (PPO) were measured during fruit development from a few days after full bloom until overripe at 92 days after full bloom. Cold ground samples in McIlvaine's buffer were analysed for PPO activity over a range of pH (5.0, 6.0, 6.8 and 7.2); for response to intact fruit sample pre-heating (25, 35, 45, 55 and 65 °C); for sulphite and NaCl inhibition (0.2, 0.5, 2 and 5mM) and other inhibitors (SHAM 0.2mM, cinnamic acid 2.5mM and tropolone 0.5mM). PPO activity was measured at 25°C using a Clark-type oxygen electrode with 4-methyl catechol (20mM) as substrate.

As fruit ripened PPO activity increased under all conditions tested. The increase in activity was not even with fruit development. Three common peaks of PPO activity occurred at ages 22-29 days, 57 days and for fully-ripe fruit at 85-92 days.

Optimum pH was found to be 6.8 with a wide range for all ages of fruit. PPO activity tended to be higher for more mature fruit at a higher pH of 7.2 to 8.0, whereas activity tended to be higher in less developed fruit at the lower pH of 6.0.
Catechol and chlorogenic acid showed reduced PPO activity compared with 4-methyl catechol over all development ages, however, there was a different pattern of response. Both catechol and chlorogenic acid showed greater PPO activity in the fully mature, day 92 fruit and less in the very young day 8 fruit, relative to the control 4-methyl catechol substrate. L-DOPA, as a substrate, showed a reaction lag as previously reported, and quite depressed PPO activity with no particular variation with development age compared to the control.

Pre-heating of fruit samples in air for 30 minutes resulted in increased inactivation with holding temperature (35°C - 31%, 45°C - 82%, 55°C - 97%, 65°C - 99%). Sulphite and NaCl acted as inhibitors with increasing effect as concentration increased. Added sulphite depressed PPO activity by about 30% at the level (2mM) used. This was less than the literature would suggest and it appeared that fully-ripe fruit were less inhibited than mature, non-ripe fruit. NaCl has a greater inhibitory effect on apricot PPO activity at the lower pH 5.0 tested. As NaCl added increases PPO activity decreases after an initial small rise. Again, less sensitivity to NaCl inhibition is shown by fruit of greater development age. Sensitivity to inhibition by SHAM, cinnamic acid and tropolone decreased with development age. Tropolone was the most effective inhibitor of apricot PPO.

The pattern of change in PPO activity, was consistent with physiological and biochemical changes reported by other workers as fruit develop from hard, green to soft, ripe. Regarding the existence of different PPO isozymes during development, no evidence of a isozyme based differential response with age was found within the constraints of the parameters tested.
Declaration

This thesis contains no material which has been accepted for an award of any degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give my consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

........................................    ...........................

R. B. Barrett, December, 2002

I, Andreas Klieber, consider that this thesis is, prima facia, worth examining for the award of Master of Applied Science (Agriculture).

Dr. A. Klieber ..................................        Dated .........................
Acknowledgements

My sincere appreciation goes to my supervisors, the late Dr Don Aspinall, Dr Simon Robinson and Dr Andreas Klieber for their excellent advice, patience and encouragement throughout this project and during the preparation of this thesis. In addition to my supervisors I would like to acknowledge the support of Dr John Jackson during the earlier stages prior to his retirement. Members of the Botany Department, particularly Professor Wiskich, Richard Norrish for electronic advice, Dr Ann Rathjen and Lidia Mischis for their valuable assistance and advice on the setting up of the Clark oxygen electrode. Biometrics SA staff member Helena Oakey was very helpful with statistical analysis and advice.

I wish to acknowledge gratefully the support of the University of Adelaide in providing a scholarship, which has enabled me to pursue the studies reported herein. The support of my colleagues within the Department of Horticulture, Viticulture and Oenology, particularly Professor Margaret Sedgley, has been appreciated for their continued encouragement.

Finally, I wish to express my appreciation to my wife, Angela, and to Samuel for grinding fruit, Daniel for computing and Michael for critical review. Thank you for your patience and encouragement throughout my studies.
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<th>Description</th>
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<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>A&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Available water</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>°Brix</td>
<td>degrees Brix (a refractive index measure of g solute/100 g solution)</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CA</td>
<td>controlled atmosphere</td>
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<tr>
<td>ca.</td>
<td>circa (around a given date)</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA to an RNA</td>
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<tr>
<td>conc.</td>
<td>concentration</td>
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<tr>
<td>Cu</td>
<td>copper</td>
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<tr>
<td>cv</td>
<td>cultivar</td>
</tr>
<tr>
<td>C0&lt;sub&gt;2&lt;/sub&gt;</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
</tr>
<tr>
<td><em>et al.</em></td>
<td>Et alii (and others)</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GC</td>
<td>gas chromatograph</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>hr.</td>
<td>hour</td>
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<tr>
<td>k</td>
<td>first-order reaction constant for enzyme activity</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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kg    kilogram
kPa   kilo Pascal
$K_m$ equilibrium constant for enzyme activity
L     litre
L.    Linnaeus
$\mu$g microgram
mg    milligram
min.  minute
$\mu$l microlitre
$\mu$m micromole
ml    millilitre
M     molar
mM    Millimolar
mRNA messenger RNA
M wt. molecular weight
N$_2$ nitrogen
NaCl sodium chloride
nm    nanometre
NMR nuclear magnetic resonance
O$_2$ oxygen
OD    optical density
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
pers. comm. personal communication
<table>
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<tr>
<td>pH</td>
<td>negative log of hydrogen ion concentration</td>
</tr>
<tr>
<td>Pi</td>
<td>isoelectric point of enzyme or protein</td>
</tr>
<tr>
<td>PPO</td>
<td>polyphenoloxidase</td>
</tr>
<tr>
<td>ppm</td>
<td>part per million</td>
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<tr>
<td>PVP</td>
<td>polyvinylpyrolidone</td>
</tr>
<tr>
<td>RH</td>
<td>relative humidity</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
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<td>SHAM</td>
<td>salicylhydroxamic acid</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>spp.</td>
<td>a number of species</td>
</tr>
<tr>
<td>syn</td>
<td>synonym</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>TA</td>
<td>Titratable acidity</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tween</td>
<td>polyoxyethylene-sorbitan monolaurate detergent</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet (UV-vis for ultraviolet and visible wavelengths)</td>
</tr>
<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum velocity of enzyme-catalysed reaction (substrate saturated)</td>
</tr>
<tr>
<td>V/V</td>
<td>volume/volume</td>
</tr>
<tr>
<td>WWW</td>
<td>world wide web</td>
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Figure 2.10: Some potential inhibitors of PPO. Macheix et al., 1990.

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Figure 7.2: Averaged PPO activity of apricot (cv. Moorpark) in the presence of sulphite by development ages. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Error bars are standard error of means (n=10).

Figure 7.3: Effect of different concentrations of sulphite addition as sodium metabisulphite on apricot (cv. Moorpark) PPO activity. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Means of 10 fruit sampled by 12 developmental ages. Error bars are standard error of means (n=120).

Figure 7.4: PPO activity of apricot (cv. Moorpark) in the presence of sulphite by development ages. Effect of sulphite level for 12 development ages showing trend for ages for the two highest SO2 levels applied. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Error bars are standard error of means (n=10). Note that the Y axis scale is expanded here compared with figure 7.2.

Figure 7.4: Distribution of species of $\text{H}_2\text{SO}_3$, $\text{HSO}_3^-$, $\text{SO}_3^{2-}$ as a function of pH showing molecular SO2 exists in very acid conditions with a change to sulphite at approximately pH 6.5. (Bollion et al., 1996)

Figure 8.1: PPO activity of apricot (cv. Moorpark) assayed for a range of sodium chloride concentrations at pH 6.8 for five replicates at each concentration using a bulk sample of ripe fruit (age 85 days). Note the lower range used here is 0.01 and 0.05 mM whereas the main experiment used 0.2 and 0.5 mM. Error bars represent standard error of means (n=5).

Figure 8.2: Effect of different concentrations of NaCl on PPO activity of apricot (cv. Moorpark). Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Means of 10 fruit sampled by 12 developmental ages. Error bars are standard error of means (n=120).

Figure 8.3: Effect of two concentrations of NaCl (33 and 66 mM) at a range of lower pH levels on apricot (cv. Moorpark) PPO activity for firm-ripe fruit. Activity was assayed with 2 mM 4-methyl catechol and pH buffered apricot suspension containing PPO enzyme at pH indicated. Means of 3 fruit at 71 days age. Error bars are standard error of means (n=3).

Figure 8.4: Effect of 4 different concentrations of NaCl by 4 ages of fruit on apricot (cv. Moorpark) PPO activity. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme and various NaCl levels. Early development indicated as 'day1', 'day8' and later development as 'day85' and 'day92'. Log means of 10 fruit sampled by 4 developmental ages. Error bars are standard error of means (n=10).

Figure 9.1: PPO activity for whole apricot (cv. Moorpark) samples incubated at the heating temperature indicated prior to testing. Samples were held submerged in water baths at the indicated temperatures in Eppendorf tubes. Tissue was subjected to these conditions whilst intact with a small amount of air surrounding the sample in the tube. No buffer or liquid was added to the tube prior to heating for 30 minutes. Samples removed from the water bath were immediately cooled with running water at 10°C. The samples were then ground with liquid nitrogen and prepared for testing at 25°C with 4-methyl catechol (20 mM) as substrate as per the standard method. Bars are standard error of means (n=120).

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Figure 9.3: PPO activity for apricot (cv. Moorpark) fruit samples incubated at the heating temperature of 65°C prior to testing. Tissue was subjected to 65°C whilst intact with a small amount of air surrounding the sample in the tube. No buffer or liquid was added to the tube prior to heating for 30 minutes. Samples removed from the water bath were immediately cooled with liquid nitrogen and prepared for testing at 25°C with 4-methyl catechol (20mM) as substrate. Bars represent means for 10 fruit samples from the specified age of fruit. Note the figure has the Y-axis scale expanded compared with Figure 9.2.

Figure 10.1: Effect of development age on response of apricot (cv. Moorpark) PPO to a fixed level of inhibitor - SHAM (Salicylhydroxamic acid), 0.2mM. Control is pH 6.8 buffered apricot suspension using 20mM 4-methyl catechol as substrate in a Clark oxygen electrode. Error bars are standard error of means (n=10).

Figure 10.2: Effect of development age on response of apricot (cv. Moorpark) PPO to a fixed level of inhibitor - Cinnamic acid, 2.5mM. Control is pH 6.8 buffered apricot suspension using 20mM 4-methyl catechol as substrate in a Clark oxygen electrode. Error bars are standard error of means (n=10).

Figure 10.3: Effect of development age on response of apricot (cv. Moorpark) PPO to a fixed level of inhibitor - Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), 0.5mM. Control is pH 6.8 buffered apricot suspension using 20mM 4-methyl catechol as substrate in a Clark oxygen electrode. Error bars are standard error of means (n=10).
1.1 The apricot industry

In 2000, the dried apricot industry in Australia produced $17.6 million worth of product from about 1,600 t of dried fruit, mainly in the Riverland with small amounts grown in the Barossa Valley and Mypolonga (Witherspoon, 1998).

The quality of the dried apricots received in bulk bins at processing plants during the season is variable. One currently undetectable variation in quality is the likelihood of a 500kg bulk bin's contents discolouring (browning) during storage prior to processing/packing. This quality loss may cost industry tens of thousands of dollars per season. This investigation was prompted by the need to examine the characteristics of the enzyme, polyphenoloxidase, considered the main cause of the browning (Haslam, 1998).

1.2 Apricot development

Our knowledge of apricot fruit development is limited. Brady (1993) outlined biochemical and molecular methods which could be used to study development of fruits whilst others, notably Ahmed & Labovitch (1980) and Haslam (1998a), described the changes in phenolic compounds in fruit with ripening with emphasis on loss of astringency with ripening. For apricots, Jackson & Coombe in 1966 described the morphological development, including ripening, of *Prunus armeniaca*
L. cv. 'Moorpark' under South Australian conditions. Gathercole (1988) described apricot cultivar characteristics, including cv. 'Moorpark', for the Australian situation. Later, Lichou et al. (1995) undertook major studies of developmental transformation in the French cultivar 'Rouge de Roussillon' confirming the morphological development pattern described by Coombe (1976) earlier.

### 1.3 Post-harvest drying of cut fruit

Commercially apricots are preserved by treating the freshly cut halves of ripe, de-stoned fruit with sulphur dioxide ($\text{SO}_2$) gas to 'fix' the colour of the fruit to prevent browning during the drying process. Drying of fruit may be categorised into natural (sun) and machine drying (dehydration) with type of drying used affecting browning (Cecil et al., 1991). Other causal factors may be cultural such as nitrogen fertiliser levels (Gathercole, 1988; Dahlenberg, 1994). Both cultivar variation and cultural effects on browning have been observed (Vamos-Vigyazo et al., 1984).

Drying is normally achieved in Australia by sun drying on wooden trays for up to ten days. Fruit are then stored in wooden bulk bins under ambient conditions that prevail within a storage facility. During this time some batches of dried apricots discolour by browning reactions. Higher temperatures generally hasten discoloration (Figure 1.1).
Figure 1.1: Development of darkening in dried, sulphured apricots during storage at two temperatures, 25°C and 35°C. Fruit with an optical density reading beyond 0.3 is considered unacceptably dark and thus unmarketable. *Dahlenburg et al., 1990e.*

### 1.4 Enzymic browning

Enzymic action is the most important factor in browning of both fresh and dried apricots. Enzymic browning results from oxidation, in the presence of oxygen, of phenolic compounds by polyphenoloxidase (PPO)(Haslam, 1998). Phenolic compounds are oxidised to quinones. These quinones may polymerise into coloured, usually brown, products (Nicolas *et al.*, 1994; Amiot *et al.*, 1997). Rate of enzymic browning depends on whether the responsible enzyme is present, active and physically available. For example, activity might be constrained by low temperature.
Enzyme action, particularly polyphenoloxidase (PPO, E.C. 1-13-2), may be modified by ascorbic acid content (Mayer & Harel, 1979), sulphite content and finally, fruit structure breakdown associated with degree of ripening (Jackson, 1965). Enzymes other than PPO such as laccase, peroxidase and b-galacturonase are also reported to be involved in browning in a minor way (Mayer & Harel, 1979; Jones, 1994).

The oxidised phenolics and proteins produced when fruit tissue such as peach or cocoa is wounded reduces the digestibility and palatability of that tissue (Maness et al., 1992; Amorim & Melo, 1994). For humans it also makes the product less marketable due to the dicolouration in fresh or dried fruit. It has been suggested that high levels of PPO activity, together with high levels of phenolic substrate early in fruit development may act as a defence mechanism by protecting seeds from infection or predation prior to maturity (Mayer & Harel, 1979; Knee et al., 1994). Birds and insects usually cause this predation wounding. The high PPO activity measured in soft meristematic tissues of apricot fruit may serve a similar role in defence against predation. As a fruit it remains vulnerable to predation wounding and therefore browning.

Developmental expression of PPO has been studied in a number of fruits, particularly in peach (Cary et al., 1992; Shahar et al., 1992; Hunt et al., 1993; Steffens et al., 1993). Most studies were initiated with a view to commercially reducing browning in the fruit or vegetable.
Chapter 1. General Introduction

1.5 Apricot polyphenoloxidase

In various fruits, sulphur dioxide is used to reduce browning during drying as an aid to preservation (McBean, 1966). Considerable difficulty is encountered by those researchers who attempt to determine the chemical and enzymatic pathways associated with browning in sulphured, drying fruit due to the complex nature of sulphur chemistry involved (Vamos-Vigazo et al., 1983; Spanos & Wrolstad, 1992). The major thrust for browning reduction has been to examine the conditions under which sulphur is applied and also the condition of the fruit at the time of application (McBean, 1950a, 1966, 1967b, 1985; Dahlenberg, 1976; Rettke, 1993). Factors such as ripeness, sugar content, pH, flesh characteristics and sulphur uptake have been examined for their relation to browning. Dahlenberg et al. (1990e) has reported that while there is a correlation between fruit nitrogen levels and browning \( r^2 = 0.67 \) there are low correlations (mean \( r^2 = 0.15 \)) for browning with sulphur dioxide level, \(^\circ\)Brix, pH and titratable acidity. High level of SO\(_2\) addition (1500 to 3000ppm) is still the main procedure used commercially for browning reduction.

In apricot, Dijkstra & Walker (1991) reported that there are two types of PPO present, a laccase and a catechol oxidase. Kumar (1992) found from isozyme studies of apricot fruits that there are a number of isozymes of PPO which are expressed at different times during fruit development. These finding were supported by Byrne (1989) who found evidence suggesting up to four isozymes of PPO.
1.6 General objectives of this research

Characterisation of the PPO enzymes in apricot is important from a processing viewpoint, where manipulation of the harvest and drying conditions could reduce the activity of the enzyme or its substrate levels and so inhibit browning in dried fruit or enable use of lower sulphur dioxide levels.

This study is aimed at determining the expression and activity of PPO during apricot fruit development together with the biochemical characteristics of the extracted crude enzyme at different development stages. The effects of biochemical factors to be considered include pH, salinity, sulphur dioxide level and other inhibitors such as salicylic acid derivatives, tropolone and aromatic organic acids.
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2.1 Development and ripening in Prunus and apricot

Stone fruit are a diverse group, mostly of the genus Prunus, with a characteristic lignified endocarp, a fleshy mesocarp and a thin exocarp or skin. It was noted in the review of stone fruit by Romani and Jennings (1971) that, despite their commercial importance and their culture in many countries, there were relatively few detailed studies of their biochemistry and physiology.

The apricot species that are well known commercially have their origins in Asia or southern Europe. Mume fruit, Prunus mume Sieb. and Zucc. is a native of Japan, while the apricot, Prunus armeniaca Linn. is native to Armenia, Arabia and the higher regions of Central Asia (Guerriero & Watkins, 1984). There are references to wild types near Kabul, in Siberia, and in northern China (Audergon et al., 1991). There are over 400 cultivars or hybrids reported (Audergon et al., 1991; Guerriero & Watkins, 1984) many with very specific climatic niches (Gathercole, 1988) which are often associated with a high degree of inbreeding as shown by isozyme studies (Byrne, 1989; Battistini & Sansavini, 1991).

2.1.1 Fruit development

Stone fruit have a distinct pattern of development that was well described by Tukey (1936). Three stages of development are recognised, describing growth in terms of a
double sigmoid pattern (Jackson, 1965). In stage I, cell division is rapid and accompanied towards the end of the period by cell expansion with a rapid increase in pericarp volume; stage II is a period of relative quiescence in the pericarp and rapid development of the embryo; in stage III, the endocarp completes its development and the pericarp resumes a rapid increase in volume which is predominantly, due to cell expansion (Fig. 2.1 and Fig. 2.2). In early maturing varieties, including those varieties with a low requirement for winter chill, stage II is compressed and endocarp closure may not be complete when the pericarp is mature (Lilleland, 1930 and 1935).

Figure 2.1  Development stages in *Prunus* spp. showing derivation of the full drupe from the embryo at 35 days to full maturity at 130 days. *Jackson, 1974.*

Jackson (1965) and Jackson & Coombe (1966 a,b) reported on the development of apricot fruit in South Australia following from the work of Sterling (1935). They
confirmed that growth of the mesocarp was a product of cell expansion from anthesis to 15 days after anthesis. This is similar in pattern to other stone fruit. They reported differences in cell shape between those close to the endocarp (radial expansion) versus those at the periphery of the mesocarp (tangential expansion). They found the cell volume to be similar. This is significant when one considers that PPO is thought to be membrane bound (Haslam, 1989). This infers that equal amounts of PPO enzyme would be present from an equal volume sample from close to the endocarp compared with a sample near the skin of the fruit.

Figure 2.2 Growth stages for the developing apricot fruit. Note double sigmoid growth curve associated with pit-hardening (sclerotisation of the endocarp). Adapted from Jackson, 1965. A Study of Growth in Apricot Fruit.
Ripening is used to describe the effects of the biochemical processes which make the fruit attractive to the seed dispersal mechanism (Souty, 1991). Fruit quality varies with colour, aroma and texture differences in fruit due to the nature of cropping conditions and the fact that fruit is perishable. Maturity and ripeness are reported to be important predictors of browning in fruit that are dried (McBean, 1950 and 1985; Dahlenberg, 1976). Unripe fruit is more likely to show browning when dried (McBean, 1985).

Stutte (1989) studied quantification of net enzymatic activity in developing peach fruit using computer video image analysis. Changes in oxidative browning of developing cv. 'Redskin' fruits were quantified. Oxidative browning of endocarp tissue occurred rapidly at the onset of Stage I (described below) and decreased in rate and intensity during fruit development, with little or no browning occurring by the time endocarp sclerification began at the onset of Stage II. Conversely, little or no browning occurred in mesocarp tissue during early development, but browning increased in rate and intensity through endocarp sclerification. Net oxidative browning was correlated with net polyphenoloxidase and peroxidase (EC 1.11.1.7) enzyme activity of the tissues as quantified by image analysis after staining. Image analysis revealed localised areas of activity within the tissue as is also reported for apple.

2.1.2 Ripening events in *Prunus*
Chapter 2. Literature Review

A study of the character of PPO during ripening requires a definition of maturation and ripening. Commercial maturity is used to describe the state of a fruit when it may be harvested and then is sufficiently advanced to allow ripening (Wills, 1987).

Physiological maturation may be defined here as the period between flower carpel fertilisation through to a point where fruit will ripen if harvested. Ripening is therefore the post-maturation period through into senescence. In some fruit such as grape (Coombe, 1997) and apple (Boss, 1996) maturation-ripening may be divided into pre- and post-veraison periods.

The nutrient composition of commercial stone fruit is well described in food composition tables. As in other Rosaceae, stone fruit translocate carbon as sorbitol as well as sucrose (Marino et al., 1993), and the fruit contain significant levels of sorbitol. Young fruit store some carbon as starch, but this is used before the fruit enter stage III and starch to sugar conversion is not involved in ripening. Ripe fruit contain significant amounts of free galacturonic acid, presumably a product of pectin breakdown (Ash & Reynolds, 1954). The fruit are decidedly acid, with acid levels in excess of 1 percent as malate and a sap pH sometimes below pH 3.5. Reid and Bielski (1974) in New Zealand found that malate level decreases, and the citrate concentration increases through ripening, but the overall acid level declines and the pH rises slightly. Ripening is associated with softening due to polygalacturonase activity (Ahmed & Labavitch, 1980).
Alcohol-insoluble solids in the mature fruit constitute 2.2% to 2.5% of the fresh weight, and this is mostly cell wall material. In ripe fruit, 24% to 29% of the alcohol-insoluble substance was found to be pectin in four peach cultivars (Shewfelt et al., 1993). This percentage did not change consistently through ripening, although the chelate-soluble proportion may increase 2.5-fold.

The content of phenolic compounds is greatest through stage I and then declines (Kumar, 1987; Bassi et al., 1996). Cultivars of low quality had distinctly higher phenolic content. The prominent phenolic compounds were reported as chlorogenic acid, epicatechin, catechin, cyanidin and caffeic acid derivatives (Bassi et al., 1996).

A low protein content, the presence of phenolic compounds and a very high content of soluble pectic compounds present difficulties for those who would study apricot fruit enzymes, and there have been relatively few detailed studies reported. The glycolytic, gluconeogenic and mitochondrial enzymes which could shed light on ripening breakdown mechanisms have not attracted detailed study either.

The breakdown of cell walls and pectin substances during ripening or pathogen attack in fruit has been studied in depth by Fishman et al. (1992, 1993a,b) reporting the release of calcium ions during the process. Dawson et al. (1992); Bouranis & Niavis (1992); Von Mollendorf et al. (1993), report similar work for nectarines and other stone fruit. Haslam (1998b), noted that pectin acts as a sequester of phenols so that PPO cannot interact with it. This applies mainly to ripening of persimmons, but
reference is included for Prunus spp. He suggested either encapsulation or formation of polyelectrolytic protein-polyphenol complexes.

During normal ripening, polygalacturonase activity increases as the fruit soften (Ahmed & Labavitch, 1980; Alonso et al., 1996). As the enzyme accumulates, the solubility of the pectins increases (Knee et al., 1994) suggesting, but not establishing, a causative connection. While Cutillasituralde et al., (1993) on the other hand, suggested that browning only occurs in cells of ripe fruit due to pectin solubilisation, and is unrelated to polygalacturonase activity.

When bisulphite ions are added to peroxidase they complex with the iron in the molecule and form a stable complex (Embs & Markakis, 1965). Peroxidase thus complicates the observation of PPO action especially if sulphite has been added since only the PPO reactivates as the SO$_2$ is lost.

### 2.1.3 Tannins and loss of astringency

Loss of astringency is one of the major changes which takes place during fruit ripening of many edible fruits (Gustavson, 1956). It is generally agreed (Porter, 1988; Haslam, 1989) that this property is due to the presence of tannins. Although some astringent fruits such as peaches show a reduction in tannins during ripening (Reeve, 1959), others such as some persimmon do not (Haslam, 1994). Prunus species appear to belong to the majority of fruits where it is found that tannins,
particularly astringent types, decrease as the fruit nears maturity (Reeve, 1959; Yonimori et al., 1994; Arakawa & Ogawa, 1994).

Chang & Hwang (1990) proposed that the stability of banana PPO was likely to be affected by the change in water-soluble tannin, during different stages of development, but they were unable to quantify the effect due to equipment limits. There are no similar reports for apricot.

2.2 The substrates of polyphenoloxidase

PPO isozymes appear to have a range of substrates. This section aims to establish the range and relative PPO activities that have been reported in apricot, Prunus spp. and related fruits. Phenolic compounds are examined in this section and are the principal substrates for PPO, with anthocyanins as minor substrates (Haslam, 1989).

2.2.1 Molecular bonding

In plants a large number of o-diphenolic compounds are present, which are more or less oxidisable by PPO as seen in Figure 2.3. Naturally occurring substrates include 3,4-dihydroxyphenylalanine, the chlorogenic acids, adrenaline, phenylalanine, caffeic and gallic acids, and flavonoids (Mayer & Harel, 1979). Other substrates for this enzyme that do not occur naturally have been found, sometimes with apparently higher affinities. An example is 4-methyl catechol (Brown & Walker, 1990).
Figure 2.3 Some phenolic compounds found in fruits. *Hagerman, 1992*

Considerable speculation exists regarding the expression and activation of the various enzymes during phenol biosynthesis (Lee & Kang, 1988; Ju *et al.*, 1992;
Miles & Oertli, 1995). It would appear from the work of Miles & Oertli (1995) and others that PPO is activated in disease defence by stress or wounding, possibly in association with changes in ethylene production.

Phenolic compounds have a specific reactivity due to the interaction of the hydroxyl groups and the aromatic ring not found in either the aromatic or hydroxy group alone (Allen, 1997). This is due to the freedom of two electrons from the oxygen atom being able to partially shift. This increases electron density of the ring at the expense of the oxygen atom. The phenolic molecule becomes more reactive due to this electron shift. In particular, the withdrawal of electron charge from the oxygen tends to make the -OH group more likely to form a hydronium ion, thus increasing the acidity of the molecule. The electron shift also leads to more hydrogen bonding possibilities. This hydrogen bonding is what gives the phenolic its ability to bind with proteins via the weak hydrogen bonding force. Such bonding often results in precipitation of the combined molecules (Allen, 1997).
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Figure 2.4: Phenol structure of the two common dihydroxy phenolic structures associated with phenolic oxidases in plants. On the left a basic phenolic structure consisting of an aromatic ring with a single hydroxyl group. A second hydroxyl may occupy either an *ortho* or *para* position as indicated. On the right are illustrated a 1,3 di-hydroxy phenolic and the 1,2 dihydroxyphenolic at the ortho position. The 1,2 substitution is more reactive. *Allen, 1997.*

When a phenolic ring (Figure 2.4) has two adjacent hydroxyl groups they may oxidise to a quinone. Figure 2.5 and 2.6 show such a dihydroxyphenyl system oxidised to the corresponding quinone. Once formed the quinones may also react with electron rich compounds (negative charge). Often these are other phenols.

Figure 2.5: A dihydroxyphenol (a) molecule may be oxidised to the corresponding quinone (b). The quinones so formed are very reactive. The end result is often the formation of coloured products (Figure 2.6). *Allen, 1997.*
Figure 2.6: The oxidation of a mono- and di-hydroxy phenol compound (partly shown) to the equivalent 1,2-quinone. *Lee and Whitaker, 1995.*

An example is the oxidation of (+)-catechin to the 1,2-quinone. The quinone formed is now electron deficient (positive charge) and may react with another (+)-catechin to form a dimer. Because of the nature of the phenolic ring structure, a regeneration of the original reactive sites may occur to a hydroxyphenol. This allows more oxidation of the dimer (as if it were the original (+)-catechin monomer). The formation of a quinone dimer follows allowing the cycle to begin again. One of the reaction rate limiting factors is the availability of oxygen in the solution and suitable catalysts, usually an enzyme to initiate the first oxidative step.
2.2.2 Two types of phenolics

The two main types of phenolics are the hydroxycinnamic acids and the flavonoids. Hydroxycinnamic acids consist of the basic phenol group with one hydroxyl on a single aromatic ring to which are added three carbon atoms. The flavonoids are derived from the hydroxycinnamic acids in that a further six carbon atoms are added to the three carbon chain (Figure 2.7). This is cyclised to form effectively a three ring phenolic structure. The combination of three rings allows a larger more dispersed positive charge for flavonoids which may in turn allow them to absorb light energy lower than the ultra-violet normally associated with the phenol ring. Thus some flavonoids, particularly if positively charged, are coloured. An oxidised mixture of hydroxycinnamic acids and flavonoids may appear brown in colour due to the mix of flavonoids plus the oligomers associated with quinone condensation of phenolics.

The hydroxycinnamic acids besides having an aromatic ring also have a C=C double bond plus a carboxylic acid at the end. More commonly the aromatic ring has two hydroxyl groups as a 1,2-dihydroxyphenol system. As with the catechin mentioned above the dihydroxyphenol may oxidise to a quinone leading to similar polymerisation. Additionally, the carboxylic acid may be linked to another hydroxyl group from another molecule. This may be another acid such as tartaric acid or quinic acid. In nature, fruits contain a limited range of these hydroxycinnamic based phenols or derivatives as shown in Figure 2.3 above.
Figure 2.7: Compounds thought to be the major substrates for PPO in fruits. DOPA is an example of an ortho-dihydroxyphenol. The most common Prunus PPO substrate, caffeoylquinic acid (chlorogenic acid) is shown as a hydroxylated aromatic ring with a carbon framework (originally caffeic acid) ending in a carboxylic acid, which has been replaced with a quinic acid moiety. *Macheix et al., 1990.*

2.2.3 Tannins and PPO activity

Tannins are flavonoid polymers. Haslam (1981 and 1994) stated that the common feature of tannins was an accumulation of unconjugated phenolic groups in a moderate sized oligomer. In some fruit, loss of astringency has been attributed to insolubilisation of tannins due to acetaldehyde-induced polymerisation of tannins with flavan-3-ols, leading to coloured complexes (*Fulcrand et al.*, 1996).
These complexes may have a role in PPO activity via inhibition of either substrate availability or enzyme inhibition (Matsuo & Itoo, 1982). Chlorogenic acid, a major PPO substrate in *Prunus* spp., is sometimes included in the definition of “tannin” (Joslyn & Goldstein, 1964; Rouseff, 1990; Okuda et al., 1993); thus loss of astringency (as chlorogenic acid) becomes of interest with respect to PPO activity.

### 2.2.4 Phenolic levels in fruits

Levels of phenolic substrates reported by Cilliers et al. (1990) were related to total chlorogenic acid levels measured by Folin-Ciocalteu colorimetry and to caffeic acid by reverse phase HPLC (280nm detection). Concentrations of total phenols between 8 and 1012 mg/L were found in various apple cultivar juices. The total chlorogenic acid proportion, as measured by HPLC, was only 6 to 11% of total polyphenols found by Folin-Ciocalteu assay. Prabha & Patwardhan (1985) found that total phenolic level does not provide a clear indication of PPO substrate availability and suggest levels of fruit specific phenolics could be a more useful measure. Compartmentalisation and cell wall/membrane integrity may also limit availability as mentioned on page 12.

Villanua-Marti et al. (1992) in Spain measured polyphenoloxidase in six cultivars of apricot using a HPLC and found the dried apricots contained about 0.5% fresh weight as polyphenols, mainly 3,4 dihydroxybenzoic acid, vanillic and chlorogenic acid. The most abundant flavonol was quercetin and its glycosides -
rutin and hyperoside. Postyrik & Peterkova (1993) had reported a comparison of polyphenol levels (as pyrocatechol) in the flesh of European versus Chinese apricots. Of 31 apricot cultivars examined levels ranged from 0.24 to 1.84 mg\textsuperscript{-1}g fresh weight, with no consistent difference between European and Chinese cultivars in relation to polyphenol content or o-diphenol-oxidase activity.

Distinct composition differences between samples are found on the basis of the relative proportion of phenolic acids, flavonols and their glycosides in apricots (Henning & Herrman 1980; Fernandez de Simon et al., 1992); with the latter authors reporting chlorogenic acid (5'-caffeoylquinic acid) as the main phenolic in apricot puree (0.65-3.09 mg L\textsuperscript{-1}) with a 3'-caffeoylquinic acid next most common (0.36-1.06 mg L\textsuperscript{-1}). Previously, both El Sayed and Luh (1965) and Risch and Herrman (1988) reporting on the content of hydroxycinnamic acid derivatives in stone fruits, including apricots, confirmed caffeoylquinic acid (3-isomers) and chlorogenic acid were the most common phenolics.

In other Prunus spp., Risch & Herrmann (1988) found caffeoylquinic acid predominated in peach. Wang et al. (1993) examined a number of peach cultivars during the ripening process for malic, quinic and citric acid content, finding most had an increase in malic acid but not in citric or quinic acids during ripening. It is not clear whether the extraction method they used would hydrolyse chlorogenic acid resulting in increased quinic acid, thereby altering the apparent free quinic acid levels.
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The evidence is that the major phenolic substances are cinnamic acid derivatives such as chlorogenic acid and epicatechin, while the major flavonols are quercetin based. However, it appears that the enzyme conformation has not been elucidated to date, so that the substrate limitations cannot be defined. There are clearly a large number of substrates for PPO with differing apparent equilibrium constants ($K_m$). In a number of cases the PPO activity appears to be substrate rather than enzyme limited with higher activity when higher levels of phenols, or specific phenolic ratios, are present (Robertson et al., 1988; Zhou & Feng, 1991). This may be due to the physical situation of the enzyme in terms of its attachment to membranes (Dijkstra & Walker, 1991).
2.3 Polyphenoloxidase in *Prunus* and apricot

Generally all fruits lose quality following harvest at a rate that is dependent on varietal characteristics, maturity at harvest, and conditions during harvest, packing and storage (Wills *et al.*, 1987). The principal reactions leading to quality loss, which are consequently the principal targets for the effective use of preservatives and other forms of control are known, but not generally understood. They can be divided into enzymatic and non-enzymatic reactions. For example, enzymatic browning is initiated by polyphenoloxidase, while non-enzymatic browning is commonly caused by Maillard reactions associated with heating.

2.3.1 Polyphenoloxidase (PPO) and browning in fruit

The organoleptic qualities and biochemical character of fruits may be greatly modified by the appearance of browning due to the formation of pigments that cover the ground colour of the fruit. Generally these changes are considered as quality degrading, both visually and nutritionally. Silva & Nogueira (1984) in a study of polyphenoloxidase and peroxidase activity found that polyphenoloxidase was the more important browning enzyme in fresh fruits. While some of the browning is non-enzymatic, most is caused by enzymic oxidation of phenolic compounds under the effect of one main enzyme - polyphenoloxidase (Eskin, 1991).
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Three general situations may cause browning reactions in fruit. Physiological changes associated with ripening, disorders associated with cold storage or chilling injury and thirdly, operations associated with harvesting and processing of fruit where crushing, wounding and juice extraction occur (Haslam, 1989). With increasing mechanisation and pre-processing of fruit for marketing, browning is becoming a more serious problem (Lee & Whitaker, 1995). A common feature is that phenolic compounds (substrates), which are mainly located in cellular vacuoles, interact with oxidative enzymes, generally located in the cytoplasm of the cell, in the presence of oxygen once the cell membranes become disrupted (Haslam, 1989).

Browning enzymes are generally divided into laccases (E.C. 1.10.3.2) mainly associated with procaryotes, and the cresolase/catecholase group (E.C. 1.10.3.1) found in higher plants (Vaughn et al., 1988). Both groups may be loosely referred to as "polyphenoloxidase" in literature and in that case they have a joint designation as E.C. 1.14.18.1 (Mayer & Harel, 1979). In this thesis "Polyphenoloxidase" (PPO) will only be used to refer to the cresolase/catecholase higher plant group.

Laccase is common in fungal infection hyphae (Mayer & Harel, 1979), where it is reported to oxidise only polyhydric phenols (Sherman et al., 1995). In apricot laccase may be either innate or introduced by fungi (Dijkstra & Walker, 1991). Laccase has been reported from apples, apricots, potatoes, cabbage, and sugar beets (Cai et al., 1993; Haslam, 1998b) assuming it to be innate. Laccase has not
received a great deal of attention in fruit (only 16 of 123 papers found) except perhaps grapes, where the action of *Botrytis cinerea* is of great interest to winemakers. For example, Doneche (1991) reported high sugar content of grapes inhibited laccase activity. Goodman *et al.* (1992) reported on *Botrytis* and laccases in raspberry infection and Maas & Line (1994) found that *Botrytis* which released laccase in strawberry fruits resulted in damage quite unlike that due to mechanical wounding. Robinson *et al.* (1993) found sapburn on "Kensington" mango skin cell disruption primarily due to catecholase-type PPO causing skin discoloration during harvest and storage although a laccase PPO was also found in the sap.

When fruit is considered "Polyphenoloxidase" normally refers only to the cresolase/catecholase enzyme (Arora & Sandhu, 1986; Rathjen & Robinson, 1992; Boss *et al.*, 1995). Thus polyphenoloxidase is extensively distributed in plants. It is found in such plant materials as squashes, roots, citrus fruit, plums, bananas, peaches, pears, melons, olives, tea, mushrooms, and others (Mayer & Harel, 1979).

Polyphenoloxidase can catalyse two different sub-reactions. One of these is the hydroxylation of certain o-mono-hydroxyphenols to dihydroxyphenols, an example of which is the hydroxylation of p-cresol to 3,4-dihydroxytoluene - the "Cresolase" activity. The other reaction is the oxidation of o-dihydroxyphenols to o-quinones, illustrated by the oxidation of catechol to o-benzoquinone. This second is termed "Catecholase" activity. The combined reaction in fruits is still
commonly termed ‘Polyphenoloxidase’ (Singleton & Trousdale, 1992; Mayer, 1987) despite the potential for confusion this may create.

Figure 2.8  Overview of the proposed action of *Neurospora* polyphenoloxidase reaction in *Robinson & Eskin (1991)*. Both monophenolase and diphenolase activities are depicted. Circled numbers 1 to 5 indicate the diphenolase steps whilst numbers 1,2,6,7,8,4,5 indicate the monophenolase steps. N=protein ligand; R=endogenous protein bridge; L=exogenous ligand.

A certain amount of o-dihydroxyphenol is normally always present during the oxidation of monophenols by cresolase. Catecholase oxidises the o-dihydroxyphenols at a faster rate than the corresponding monohydroxyphenol.
Monophenols and o-dihydroxyphenols can thus be oxidised to the potential browning precursors by this dual action of PPO (Sherman et al., 1995).

PPO has a molecular weight of ca 42,000 and contains two molecules of copper (Cu) in each enzyme molecule (Rathjen & Robinson, 1992b). Freshly prepared enzyme contains copper in the cuprous form, but it slowly oxidises to the cupric form on ageing (Rigaud et al., 1991). This change does not result in the loss of any activity. The apoenzyme, which is free of copper, is not active, but its activity can be restored by the addition of cupric ion (Espin et al., 1997). Hudson and Gardner (1988) reported PPO in the chloroplast genome of kiwifruit. While polyphenoloxidase is a nuclear encoded protein, it has been found to be located in the chloroplast with speculation as to its function; whether it is required in the chloroplast for an anti-oxidant role, perhaps defence or that it is just an evolutionary left over (Sharman et al., 1991).

PPO in the pure form is colourless, whereas purified laccase is blue (Mims & Davis, 1984; Mozhaev et al., 1989). Concentrated solutions of PPO have been found to be most stable near a neutral of pH 5 to 7. Heating for a short time at 60 to 85°C inactivates the enzyme in most cases. Concentrated solutions of this enzyme in dilute phosphate buffer at a near neutral pH can be held at 1°C or frozen at -25°C without loss of activity for several months (Mayer, 1987). However, it loses activity on prolonged storage. PPO is inhibited by substances which form stable complexes with copper such as H₂S, KCN, CO, and p-aminobenzoic acid. Reagents which react with sulphydryl groups do not inhibit
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the enzyme (Mayer & Harel, 1979, Marques et al., 1995). Selective inhibition of catechol oxidases by salicylhydroxamic acid (SHAM) has been reported by Allan & Walker (1988) and also Rathjen & Robinson (1992b) as a method for discriminating between laccases and catecholases.

Kumar, 1986 (abstract) and 1987, is reported as having found six polyphenoloxidase isozymes in apricot at fruit set with the number increasing during ripening to ten at yellow to orange colour change. In addition to the number of isozymes a shift in optimum pH from 7.0 to two optima of 6.6 and 7.4 was recorded as the fruit ripened. It is implied that the latter polyphenoloxidase isozymes which are activated are the ones involved in any browning reactions. No confirmatory reports were found for apricot.

2.3.2 Oxidation of monophenols (cresolase activity)

Hydroxylation of certain monophenols to o-dihydroxyphenols, the reaction catalysed by Cresolase, is brought about by the same enzymic molecule that produces Catecholase activity (Rathjen & Robinson, 1992a). This enzymatic hydroxylation has some very unusual characteristics. The induction period of this reaction is rather long, and increases with the amount of purification of the enzyme. The usual induction period is a fraction of a second, but when pure enzyme is used the period may be several minutes. However, this induction period can be eliminated or reduced by the addition of a little o-dihydroxyphenol, and the rate of oxidation after induction is linear (Mayer & Harel, 1979). Rigaud
et al. (1991) confirm the general view of a lag phase observed prior to o-hydroxyphenol oxidation. They suggest the lag phase commonly observed in extracts is due to fast coupled oxidation of ascorbic acid by o-quinones with simultaneous reduction of the o-quinones back to dihydroxyphenols. Cilliers & Singleton (1992) suggest that coupled oxidation of a quinone dimer facilitates the main enzymic reaction as seen in Figure 2.14 below.

Figure 2.9: Coupled auto-oxidation of quinone dimers (Cilliers & Singleton, 1992) shown by the dotted line. This reaction is thought to require a specific molecular alignment of the two quinone moieties involved. Disruption of the alignment could lead to inhibition of the browning which results.
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2.3.3 Oxidation of dihydroxy phenols (catecholase activity)

The action of Catecholase on o-dihydroxyphenolic compounds is complex. Since copper is the prosthetic group of the enzyme, it is thought that the Catecholase activity is based on the change of the copper from the cupric to the cuprous state (Rigaud et al., 1991). When the enzyme is isolated, the copper is known to be in the cuprous state. The presence of o-dihydroxyphenols brings about the oxidation of the copper to the cupric state. These reactions are;

\[ 2 \text{Cu}^{++} (\text{enzyme}) + \text{catechol} \rightarrow 2 \text{Cu}^{+} (\text{enzyme}) + \text{o-quinone} + 2 \text{H}^{+} \]

\[ 2 \text{Cu}^{+} (\text{enzyme}) + 2 \text{H}^{+} + \frac{1}{2} \text{O}_{2} \rightarrow 2 \text{Cu}^{++} (\text{enzyme}) + \text{H}_{2} \text{O} \]

By losing two electrons and two protons the substrate is oxidized. The copper of the enzyme takes up the two electrons and changes into the cuprous state. The two electrons are rapidly transferred to oxygen forming water and two protons are liberated. This release of water is important when the browning of dried foods of low water activity (A_w) is being considered in relation to browning, as rehydration of the enzyme occurs. The enzyme then returns to the cupric state and is ready to repeat the cycle.

The reaction of polyphenoloxidase most extensively studied has been the indirect oxidation of a reducing agent by a polyphenoloxidase with an o-dihydroxyphenol substrate. Equations (i to (iii represent the changes which take place.
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\[ \text{o-dihydroxyphenol} + \frac{1}{2} \text{O}_2 \rightarrow \text{o-quinone} + \text{H}_2\text{O} \]  

(i)

\[ \text{o-quinone} + \text{RH}_2 \rightarrow \text{o-dihydroxyphenol} + \text{R} \]  

(ii)

\[ \text{RH}_2 + \frac{1}{2} \text{O}_2 \rightarrow \text{R} + \text{H}_2\text{O} \]  

(iii)

RH$_2$ (the reducing agent) may be a hydroquinone, protein (McGookin, 1991), ascorbic acid (Abukharma & Woolhouse, 1966), glutathione (Cheynier & Van Hulst, 1988), or a reduced phosphopyridine nucleotide. R is the oxidised form, p-quinone, dehydroascorbic acid, or oxidised phosphopyridine nucleotide.

If the polyphenoloxidase reaction begins in the presence of ascorbic acid, in particular, the reaction may oscillate back and forward for some time until all the ascorbic acid is oxidised (Cilliers & Singleton, 1990). The reaction is usually more complex due to the presence of other redox compounds and substrates such as flavonoids within the cell or medium.

2.3.4 Ortho-quinones

When the surfaces of fruit and vegetables are cut, the immediate browning is caused by reactions of the o-quinones produced through the previous enzymic reactions. O-Quinones are catalytically formed by the action of the PPO. While the o-quinones have little colour, they are very reactive (Eichner, 1983; Chang et
al., 1994). The orthoquinones easily polymerise and are subject to rapid, non enzymic oxidation, the result of which may be dark brown, slightly soluble polymers. PPO does not, of itself, produce brown products (Mims & Davis, 1984; Arora & Sandhu, 1986; Milstein et al., 1989; Doneche, 1991).

Amines and quinones react with each other to form 4-N-glycyl-o-benzoquinone which can cause the deamination of glycine and which at the same time forms coloured pigments. O-quinones formed from o-dihydroxyphenols in the presence of PPO also react rapidly with naturally occurring sulfhydryl reducing compounds like cysteine and glutathione (Cilliers & Singleton, 1992). These reactions take place in addition to the oxidation-reduction reactions associated with PPO.

The o-quinones may also react with proteins to form phenol-protein conjugates as outlined in Miles & Oertli (1995) who state "It is suggested that effective defence by the plant requires oxidation of phenolics at a controlled rate that maintains a deterrent titre of the monomers (monomeric o-quinones), while allowing a well ordered deposition of sealants". Sealant here refers to the sealing of the plant tissue wound caused by insect attack. The concept of a plant defence mechanism that aims to maintain a type of buffered deterrent titre has implications for the control of fruit browning, since it is generally held (Mayer & Harel, 1979) that the reaction is substrate limited. In some cases the PPO product is inhibitory only in certain forms (Janovitz-Klapp et al., 1990).
2.3.5 Polarographic method to estimate PPO activity using the

Clark oxygen electrode

The polarographic method was popularised to some extent by the colourful documentation of Deleiu and Walker during the 1970's (Deleiu & Walker, 1972). Subsequently reports of the estimation of PPO present in plant tissues directly using catechol and 4-methyl catechol have been reported (Tu, 1993; Botre et al., 1991; Tillyer & Gobin, 1991).

Polarographic determination of the oxygen potential of liquids may be used as a direct indicator of PPO activity, since oxygen is directly used up in the reaction to produce o-quinones and water (Mayer et al., 1996). A Clark-type electrode is used consisting of a small, relatively gas-tight chamber with a semi-permeable membrane at the bottom. The solution is agitated to eliminate any oxygen gradient, and the membrane allows a small amount of oxygen to cross and to donate electrons to platinum electrodes. The resulting change in current detected is a direct indicator of the level of oxygen in solution (Dixon & Kleppe, 1965; Delieu, 1972; Pearcy et al., 1989). A disadvantage of the polarographic method is a run time of some minutes per sample to get a result. Another disadvantage is the possible contamination of the electrode with sulphite over time. PPO activity estimation using sulphur-treated fruit requires constant membrane replacement, electrode cleaning and recalibration of the electrode to prevent error due to this contamination. The electrode itself is protected to a degree from 'poisoning' in the Clark design with the use of a Teflon membrane separating the KCl and
electrodes from the solution being tested and this allows for less frequent cleaning.

Mayer et al., (1966) compared manometric, polarographic, chronometric and spectrophotometric methods for determination of PPO activity. They make the point that for PPO either the transformation of the oxygen or phenol should be measured, noting that the direct transformation of the o-diphenol is not measured. They recommended the oxygen electrode as being both the most accurate and convenient. They used 4-methyl catechol as the substrate, as 4-substituted orthoquinones are more stable in water than orthoquinone, which tends to reduce secondary quinone reactions.

Mayer et al., (1966) also note that the PPO enzyme has a low affinity for oxygen. High rates of solution agitation are thus required to maintain oxygen levels at the enzyme in equilibrium with the remaining solution when using polarography.

Delieu & Walker (1972); Mayer & Harel (1979); Walker & McCallium (1980); Dijkstra & Walker (1991) and Robinson et al. (1993) together with many others recorded the use of oxygen electrodes for measuring the activity of either crude or purified PPO extracts. In many cases the substrate has been selected to ensure high substrate specificity to catecholase PPO in an endeavour to exclude interference from any laccase present. Most commonly this substrate is 4-methyl catechol.
Given excess substrate with appropriate buffering and temperature control, the oxygen consumption in a solution is proportional to the PPO activity (Dijkstra & Walker, 1991). It is also required that no other oxidase or auto-oxidation chemicals be present in the solution.

The final percentage of oxygen in solution is often at an apparent level of 3-5%. This apparent residual is due to membrane thickness and ‘residual current’ (Wise & Naylor, 1985). The electrode measures the activity of oxygen and not its concentration. There is a linear relationship between activity and concentration of oxygen so that the concentration is determined from calibration at 100% oxygen saturation versus 0% oxygen using dithionite solution (Delieu & Walker, 1972). The slope of the line indicates rate of oxygen use, which corresponds to PPO activity.

Polarography was used by McBean (1967) for examining PPO in moist pack apricots and its use for PPO activity estimation is reported for a number of fruit by Goodenough et al. (1983); Janovitz-Klapp et al. (1990); Richard-Forget et al. (1992a); Cai et al. (1993 on laccases); Underhill & Critchley (1993); Kermasha et al. (1993); Singleton & Cilliers (1995) and Underhill & Dahler (1996).

Polarography is thus the technique of choice for PPO activity estimation for the reasons outlined above and that is why it is used in the following experiments.
2.4 Factors influencing browning in fresh fruit

In apricot variation in response to temperature, storage time, water status and cultivar with respect to browning have been reported. Over 400 cultivars or hybrids have been reported (Audergon *et al*., 1991; Guerriero & Watkins, 1984), and many apricots tested in Australia have very specific climatic niches (Gathercole, 1988) which are often associated with a high degree of inbreeding as shown by isozyme studies (Byrne, 1989, Battistini & Sansavini, 1991, Wang *et al*., 1991 and Layne, 1991).

Gajzago *et al*. (1986), investigating enzymatic browning of 10 apricot cultivars, found the lowest rate of browning occurred in the ‘Rakovszky’ cultivar. Annual variations in browning tendency were noted in most cultivars. Storage for 10 days at 5°C decreased the rate of browning, the extent of the decrease being cultivar dependent. Sharma *et al*. (1993) reported on colour changes during drying of apricot. They reported that non-enzymatic browning showed statistically significant differences among varieties and treatments.

PPO substrates also vary in different apricot cultivars. Villanua-Marti *et al*. (1991) found that Spanish apricot products were of two groups - Spanish and Turkish apricots, with a predominance of phenolic acids and flavonones, respectively.

Compositional differences affect browning and these are often associated with variety or clone. Lee *et al*. (1990) compared major peach cultivars for their
polyphenoloxidase activity and correlated that with browning levels. They found that both phenolics and PPO activity declined toward maturity. Large seasonal and cultivar variation was recorded. Browning was closely matched to polyphenoloxidase activity and phenolic content.

Vaughn *et al.* (1988) suggested chloroplast DNA is of importance, due to the existence of polyphenoloxidase within the chloroplast without an obvious function. Others have suggested that low-browning, low polyphenoloxidase peaches might be selected on the basis of selection against polyphenoloxidase associated with the leaf chloroplast genome (Uematsu *et al.*, 1991).

Cultural conditions may have an effect on the degree of browning observed in fresh and dried fruit (Stembridge *et al.*, 1972; Wills, 1987) partly due to their effect on °Brix level (Hill, 1988) and maturity (Fourie *et al.*, 1992; Cecil *et al.*, 1991). McBean (1950b) associated higher °Brix in dried apricot fruit with decreased browning. Peach trees that have been girdled produce fruit with higher TSS, total phenols and high molecular weight phenols (Kubota *et al.*, 1993). They also mature earlier and this has been found to be associated with higher polyphenoloxidase activity in picked fruit. In a separate study, Kubota *et al.* (1986) determined that *P. tomentosa* rootstock gave higher total phenols in ripening fruit than *P. persica* rootstock for plums.

Environmental stress may increase PPO activity level in apricots (Nover *et al.*, 1989) although a mechanism has not been ascribed to the effect.
Goto et al. (1988) report that mume (Japanese apricots, *Prunus mume*) are more susceptible to chilling and frost injury when they are less mature. Chilling injury and the associated membrane permeability changes may increase the amount of polyphenoloxidase that comes into contact with substrate within the fruit during harvest or after chilling. It might be noted that Morikian (1988) reports Eurasian apricot culture is conducted in areas where the temperatures range from -7.2°C to 26°C and where frosts on young fruit are not uncommon although no mention was made of the extent of browning in fruit.
2.5 Control of PPO activity

Control of enzyme browning activity generally assumes that the enzyme is PPO or one of its isozymes. It should be noted that elsewhere it is suggested that other enzyme systems may cause browning; however, the work of Silva & Nogueira (1984) on carrot, potatoes, peaches, grapes, mangoes, and apples makes it clear that PPO is the primary cause of enzyme browning in most common fruits and vegetables.

Methods to control browning may be grouped as follows;

- Sulphur dioxide
- Chemicals other than sulphur dioxide
- Inhibition by methylation of substrates
- Thermal inactivation
- Molecular expression controls

Treatments for the control of browning may act on both enzyme and non-enzymic processes, so they are considered together. In practice, methods of prevention of browning in foods are limited due to the need to apply only non-toxic additives or treatments that do not detract from the existing organoleptic properties of the food under consideration. Some consideration here of other inhibitors used in the laboratory situation such as tropolone (Bryant et al., 1953; Khan, 1985; Shin et al., 1997 and Halder et al., 1998) and SHAM (Allan et al., 1988; Bucheli & Robinson, 1994) may, however, point the way to new, equivalent inhibitors.
In this section the inhibitors are considered from the purely chemical to the oldest natural inhibitor, temperature.

### 2.5.1 Sulphur dioxide inhibitor

*Sulphur dioxide and the sulphite ion*

Burning sulphur produces gaseous sulphur dioxide, which is distributed to and taken up by fruit tissue and spoilage micro-organisms forming bisulphite. It is also possible to use other sulphur dioxide releasing compounds. Chief among these are sodium or potassium metabisulphite used as a salt which is added to a water based medium.

Sulphur dioxide (SO$_2$) is the oldest chemical inhibitor of PPO in use. Very potent as an inhibitor, it is also very inexpensive to use. Since sulphur dioxide also combines with carbonyl compounds, excess must be added to react with any carbonyl compounds present, as well as to inhibit the PPO. The inhibition of PPO by SO$_2$ is partially reversible, so that later removal of excess SO$_2$ will result in some regeneration of the PPO activity (Imberty & Goldberg, 1984; Gould, 1989; Valero et al., 1992).

Sulphur dioxide may be added to tissue in two main ways, as sulphite solution or as SO$_2$ gas. In order to be effective the SO$_2$ must penetrate the fruit tissue quickly before any oxygen inside the tissue reacts with quinones formed near cut surfaces. One way to do this is to apply the SO$_2$ in solution under vacuum conditions and...
release the vacuum so that the solution is forced into the fruit. Generally, it is better and more controllable to use SO\textsubscript{2} as a gas rather than a sulphite solution (McBean, 1967b). Since it has a very low liquefaction pressure it is convenient to use the weight or volume of liquid SO\textsubscript{2} as an accurate measurement method prior to its release whereupon it forms a gas (Air Liquide, 1992). Gaseous SO\textsubscript{2} penetrates fruit tissue more quickly and evenly than a sulphite solution (McBean et al., 1963; McBean & Wallace, 1967; Embs & Markakis, 1965; Cape, 1984; Russell & Gould, 1989). Once in tissue or juice the SO\textsubscript{2} is generally in the sulphite ion form due to the low pH of fruit tissue (Embs & Markakis, 1965).

At the present time, the use of sulphur dioxide or sulphites is the most effective method of inhibiting browning in commercial use. Post-sulphuring loss of SO\textsubscript{2} is an issue commercially as it is desirable to retain SO\textsubscript{2} during storage but have low levels in the marketed food. When dried apricots and potatoes were analysed (McBean et al., 1963), it was found that in most cases about 50 percent of the sulphite was lost before any significant amount of browning developed (Monzini & Gorini, 1991; Hauser et al., 1993). The temperature of storage and the moisture content of the product also influence the loss of tissue sulphite during storage (Monier-Williams, 1927; Cape, 1984; Karolewski, 1986; Dahlenburg et al., 1990a; Valero et al., 1992; Baritaux et al., 1991; Wedzicha & Herrera-Viloria, 1991; Diehl, 1992; Swales & Wedzicha, 1992; Edberg, 1993).

Cysteine, a sulphur containing amino acid, is widely reported as an inhibitor of polyphenoloxidase (Richard-Forget et al., 1991, 1992, 1992b; Imberty &
Goldberg, 1984; Janovitz-Klapp et al., 1990; Friedman et al., 1992; Richard-Forget et al., 1992a; Sidiq et al., 1992). None of these report or suggest cysteine's use as an inhibitor commercially although it is found in foodstuffs.

Glutathione

Glutathione, mentioned in connection with polyphenoloxidase characterisation and sulphite binding has been considered by a number of workers as a browning prevention measure (Salgues et al., 1986; Rigaud et al., 1991a,b; Friedman et al., 1992; Gisselmann et al., 1992). Friedman et al. (1992) for example, working with potato browning, suggest that glutathione is an excellent preventative for browning, approaching the effectiveness of sulphite. They recommended glutathione evaluation for food. In a later paper, Friedman (1994) advocates the use of S-amino acids and glutathione to stop free radical food problems in connection with cancer incidence and, incidentally, browning. It should be noted that glutathione level in tissues of fruits is not directly related to the browning potential (Cheng & Crisosto, 1995) as has been suggested (Rigaud et al., 1991; Cheynier et al., 1991).

Kinetic studies (Richard-Forget, 1992; Richard-Forget et al., 1992b; Goupy et al., 1995) using model solutions of cysteine and cysteine-quinone addition compounds (unspecified) on apple polyphenoloxidase indicate that the quinone addition compounds can co-oxidise with o-quinones leading to regeneration of coloured phenols. Thus inhibition of polyphenoloxidase alone is insufficient to
prevent discoloration in these model solutions at least. The Richard-Forget group (1992d), also examined apple polyphenoloxidase and the use of chlorogenic acid, catechins and 4-methyl catechol as substrates in relation to inhibition of the enzyme, noting substrate affinity is important in the functioning of inhibitors. In this context, Billaud et al. (1996) point out that the affinity is partly a function of protonation, lower pH causing more potent inhibition.

Important contributors in the wine area are Rigaud et al., who in 1991 reported on the influence of must composition on phenolic oxidation kinetics. White grape musts with added sulphur dioxide, ascorbic acid, glutathione, copper or flavanols were prepared and oxidised using known amounts of oxygen. Sulphur dioxide and ascorbic acid delayed caffeoyltartaric acid oxidation, 2-S-glutathionyl caffeoyltartaric acid formation and browning development. The glutathione content determined the quantity of 2-S-glutathionyl caffeoyltartaric acid formed, browning was prevented when all the caffeoyltartaric acid quinones generated by enzymic oxidation could be trapped as 2-S-glutathionyl caffeoyltartaric acid. Previously Salgues et al. (1986) had also described the oxidation of 2-S-glutathionyl caffeoyltartaric acid by Botrytis cinerea laccase. Coupled oxidation of flavanols by the caffeoyltartaric acid quinones also led to increased discolouration. It is not clear whether this mechanism also operates with other phenolics such as caffeoylquinic acid, which does occur in apricot. Copper, which may lower the concentration of available glutathione, was found to enhance must browning. Similar findings are reported by Yokotsuka et al. (1991).
Copper-based sprays may therefore be implicated in increased browning in stone fruits.

### 2.5.2 Chemicals other than sulphur dioxide

Inhibition of enzymes by various chemicals will be considered. The reaction kinetics of PPO suggests that it is difficult to differentiate between suppression of activity per se and formation of product-enzyme complexes that behave as if an inhibitor was added. Valero et al. (1991) reported that tropolone inhibits grape polyphenoloxidase in a non-classical manner. Enzyme kinetics studies show a decrease in initial velocity to a steady-state PPO inhibition velocity on a time scale of minutes rather than parts of seconds. Kinetic data obtained correspond to those for a postulated mechanism that involves rapid formation of an enzyme-inhibitor complex that subsequently undergoes a relatively slow reversible reaction. The potential for the use of this compound in some commercial applications was investigated due to the low levels of inhibitor required (Bryant, 1953).

Chen, Wei & Marshall (1991a) reported on the mechanism of kojic acid in inhibiting polyphenoloxidase by interfering with oxygen uptake. Polarography, spectrophotometry and chromatographic techniques were used to show that the kojic acid was capable of reducing the o-quinones to diphenols thus preventing the melanin (brown) pigment forming following PPO activity. In a related report
Chen et al. (1991b) showed the inhibition of apple polyphenoloxidase by kojic acid with 4-methyl catechol substrate to be due to competitive inhibition.

Salicyl-hydroxamic acid (SHAM) was used by Allen & Walker (1988) for the selective inhibition of catechol oxidases compared with laccases. Mayer et al. (1966), Steffens et al. (1993) and Bucheli & Robinson (1994) also describe this use of SHAM.

Aromatic carboxylic acids inhibit PPO activity (Figure 2.10). These acids, such as cinnamic, benzoic and phenylpropionic acids, act as pure competitive inhibitors of polyphenoloxidase with inhibition enhanced by p-hydroxy substitutions and greatly decreased by m-methoxy substitutions (Iyengar & McEvily, 1992; Sherman et al., 1995; Vamos-Vigyazo, 1995).

Halides have been reported as PPO inhibitors by Janovitz-Klapp et al., (1990) where NaF, NaCl, NaBr and NaI are decreasingly effective. They reported that the fluoride ion was by far the most potent inhibitor of polyphenoloxidase at levels down to 4µM. Fluoride ions appear to be more effective at pH 3.6 rather than pH 5.0. One presumes this is in dilute solutions.
2.5.3 Inhibition by methylation of substrates

Activity of PPO can be reduced by in-situ methylation of the natural substrates of PPO. The enzyme catechol o-methyltransferase is capable of methylating the 3-position of 3,4-dihydroxy aromatic compounds with the o-methylation being irreversible (Amiot et al., 1997). Caffeic acid yields ferulic acid when this occurs (Xu et al., 1993; Tilbury, 1980). PPO causes little if any oxidation of monophenolic ferulic acid. Therefore, the in situ conversion of all the natural substrates (including the dihydroxyphenolic compounds formed from monophenols as catalysed by cresolase) could be a way to prevent enzymatic browning. Xu et al. (1993) found that an anaerobic methylation treatment with catechol o-methyltransferase system at pH 8 permanently prevents oxidative darkening of apple juice and fruit sections, because it modifies their PPO substrates.
2.5.4 Thermal inactivation

There is a complex relationship between temperature treatments, substrate and additives (Ng & Soleha 1991). Various papers, such as that of Remacha et al. (1992) record low temperature storage of fruit, but more commonly pulp, are of use to control non-enzymic browning. Von Mollendorf et al. (1992) recorded that storage of fresh nectarine fruit at -0.5 °C resulted in less browning of mesocarp tissue than those stored at 3 °C. A 10 minute heat treatment of fresh apricots decreased the browning rate exponentially with treatment temperature, with browning being eliminated at 95°C (Gajzago et al., 1986).

Remacha et al. (1992) tried kinetic models to describe the effects of temperature. They found an activation energy of 17.19 kcal mol\(^{-1}\) for peach versus 11.15 and 5.45 for plum and apricot, respectively. No reason was suggested for the low apricot figure.

Vegetables packed for freezing preservation are 'blanched' whereby boiling water or steam is used to inactivate the enzymes and control enzymatic browning. This method may not be satisfactory for fruits because of the development of cooked flavour and also a softening of the tissue (Eskin, 1991). Pasteurisation can be used for fruit juices, but these should be held at the proper time-temperature to inactivate the enzymes. Usually the minimum time-temperature gives the best results, cutting down any undesirable changes that might result from excess
heating. A temperature of 85°C is often used (Komatsu et al., 1993).

Peroxidases may be more heat stable than PPO and may retain activity even though the PPO is heat inactivated (Bucheli & Robinson, 1994). Extreme pressure has been used to deactivate enzymes in fruit purees and whole fruit as a means of preservation although no PPO activity specific research using this method has been reported (Wrolstad, pers. comm., 1998).

### 2.5.5 Molecular expression controls

Molecular biology techniques offer the prospect of controlling the expression of the PPO enzyme by controlling the production of the enzyme as a protein being fabricated within the cell. A number of techniques are available, but the principal current method revolves around the production of an ‘antisense’ copy. For example, Jones et al. (1995) reported on antisense inhibition of polyphenoloxidase gene expression using appropriate promoters to express antisense PPO RNA, so that melanin formation can be specifically inhibited in the potato tuber. This opens the possibility of preventing enzymatic browning in a wide variety of food crops without resorting to treatments such as heating or the application of antioxidants. No reports of the use of molecular expression control in apricot PPO were found.
2.6 Conclusion

In fruits and apricots variation in response to substrates, temperature, storage time, water status and cultivar with respect to PPO activity have been reported.

The evidence supports a view that apricot fruits contain PPO (Gajzago et al., 1986; Mayer & Harel, 1979) which can lead to browning. Laccase is reportedly present in apricot also which is unusual for a fruit (Dijkstra & Walker, 1991). The degree of relative activity of PPO versus laccase leading to browning is not reported (Vamos-Vigyazo et al., 1984). Studies, which relate net enzymic activity to location within the developing apricot, such as those in peach by Stutte (1989), are still required to attempt to establish the cellular location of browning.

A large number of substrates exist for PPO (Mayer et al., 1966). Of the substrates required for the enzymes to act on, thereby causing browning in apricots, the evidence is that the major phenolic substrates are cinnamic acid derivatives (chlorogenic acid, epicatechin and catechins), while the major flavonols are quercetin based. In apricot, chlorogenic is cited as being one of three main phenols (Villanua-Marti et al., 1992) supporting PPO activity. In a number of cases, activity seems to be substrate rather than enzyme limited with higher browning when more phenol is present initially (Souty et al., 1991). Dijkstra & Walker, (1991) thought this may be due to the physical situation of membrane-attached enzyme being isolated from substrate in fruit extracts.
Chapter 3. General Materials and Methods

3.1 Plant materials

Apricot fruit (*Prunus armeniaca* L.) cv. "Moorpark" on peach rootstock were harvested from a single tree within the experimental orchard, Waite Research Precinct, Adelaide during the summer of 1995-96. Trees were subjected to normal cultural control including the use of glyphosate as herbicide. No non-organic chemicals were used for disease or pest control from initiation to maturity. No fertilisers were applied during the harvest period.

*Ripening*

Apricot fruit growth is described as double sigmoid with three growth stages (Denne, 1963). Stage I is associated with cell division following the fertilisation of an egg in the carpel which is to become the fruit. Slow initial growth is followed by a period of exponential expansion, as weight or volume, which then slows down again as the fruit enters Stage II. This second stage is associated with scleratisation of the endocarp, the original inner layer of the carpel. During this stage the fruit typically does not increase in cell number or cell volume as the hardening of the endocarp takes place. Stage III begins slowly with the accumulating of water and solutes into the mesocarp in particular. A maximum rate of expansion is reached followed by slowing of water accumulation as the fruit goes from maturity to ripening. Stage III is associated with fruit softening and transformation of reserves into sugars, colour
and flavour compounds (Denne, 1963). These stages are indicated in Figure 3.1, for comparison with sampling weights.

**Sampling**

Ten fruit were harvested into crushed ice at weekly intervals from a few days after full bloom when the blossom falls off, usually termed 'capfall', until full maturity was reached. Fruit were sampled randomly from all sides of the tree within a band 60cm to 240cm in height from uniform positions with respect to other fruit and shading effects. Fruit were visually assessed for uniform size prior to sampling from the tree.

These cooled fruit were weighed and halved. A wedge of tissue was taken from the pedicel to the distal end of one fruit half. Wedge samples did not include suture material. In the case of non-uniformly ripened fruit the side most coloured was used. The wedge was approximately 20 to 50% of a side depending on the size of the developing fruit. In early sampling it was not possible to dissect the endocarp from the mesocarp and pericarp and after stone hardening stage it was not practical to sample the endocarp with the flesh and skin. Skin was included in the wedge.

Each chilled wedge was cut into 2 to 4mm slices prior to placing into sample tubes followed by addition of liquid nitrogen within two minutes of fruit cutting to precool the samples and also exclude oxygen. Tubes were held at -18°C for up to one hour while other fruit sampling took place.

A drop of juice, taken prior to freezing, was used to estimate sugar content as °Brix with a hand-held ATAGO, N1 refractometer (Atago Inc., Kirkland, WA, USA) with
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allowance made for ambient temperature. Samples were then placed in a -70°C freezer until sample preparation (see section, 3.2) and extraction began. No buffers, solvents or anti-oxidants were added to samples. Whole fruit weights, sample weights for each side, °Brix and comments regarding seed disposition and appearance vis-a-vis fruit shape are included in Appendix I.

Mean weight by time is shown in Figure 3.1 and a double sigmoid growth curve, characteristic of Prunus spp. (Denne, 1963), was observed.

Figure 3.1: Fresh weights of sampled apricot fruit (Prunus armeniaca L. cv. “Moorpark”) by age showing double sigmoid growth curve characteristic of Prunus spp. Bars represent standard error of means, n=10 (fruit weight only).
3.2 PPO Activity measurement

Preparation of Crude Extract

Fruit samples were powdered by grinding in liquid N\textsubscript{2} and then adding 3 ml of extract buffer mix per frozen apricot sample. The McIlvaines’s buffer used was made up of sodium hydrogen phosphate and citric acid to a final concentration of 0.1 M. These buffers were adjusted to a constant ionic strength by addition of potassium chloride as required. This adjustment is required since the activity of certain types of organic compounds depend strongly on the ionic strength of the solution (Clark, 1956; Perria & Dempsey, 1974). McIlvaine’s buffer solution was used based on its preferential use by other researchers of PPO activity (Irwin \textit{et al.}, 1994). This buffer is unstable and fresh solutions needed to be prepared each day.
3.3 Polarography - Experimental

A 1000 µl sample extract was added to the 3 ml Clark electrode cell (Rank Bros., Bottisham, U.K., Figure 3.2) together with 1950 µl of 50 mM sodium phosphate ‘reaction’ buffer, at pH 6.8 as a default, and was used for polarography. The reaction buffer, containing sodium dodecyl sulphate (SDS, 1% w/v, Sigma cat. L4509), was used to activate PPO during assays (Moore & Flurkey, 1990).

In preliminary work it was determined that sulphured, dried apricots had an apparent residual PPO activity of 12 nmol oxygen consumed min⁻¹ g⁻¹ dried wt. The oxygen
Chapter 3. Materials and Methods

meter and cell used (3 cm$^3$) have a working sensitivity limit of 4 nmol O$_2$ consumed min$^{-1}$ g.fwt.$^{-1}$ (Model 3, Rank Bros., Bottisham, UK.).

Each sample group of approximately 0.20g fresh weight was weighed when frozen. At testing each sample was removed from the freezer and ground with N$_2$ in a pre-cooled mortar and pestle.

The oxygen meter was set up according to Rank Bros. directions for the instrument use with advice from Fox (1992) in regard to spin rate of the magnetic stirrer and bias voltage (0.65Volts). The diagram of the system is shown in Figure 3.2. Fresh carrot cortex scrapings were used to verify the functioning of the cell using 4-methyl-catechol as a substrate (Chubey & Nylund, 1969). Sodium dithionate was used to zero the cell (Delieu & Walker, 1981; Williams & Wilson., 1993).

The cell water jacket was maintained at 25°C (Walker, 1963). Upon stabilisation of the open electrode at 100% oxygen (1 minute) it was closed with the plunger and 50µl of ice-cold, 100 mM 4-methyl catechol (Sigma M6636, 4-Methyl-1,2-benzenediol), prepared daily, was added via the syringe hole with a micropipette to initiate the reaction.

Initial slope of the reaction, prior to product inhibition of the enzyme, was determined from the output of a Houston plotter (Bausch & Lomb, Houston Instrument Division, Austin, Texas, Model EB5136) using a 1 volt input range. Unstable oscillation of the magnetic stirrer 'flea' was observed to cause ca 10%
variation of the plot line occasionally. The average slope line was, however, very clear (Figures 3.5, 3.6).

A similar instability could be observed if i) the flea was running too slowly as predicted by Mayer et al. (1966) or ii) the cell contained particulate matter as was the case when coarse, non-ground, apricot puree was tested.

Variation in the volume of 100 mM 2-methyl catechol from 20 to 50 microlitres (required to deliver 2 mmoles for reaction with available oxygen) or varying its temperature from 0°C to 20°C also had no effect on the slope recorded. In this connection it should be noted that the reaction appeared to be quite sensitive to the water bath temperature. Variation in the temperature of the reaction cell contents from 25°C to 23.5°C resulted in a marked decrease in the initial rate of reaction as predicted by Delieu & Walker (1981). Great care was therefore taken to maintain the reaction cell water jacket temperature at a constant 25°C.

### 3.4 General method used with Clark electrode

Two buffers were used - an extraction buffer and a reaction buffer. The extraction buffer at pH 6.8 was used during grinding and suspension of the sample. Because the polarographic half-wave potentials of certain types of organic compounds depend strongly on the ionic strength of the solution, Perria and Dempsey (1974) suggest the use of constant ionic strength McIlvaine citrate/phosphate buffers covering the range of pH 2.2 to 8.0 at 25 °C noting also that surfactants, such as SDS, do not upset
citrate-phosphate buffer operation. The general method used to measure PPO activity with the Clark electrode is shown in Table 3.1 and Figures 3.3 and 3.4.

Table 3.1: Actions required for each apricot sample PPO activity assay when using the Clark oxygen electrode.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1:</td>
<td>Add 1950 µl of prewarmed (25 °C) Reaction buffer (including SDS) to reaction cell</td>
</tr>
<tr>
<td>Step 2:</td>
<td>Remove sample from -18°C freezer</td>
</tr>
<tr>
<td>Step 3:</td>
<td>Identify tube and record details</td>
</tr>
<tr>
<td>Step 4:</td>
<td>Place frozen fruit sample into chilled mortar (using liquid nitrogen)</td>
</tr>
<tr>
<td>Step 5:</td>
<td>Grind “dry” to a coarse paste (3 seconds)</td>
</tr>
<tr>
<td>Step 6:</td>
<td>Add 1000µl of extract buffer (no SDS)</td>
</tr>
<tr>
<td>Step 7:</td>
<td>Grind sample into a slurry</td>
</tr>
<tr>
<td>Step 8:</td>
<td>Add 2000µl of extract buffer</td>
</tr>
<tr>
<td>Step 9:</td>
<td>Grind to a final fine slurry (the “extract”)</td>
</tr>
<tr>
<td>Step 10:</td>
<td>Add 1000 µl of the extract to the reaction cell</td>
</tr>
<tr>
<td>Step 11:</td>
<td>Set Clark electrode output to 98% with SENSITIVITY control</td>
</tr>
<tr>
<td>Step 12:</td>
<td>Allow temperature to stabilise to 25 °C</td>
</tr>
<tr>
<td>Step 13:</td>
<td>Turn chart recorder on</td>
</tr>
<tr>
<td>Step 14:</td>
<td>Add 50 µl of 100 mM 4-methyl catechol to initiate reaction</td>
</tr>
<tr>
<td>Step 15:</td>
<td>Record run data on chart output</td>
</tr>
<tr>
<td>Step 16:</td>
<td>At completion remove plug and wash</td>
</tr>
<tr>
<td>Step 18:</td>
<td>Tip reactants out of cell, including the magnetic flea, wash flea</td>
</tr>
</tbody>
</table>

An example plot is shown in Figure 3.5. In this example the 50 µl of 4-methyl catechol addition is shown. Following addition there is usually a rapid (1 to 5 seconds) development of a linear slope which degrades into a slower rate curve associated with auto-inhibition.
Figure 3.3: Adding extract to the reaction cell from chilled grinding mortar (Step 10).

Figure 3.4: Adding 50 µl of 100 mM 4-Methyl Catechol substrate to Clark Electrode reaction cell containing apricot extract sample (PPO source) to initiate the reaction (Step14).
Figure 3.5: Sample output from the Clark oxygen electrode. Note the start point at the top is equivalent to 100% oxygen content in the reaction cell. The introduction of the 4-methyl catechol causes a “blip” (which may take a few seconds to stabilise). Slope of the initial reaction indicates PPO activity. These two samples show different initial reaction rates.
Figure 3.6: Example output from the Clark oxygen electrode. In this example, following addition of substrate, there is a rapid development of a linear slope, which degrades into a slower rate curve associated with auto-inhibition. The straight line was used to show the slope of the initial reaction. At completion the slope levels out to an apparent 2 to 3% residual oxygen due to the electrode membrane characteristics. The vertical scale represents approx. 110% oxygen content down to approx. 10% (the plot is electrically offset to allow the recorder pen to accommodate the 'blip' associated with the addition of the substrate). Major horizontal scale is in centimeters, forward speed is 5cm min\(^{-1}\).
Stardardising of measurement over the period of the experiment was achieved with the use of a standard sample which was run at the beginning and end of each experimental session of either a day or half day. The standard sample was prepared from six chilled fruit, homogenised in a Waring-type blender and subdivided into vials, which were frozen at -70\(^\circ\)C, thawed each session and used for all experiments. Data was normalised to this standard.

### 3.5 PPO solubility and activation

Despite possible experimental artifacts, the weight of evidence indicates that polyphenol oxidases are commonly membrane-bound, particularly in chloroplasts (Mayer & Harel, 1979). In fruit, solubilisation tends to occur with fruit ripening as seen in grapes (Sanchez-Ferrer et al., 1989) and apples (Harel et al., 1965).

Sometimes more drastic conditions are required for solubilisation of membrane-bound PPO, such as the use of detergents or limited digestion with proteases (Vaughn et al., 1988). In view of this, the solubility of the PPO enzyme in apricot was investigated with the aim of ensuring maximum extraction of PPO activity. Standard sample fruit prepared as above were used. Samples of 2 g fresh weight equivalent in 30 ml of pH 6.8 phosphate-citrate (McIlvaines) buffer were subsampled into 14 ranges each of three replicates. Sodium dodecylsulphate (SDS) was used as a membrane solubilisation agent at levels from 0 to 2% w/v. This range was chosen based on prior reports (Dijkstra & Walker, 1991; Mayer & Harel, 1994) on the use of SDS. Results for 3 replicates (Figure 3.7) indicated greatly increased activity was
achieved with the addition of 0.033% SDS and an apparent decline in that response with increasing addition up to 2% SDS. A slightly higher level than indicated with the samples tested, of 0.1% SDS was chosen on the basis of these results to use in all extractions.

![Graph](image-url)

**Figure 3.7:** Activation of PPO by addition of sodium dodecyl sulphate at various concentrations to the reaction mixture containing crude extracts of apricot fruit. Bars represent standard error of means (n=3).
3.6 Sample concentration and rate of reaction

PPO dependent oxygen uptake increased with the addition of increasing amounts of homogenate up to the maximum used of 1g (Figure 3.8). There was no inhibitory effect with the addition of excess homogenate indicating that no significant endogenous inhibitors were present in the extract. At very high levels the amount of homogenate in the 3 ml volume of the reaction cell would have been sufficient to reduce the effect of the agitating magnetic ‘flea’, thus reducing the apparent activity due to a slowing down of oxygen transfer at the membrane of the dissolved oxygen cell. An average fresh fruit weight of 0.25g in 3 ml of extract volume was used in all experiments thereafter.

Figure 3.8: Rate of PPO dependent oxygen consumption vs the addition of varying amounts of fruit homogenate containing PPO into the extraction buffer. Bars represent standard error of means (n=5).
3.7 Experimental design and analysis

Apricot trees, clonally propagated, should have a homogeneous DNA profile. Without checking for this using DNA fingerprinting it is unwise in practice to assume lack of variation for *Prunus* (Coombe, 1976; Granger *et al.*, 1993). For this reason a single, large, well established, mature tree was chosen as a single parent source of samples to eliminate maternal tree-to-tree variation.

Ten fruit were sampled from this one tree on a weekly basis for 12 weeks, except for fruit in week 9 where only eight fruit were sampled, forming 12 age groups from 'Day 1' to 'Day 92'. Due to the bilateral asymmetry of apricot fruits (Brady, 1993) one side (the more coloured, normally larger) was consistently used.

During the development of the fruit, represented by different fruit ages, a number of effects may exist which are not simply a function of physiological ripening. For example, variation in air temperature and/or disease challenges occurred during the time from Day 1 to Day 92.

From the half used, thin wedge samples were taken for use in treatments relating to buffer pH (chapter 5), substrate concentration (chapter 6), sulphite (chapter 7), salt (chapter 8), and heat inactivation (chapter 9).
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The null hypothesis was that there is no variation in the PPO activity level with age or treatment and furthermore that there is no variation between fruit sampled at each age.

The hypothesis is based on inference. The inference assumes a model something like;

\[ y = \alpha + \beta \times x + \varepsilon \]

Where y is the dependent variable, \( \alpha \) is the intercept parameter, \( \beta \) a slope parameter and 'x' is the independent variable and \( \varepsilon \) the error term.

The ANOVA assumes that errors in the residuals are normally distributed that the errors in the residuals have constant variance, and that the errors are independent.

Considerable effort was expended in analysing data statistically. A number of statistical packages were utilised for their particular capabilities. GENSTAT®, (Lawes Experiment Station, Rothamstead, version 5), for its residual values plots and split-plot Analysis of Variance capability, Plotit® (Scientific Programming Enterprises, Haslett, MI, USA, version 3.20f) for its graphics, multiple variance analysis and computational speed and Excel® (Microsoft Excel 97, version SR-2) for spreadsheet development. The need to transpose large arrays (600 x 5) for GENSTAT was a limiting factor.

Data were handled independently for each experiment. The data were examined more extensively for the pH and heat inhibition experiments in order that general
assumptions of data trends and independence could be tested. Initially, split-plot analysis of variance was proposed for data analysis. This was based on the advice that the age of the fruit being tested may not be an independent factor for non-experimental effects such as daily temperature variations or the incidence of a disease challenge. The split-plot design allows firstly a comparison of time by experimental treatment (e.g. pH), which if found to be non-significant, then allows consideration of the higher fruit x pH comparisons.

Subsequent biometrical advice that a time-fruit factor be assigned to the data was acted on. This allowed the use of the GENSTAT® general ANOVA model rather than the split-plot model.

The General ANOVA provided more meaningful analysis. Results of the analysis with a copy of the relevant raw data are found in Appendix 2. Plotit® analysis of the heat inactivation data as factorial ANOVA for the 5 heat treatments over the 12 ages factor columns of 10 fruit was done and provided an indication of the within age variation particularly.

Generally the results indicate the data to be skewed. Efforts to transform the data using LOG, and square root transforms did not provide useful improvement over using the raw rates.

Polynomial (3rd order) plots suggest a curvilinear rather than a straight regression of percent rate of activity versus age of fruit sample.
Because some of the data is skewed it is not appropriate to assume that a low P value should lead to rejection of the null hypothesis in each case. Also the analysis of variance is very sensitive to outlier data and the normality of the data distribution. Daniel (1960) demonstrated that one outlier can reverse the conclusion of an ANOVA and showed that it is not a robust technique against outliers. Therefore, there was a need to graph points first to establish the extent of outliers and distribution of residuals. This was done using the GENSTAT statistical package.

The results show that residual values generally increase with increasing means of activity rate. Increasing means for activity occur with the increase in the ripening age of the fruit. The results obtained therefore show that while activity generally increases with the age of the fruit, so to does the variation in activity for each age. This would suggest that either the results are less reliable indicators of the population with increasing age or there is a real increasing variation in PPO activity with age of fruit.

Standardisation across the various treatments was done with the use of standard samples which were run at the same time, and thus under the same conditions, as the particular tests being conducted on each day. The standard sample was prepared from five, day 85 aged fruit at harvest. A large quantity of sub-samples were retained and held in a -70°C freezer until required. These samples were then available to 'recalibrate' the results from the Clark oxygen electrode for each day. All standard sample data were summed and result daily standards (control) data were adjusted to the common mean of the summed standards. This allowed comparison across and within treatments, at least as far as potential day-to-day variation in the
Chapter 3. Materials and Methods

Clark electrode is concerned. Variation has been reported as 'drift' due to gradual contamination of the platinum electrode and/or separating membrane (Delieu & Walker, 1972).
This chapter summarizes the level of PPO activity that develops in fruit with increasing fruit age as depicted in Figure 4.1. It is possible to distinguish between maturation and ripening in fruit, but difficult to quantify in terms of timing (Coombe, 1976). For simplicity I will refer to these processes as ‘development’.

As fruit develop, a number of changes take place both physically and chemically. In particular the cells that make up the fruit start by rapidly dividing and there is a period of high metabolic activity associated with that stage. Following the slowing down or cessation of the major cell division phase, there begins a period of consolidation of the endocarp (pit-hardening) of the fruit in the case of Prunus species. Inside the fruit a large quantity of product from the key enzyme phenylalanine ammonia lyase (PAL) is required to provide the molecules needed to form phenolic compounds and then to polymerize those compounds to form lignified tissue (Robinson et al., 1993).

Subsequent to pit-hardening there is a stage of cell expansion. With the change in water content comes change in the balance and types of carbohydrates present in the mesocarp of the fruit. Sugars form from starches and flavour compounds develop (Biondi et al., 1991). At the end of this third stage a process of cell breakdown
Chapter 4. The Effect of Ripening Age on Polyphenol Oxidase Activity

begins. Softening occurs and pigmentation changes. In the case of apricot, the fruit becomes orange-yellow in colour. This colour change is a very good indicator of full development (Brown & Walker, 1990).

Figure 4.1: Comparison of time when fruit were sampled, as day 1...day 92, with developmental stages and associated fruit appearance. Pit hardening began approximately day 22 in the experimental fruit used.

During ripening the fruit is at risk of bruising and wounding from insects (Miles & Oertli, 1995) and post-harvest cutting and drying processes. The release of phenolic compounds due to mechanical rupture of vacuoles within the cell allows the enzyme of interest, polyphenol oxidase (PPO), to act on the phenolic substrates in the presence of oxygen to form quinones which may variously react, but the general result is the formation of brown compounds that detract from fruit quality.
Prevention of browning requires first the characterisation of the PPO enzyme with respect to its expression in the fruit over various development stages. This in turn requires confirmation that level of expression of the PPO enzyme has a temporal relationship with observed enzyme activity. If one major activity peak could be found then it could be targeted for further examination on a molecular level with antisense DNA (Gray et al., 1992) to prevent browning.

4.1 Material and methods

The basic material and methodology is set out in chapter 3 and should be referred to for details. In this chapter data from various trials, as set out in following chapters, is brought together. However, only the data from treatments with standard conditions, for example, pH at 6.8, were combined. Materials and methods relating to specific conditions such as salinity are dealt with in the later, specific chapters.
4.2 Results

Fruit were sampled for a period of 92 days at approximately weekly intervals. The result for fruit development firstly, show that the growth of fruit as measured by weight follow the expected double sigmoid growth curve characteristic of Prunus spp. fruits (Figure 3.1).

It should be noted that as these drupes develop there is a period of 'pit hardening' associated with the sclerotisation of the endocarp of the fruit. It was not physically possible to separate the endocarp at very early ages and, later, it was not possible to crush and/or measure the PPO activity of the sclerotised endocarp as part of the whole fruit. This creates a number of difficulties regarding what is represented by the results because initially, the endocarp is included and later excluded from PPO activity measurement.

Figure 4.2 shows the general activity level for PPO with increasing development. The result reflects a general increase in PPO activity, as measured, with increasing development to full ripeness. Within the development ages examined it appears that, over a number of experiments described in later chapters, there are three periods of higher activity relative to the increasing plane of activity with age, these being day 22 to day 29, day 57 and day 85. The latter is equivalent to mature ripe fruit.
Figure 4.2: The general effect of "Age" (stage of development) for apricot fruit (cv. "Moorpark") in terms of activity level for PPO. Means for control samples from a number of experiments. Bars represent standard error of the mean (n=12).
4.3 Discussion

A general trend is evident (Figures 4.2) whereby the activity of PPO shows an increase with fruit age as suggested by Dilley et al. (1993). The increase with age is not linear. The effect may be due to a number of things - probably just cell wall/vacuolar state of development and relative surface area (Dilley et al., 1993), but the sensitivity shown very early from day 1 to day 15 may be associated with the degree of activation and/or release from membrane attachment, as suggested by some authors (Zhang et al., 1994). That is, the PPO may not be physically available to the substrate and so the apparent rate of its activity is lower.

Evidence for a non-linear response with age in other enzyme systems was not found in the literature. PPO’s apparent non-linear increase with age maybe an artifact associated with the methodology employed, notwithstanding the use of buffering during testing, given the changes in cytosol pH and titratable acids of different types during development (Dahlenburg et al., 1990b).

Examination of Figure 4.2 shows a curve with multiple peaks; this suggests that three isozymes may exist in apricot during development. Thus there is a peak at ages 22 to 29 days, one at 57 days together with a high peak for fully ripe fruit at 85 plus days. There is sufficient variation in the pH data (chapter 5) to question these trends, particularly for day 1, but in most of the other tests the results supported the evident trend. The question to resolve is whether these were artifacts, for example associated
Chapter 4. The Effect of Ripening Age on Polyphenol Oxidase Activity

with some other cause correlated to a development event or the normal physiological response as seen for a number of enzymes.

The apparent peaks in PPO activity were found under conditions which do not allow a direct inference on whether isozymes are present. Purified PPO may be more suitable for this purpose (Thomas & Janave, 1986; Fraignier et al., 1995), but ultimately electrophoresis or western blotting would need to be done to distinguish isozymes present (Durham et al., 1987). However, test conditions that contain all the elements naturally present, such as pectins, phenolic substrates and co-substrates, sulphhydril compounds such as cysteine and the various anti-oxidants, will give a more realistic indication of actual activities in the fruit (Fragnier et al., 1995). Some cultural actions may cause false results, one example being the increased PPO activity associated with boron sprays (Lee and Kim, 1991a,b).

Significant variation in size and physiological age exists between fruit samples (Fig. 4.1). Jackson & Coombe (1966b, 1967) found that fruits within a tree showed considerable variation in size at any stage of growth for apricots grown on the Waite campus. Jackson (1965) found significant correlation between pit-hardening and mesocarp volume in apricot fruit. He pointed out that opposing mechanisms operate in ‘early’ versus ‘late’ fruit (7 days difference) with a definite correlation between fruit size and cell volume existing in ‘early’ fruit, but in ‘late’ fruit the correlation was between cell number and fruit size. If one assumes that “Moorpark” fruit PPO is one isozyme and that it is equally distributed on the plasma membrane, then one could get two “concentrations” of enzyme distribution following from Jackson's
Chapter 4. The Effect of Ripening Age on Polyphenol Oxidase Activity

model of the “early” and “late” models of cell size distribution in apricot. This could go some way to explain the very large fruit to fruit variation found in the results obtained here as it would not be possible to identify these different types of fruit as they are picked.

A number of workers, notably Kumar (1986, 1987) and Fourie et al. (1992) have reported multiple PPO enzymes in apricot and other Prunus spp. (Torres, 1989). The evidence for apricots is not conclusive since there are insufficient confirmed reports. There is stronger evidence in other Prunus (Fraignier et al., 1995) where multiple forms of PPO (active and inactive) have been detected by SDS-PAGE stained with DL-Dopa and by Western blot, revealing multiple active forms from proteolysis of one major form, although in peaches, good justification is difficult as there are a number conflicting papers with varied experimental conditions. Whether isozymes exist in developing fruit is unknown. Partly folded or refolded protein domains, related to artifacts introduced by the extraction techniques variously used (Marques et al., 1995), suggest there may be fewer isozymes than apparent from experiments. The results obtained here do not establish, but equally do not refute, a claim for multiple isozymes of PPO in apricot.

Clearly the highest activity is associated with maximum ripeness of fruit. That would not necessarily mean that any molecular approach should target this development stage, especially since there is usually a lag between expression and activation of PPO in fruits, for example apples (Boss et al., 1995).
Chapter 5. Effect of pH on Polyphenoloxidase Activity

Optimum pH for PPO activity is of interest for a number of reasons, including the activity of the enzyme system in response to additives that can change the pH, the known change in fruit cytosol pH with developmental stage, and change of PPO activity that may occur if substrate use alters the solution pH. The pH optimum of most of the polyphenoloxidases studied to date is between 5.0 and 7.0 (Mayer & Harel, 1979). There are, however, many conflicting reports on the pH optimum, even from the same source, with differences attributed to the phenolic substrate used, the type of buffer and the purity of the enzyme (Vamos-Vigyazo, 1995).

A small range of pH levels were examined with the view to establishing an optimum for apricot PPO under the conditions applied, with a general objective of determining if the apparent optimum changed during the development of the apricot cultivar being observed. The pH of ripe apricot fruit is reported to be in the range pH 3.4 to 4.2 by Gulcan et al. (1995) who note that male parents influence ripe fruit pH of offspring. This would appear not to be ideal for PPO activity if the pH optimum activity is in the range 5 to 7 as stated above.
Chapter 5. Effect of pH on PPO Activity

5.1 Materials & methods

The basic material and methodology is set out in Chapter 3 and was that used except for the conditions outlined below. A range of pH tests were initially conducted on the buffer-enzyme system. The results (Chapter 3) confirmed the common finding of a number of workers that PPO has a pH optimum in the region of 6.5 to 7.0 (Vamos-Vigyazo, 1995; Dijskra & Walker, 1991). Fine tuning of pH around these figures indicated that a buffer pH of 6.8 gave most satisfactory results. A larger range of pH, other than 5 to 8, would be difficult to test due to the need to maintain consistent buffer capacity without a change in anion type in solution (Stauffer, 1989).

The pH of the reaction buffers used here was altered from the ‘standard’ pH 6.8 with the use of various ratios of citric acid and sodium hydrogen phosphate. Five reaction buffers with the range pH 5.0, 6.0, 6.8, 7.2 and 8.0 at a concentration of 100mM were used to span the range suggested by the literature for PPO and Prunus spp. polyphenoloxidases in particular.

The effect of temperature variation on pH is reduced due to the need to use a constant temperature water bath with the Clark electrode. However, Cruess and Sugihara in 1948 reporting on olive PPO, showed an interaction of temperature with pH on PPO activity. Several others have also reported a reduction in thermal inhibition with lowered pH (Walker, 1964; Hadziyev, 1987; Cilliers & Singleton 1989; Miller et al. 1990).
5.2 Results:

A broad response to different pH levels was shown for the five pH levels used in this experiment. A pH of 6.8 to 7.0 appears to be optimum, however the results indicated a wide pH range where the enzyme still operated in excess of 60% of maximum activity.

Figure 5.1 indicates that there is variation between fruit, at different development times. Some duplicate and triplicate testing of random fruits indicated less than 5% variation of PPO rate within fruit (results not presented).

Activity of PPO appears to change with age of fruit and this is reflected in Figure 5.2 where PPO activity shows a general upward trend regardless of particular pH of the reaction buffer. A higher rate of increase in activity with age appears to be associated with the higher pH levels except for day 92 at pH 8 which has a reduced activity compared with pH 6.8 to 7.2.

For pH 5.0, maximum rates were associated with day 57 and day 92, pH 6.0 with day 29 as well as day 57 and day 92 peaks. pH 6.8 has peaks at days 57 and 92, but also had a peak at day 1 which pH 7.2 and pH 8.0 appeared not to have. pH 8, day 29 samples had two outlier data which have not been removed from this figure.
Chapter 5. Effect of pH on PPO Activity

Figure 5.1: Mean PPO activity of apricot (cv “Moorpark”) fruit samples for all ages (1 to 92 days) at five pH buffer levels using 4-methyl catechol as substrate (20mM) in a Clark oxygen electrode. Bars indicate standard error of means (n=120). Note that whilst this figure helps to indicate the best pH to use for follow-up studies there may be different fruit development stages interacting to distort the data as indicated in Figure 5.2.

Of the pH responses achieved, pH 6.8 is shown to be most generally higher activity over all ages. There is a trend to a lower optimum of pH 6.8 for lower ages, shifting higher to as the fruit matures, then ripens (Figure 5.2). Thus day 57, 64 and 71 peak at pH 8.0 while later ages (day 85,92) peak at pH 7.2. This latter correlates with the fruit pH and sugar increase with age and a related decrease in organic acids, particularly malic (Jackson & Coombe, 1967). Since the PPO activity was tested in buffers this is only an interesting correlation.
Figure 5.2 demonstrates the high variability in responses obtained for pH for samples of various ages. In particular, higher variability is found with pH 8.0 at ages day 29 and day 64. Each data point represents 10 fruit samples, of which, some had significant outlier data points. Each fruit was allocated an informal description during testing as to its degree of colouration, ease of grinding and final reaction mix colour. These data were revisited in an attempt to explain the variability observed, but no subjective correlation could be made and the observations were not amenable to data analysis.

Figure 5.2: PPO activity of apricot (cv “Moorpark”) fruit samples for ages 1 to 92 days at five pH levels using 4-methyl catechol as substrate (20mM) in a Clark oxygen electrode. Bars indicate standard error of means (n=120).
5.3 Discussion

It is evident from the results of this trial that, as is also shown in chapter 4, the rate of PPO activity varied with the age of the fruit. This was common to all 5 pH levels tested (Figure 5.2). There was a trend for increasing PPO activity levels with age of fruit from youngest (day 1) to very ripe (day 92). This trend has not been previously reported for apricot. However, for other *Prunus spp.* like peach, this had been found (Fraignier *et al.*, 1995).

Overall, buffer pH had an effect on the activity of apricot PPO under the conditions measured. However, these results need to be treated with caution since only 10 replicates of each buffer pH by development age were tested and this may not have been sufficient given the fruit-to-fruit variation apparent.

In considering the effect of pH variation on the activity of apricot PPO it is useful to first consider the model scenario. The pH of fruit purees from a number of fruit have been found to increase with increasing maturity and development age of fruits (Macheix *et al.*, 1990).

Fruit PPOs are normally located in the cytosol or attached to membranes. In some fruit (eg peach, olive) it has been found to be attached (Macheix *et al.*, 1990; Haslam, 1989) and in others (eg eggplant) apparently not attached to membranes based on the activation of the enzyme with substances like Triton X-100 (Statler, 1993). Many reports indicate that the Pi of PPO is around 4.5, or 4.5 and 4.8 if two
Chapter 5. Effect of pH on PPO Activity

Pi’s have been determined. Similarly, the vacuolar contents are perhaps of pH 2-3 while the cytosol is about pH 4.0 (Janovitz-Klapp *et al.*, 1990). For apricots, Layne *et al.* (1996) reported a pH of 3.6 for the cytosol. Katwa *et al.* (1982) working with mango found Pi =4.1 and optimum pH = 5.5 for that PPO which is typical of a number of reports. Thus we find that, in general, the PPO optimum pH is higher than the cytosol. This suggests that substrates and enzymes are both in the protonated form under natural conditions where the enzyme is operating at less than optimum.

The anionic detergent, Sodium dodecyl sulphate (SDS), was used to improve the activation of PPO in these experiments. The effect of SDS activation has been studied in broad bean by Moore & Flurkey, (1990), who characterised the enzyme in the presence and absence of SDS. Activation of the enzyme increased in a sigmoidal manner with increasing SDS concentrations up to a maximum of 1.75 mM. SDS eliminated a low pH optimum induced by an ‘acid shocking’ effect according to Moore and Flukey (1990). ‘Acid shock’ is the process of holding partially purified PPO at neutral pH, rapidly bringing it to a low pH (e.g. 5.0) then rapidly returning it to the previous pH prior to immediate testing (Lerner *et al.*, 1972). Relative electrophoretic mobility and fluorescence of tyrosine and tryptophan residues increased in a complex way as the SDS concentration was increased with this acid shocking. Moore & Flurkey (1990) suggested the ability of SDS to activate the enzyme alters both its enzymatic and physical characteristics and this suggests that a limited conformational change, due to binding of small amounts of SDS, may induce or initiate the activation of latent enzyme. This supports the report by Marques *et al.* (1995), who examined apple PPO with and without SDS. They found SDS had an
effect on the degree of PPO folding during or prior to membrane transport.

Considering the extent of reported use of SDS there are very few papers which examine the effect of SDS per se on the PPO. An unanswered question is what the effect of SDS is on the McIlvaine’s buffer used in this experiment and in many reported PPO assays. That is, the apparent depression in activity with lower pH could be partly due to the combination of SDS and McIlvaine's buffer.

With the exception of grapes (Coombe, 1976) little is known regarding the development-specific time profile of titratable acidity in most fruits. Such data, were it available, might point to any inconsistencies arising from an examination of pH alone where the [K]$^+$ build up in fruit may have reduced organic acid concentration thus generating a misleading fruit pH picture. With the use of buffers the pH would remain stable. However, the ratio of various organic acids such as malic acid and ascorbic acid might change, altering the PPO activity in that way.

If the above PPO enzyme model is generally true for a range of fruits then the situation portrayed in Figure 5.3 could be expected to exist.
Chapter 5. Effect of pH on PPO Activity

Figure 5.3: Probable charge distribution on the PPO protein molecule at various pH regimes corresponding to those expected within normal fruit cells or fruit purees.

Alteration of PPO activity as a function of buffer pH may be caused by:

i) protonation of amino acid side chains at the active site resulting in a change in $V_{\text{max}}$ of the PPO,

ii) changes in the ionic charge of the substrate and,

iii) changes in the enzyme protein conformation and stability.

Protonation or deprotonation of the active enzyme-substrate site of the enzyme generally results in a bell shaped curve, when pH is plotted against the apparent kinetic constant for the enzyme (Stauffer, 1989). The position of the curve depends, in part, on whether the active form of the enzyme is the protonated or deprotonated form. In the case of PPO it is likely that the active form is the deprotonated species since full activity occurs at pH greater than 4.5 in most fruits (Macheix et al., 1990).

Catechol, a common substrate tested with PPO, is an example of a substrate which is active in the un-ionised form. One could expect that at some level of hydroxyl ion
concentration, protonation could occur and the substrate would become less active (Billaud et al., 1996).

In this experiment no correction for substrate ionisation was attempted. It is noted that for strawberries substrates of differing pK’s may result in different optimal pH curves (Wesche-Ebeling & Montgomery, 1990). This is likely to be the case since one is effectively changing substrate concentration in the Michaelis-Menten equation (Cilliers & Singleton, 1989) which will result in a change in the enzyme activity. The pH response was measured by extracting the sample at a specific pH and then reacting the sample at the same specified pH. This, then, was a measure of activity at that pH. It is not a record of denaturation due to pH induced ionisation state since the enzyme was not returned to one stable pH for activity testing.

It may be possible that the results obtained represent thermal denaturation of the enzyme due to pH induced sensitivity to the test condition of 25°C (Komatsu et al., 1993). This was considered unlikely since the enzyme suspension appeared to be stable for 24 hours under bench top conditions (Chapter 3).

The phosphate-citrate buffer used in this experiment binds Ca$^{2+}$ ions and although PPO is not known to require Ca$^{2+}$, there is some evidence that PPO is affected by the calcium content of its native environment (Bower et al., 1989; Souty et al., 1981). However, alternate buffers were not evaluated as the McIlvaines buffer is so commonly used in PPO studies and few problems were reported (Walker & McCallion, 1980; Milstein et al., 1989; Wesche-Ebeling & Montgomery, 1990).
Reports of optimum activity for PPO indicate complete inhibition at <pH 2.0; for example grape PPO was inhibited at pH less than 2 (Sims et al., 1991). Zauberman et al. (1991) also cite in vitro inhibition of PPO at low pH. Generally there are reports of rising activity levels between pH 2.0-4.5 (Zemel et al., 1990) and a wide optimum between pH 5-7.5 depending in the main on the fruit involved and the substrates tested.

Guyot et al. (1995) found an influence of pH on the enzymatic oxidation of (+)-catechin by PPO using aqueous buffers in the range 3-7; the nature and amount of product formed was highly pH dependent suggesting that care needs to be taken in interpreting PPO activity measured at different pH. Guyot et al. (1995) also found that in the model they used, colourless oxidation compounds were formed below pH 4.0, whereas yellow coloured dimers were formed at higher pH values. The results obtained in this experiment suggest that this may have been the case with apricot PPO as tested. This experiment was conducted above pH 4 and coloured compounds (orange-brown) were observed to form. Scant data, with the exception of Habib et al. (1998), where rising pH with fruit development is reported, is available for apricot flesh pH status, either in vitro or in vivo, during maturation and development.

The products of non-enzymatic oxidation of phenolic reactions were characterised in a caffeic acid model system similar to this experiment by Cilliers and Singleton (1989). They found that the pH-sensitive reactions indicated that the production rate of the oxidation compounds is related to the phenolate anion concentration. Richard-
Chapter 5. Effect of pH on PPO Activity

Forget et al. (1992a), examining the oxidation of chlorogenic acid in apples, proposed two pathways for the degradation of 4-methyl catechol ortho-quinones. The first one, at acid pH, corresponds to a hydroxylation followed by a coupled oxidation of another molecule of o-quinone leading to the formation of 2-hydroxy-5-methyl-p-benzoquinone and the regeneration of 4-methyl catechol. The second pathway corresponds to polymerisation reactions at higher pH values and the polymerisation reactions appeared to be dominant. Apparent PPO activity at lower pH levels may therefore be affected by the regenerated 4-methyl catechol becoming available under situations of limited substrate (Bucheli & Robinson, 1994). No adjustment to substrate level was considered necessary for this experiment given the range of pH examined.

A number of results for pH optima are available for Prunus. Siddiq et al., (1996) found that ripe Stanley plum PPO crude extract had a pH optimum of from 5.8 to 6.4 with differing substrates. Other Roseaceae fruit reportedly have higher pH optima. Oktay (1995) examined dog-rose fruit (Rosa dumalis Rechst.) PPO which had been partially purified using gel filtration, ammonium sulphate precipitation and finally, dialysis. They found the best substrate to be 4-methyl catechol with an optimum pH of 8.5 at 20°C with a $K_m$ of 8.64mM and $V_{max}$ of 432 mM min$^{-1}$. They reported the existence of two isomers using polyacrilamide gel electrophoresis. It is not clear from the paper as to whether they considered the optimum of pH 8.5 to be normally high or a possible artifact of the purification method used, such as discussed above for SDS use.
Billaud et al. in 1996 with gum arabic PPO noted that AH (neutral) and A⁻ (dissociated) forms of PPO inhibitor were active, but the AH form was the best inhibitor and had higher affinity for the enzyme or enzyme-substrate complex. That is, the inhibitors were pH dependent. This adds support to Haslam’s (1998a,b) comments on the charge of substrates leading to redox reactions of quinones, whereby quinhydrone formation depends on the presence of a hydroquinone dianion as part of a charge-transfer complex (Figure 5.4). Under more basic conditions one might expect the nucleophilic substitution of the quinone to be slowed and this is supported by the results obtained in this experiment with the less developed fruit stages.

Figure 5.4 Red-ox reactions of para-benzoquinone and the hydro-quinone dianion. Here the reduction potential of the quinones is lowered by less electron-donating substituents on the ring such as OH or Me (Haslam, 1998b).

Lerner et al. (1972) found grape PPO could have its activity changed by pre-incubation conditions, such as acid shock or urea. Generally, $V_{\text{max}}$ increased, affinity
for 4-methyl catechol decreased and affinity for oxygen increased in their studies following pre-incubation treatments. Conversely, Lourencó et al. (1992) reported for potato PPO "preincubation of the enzyme at pH 4.0-7.5 apparently had no effect on activity". No evidence of the existence of a pre-incubation effect on the activity of apricot PPO for various pH levels tested was gathered in this experiment.

Further testing to accommodate this pre-incubation hypothesis is required in future experimentation on apricot PPO. This might be investigated by using acid shock of pH 5.0 prior to testing with the base buffer of pH 6.8 used by default in this experiment which also used a non-purified extract. As Lerner et al. (1972) point out, however, one needs to allow for a change in ionic strength of the medium which pH shock may also cause.

A range of pH was examined for its effect on PPO activity. A common finding of a number of workers is that PPO has a pH optimum in the region of 6.5 to 7.0 (Vamos-Vigyazo, 1981; Dijskra & Walker, 1991). Examining a range of pH around these figures indicated that a buffer pH of 6.8 was most suited for measuring PPO activity for a range of developmental ages. Overall pH 6.8 produced the highest PPO activity, even though at some developmental stages pH 7.2 and pH 8.0 was higher. In the following chapters PPO activity needed to be examined independently of pH effects and therefore the overall best pH of 6.8 was used.
Chapter 6. The Effect of Substrate on Polyphenol Oxidase Activity

This chapter summarizes the effect of substrate level on the level of PPO activity in apricot with increasing fruit age. 4-methyl catechol was used as the basis for experiments reported in this work. There exist a number of alternate substrates upon which apricot PPO could be expected to act since substrates for other polyphenoloxidases include a range of mono- and ortho- diphenols (Fraignier et al., 1995; Siddiq et al., 1996). Apricot PPO has been reported as using both chlorogenic acid, quercetin 3-o-rutinoside and (+)-catechin as substrates (Dijkstra & Walker, 1991; Villanua-Marti et al., 1992) as well as a number of less common phenolics (Mayer & Harel, 1979).

In many instances of fruit PPO activity, the activity has been found to increase with increasing substrate concentration up to a certain level, followed by an inhibitory effect with additional substrate. This inhibitory effect is often put down to product inhibition of the enzyme, but sometimes product competition with the substrate (Mayer & Harel, 1994).

The effect of three of these substrates, L-DOPA, catechol and chlorogenic acid (Figure 6.1) on the rate of PPO activity for apricot fruit as it develops was further investigated.
6.1 Material and Methods

The basic material and methodology is set out in chapter 3 and should be referred to for details. In this series of experiments the normal substrate 4-methyl catechol has been substituted with three alternate substrates in the Clark oxygen electrode reaction cell.

DOPA, (1-DOPA, DL-beta-3,4-dihydroxyphenylalanine) is the primary PPO substrate in some fruits, notably banana (Thomas & Janave, 1986). In various fruit DOPA has been reported as having a mixed type inhibition on PPO activity. That is, both competition for the substrate and non-competitive blocking of the enzyme action occurs where a portion of the enzyme is scavenged from the catalytic turnover as a dead-end complex. This follows after the oxidation of monophenols to o-quinones as described in Sojo et al. (1998) for banana pulp PPO.

Here, DOPA (Sigma Cat. D9503, FW 197.2) was used as a substrate at a level of 2mM in the reaction buffer. This level was considered non-limiting as a substrate but not so high as to cause inhibition, based on prior reports of DOPA as PPO substrate for other fruit (Siddiq et al., 1992; Feng & Zhou, 1996; Arslan et al., 1998). A characteristic lag in PPO activity is observed following the introduction of DOPA to the reaction. Protein-bound DOPA has been implicated in mediating oxidative DNA damage due to its reducing properties in the presence of oxygen (Malovik et al., 1984) and this has been suggested as a possible reason the lag occurs.
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Catechol (Sigma C9510, 1,2-Benzenediol, FW 110.1) as a substrate is reported to show less activity with fruit PPO than 4-methyl-catechol (Wesche-Ebeling & Montgomery, 1990; Zhou & Feng, 1991; Siddiq et al., 1992). Activity was examined here using 2mM catechol, allowing direct comparison with the substrate of choice, 4-methyl catechol.

Chlorogenic acid (caffeoylquinic acid) is reported as one of the main phenolic substrates in apricot fruit (Anet & Reynolds, 1955; Garciaaviguera et al., 1994; Radi et al., 1997). Chlorogenic acid (Sigma cat. C3878, 1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid, 3,4-Dihydroxycinnamic acid, FW 354.3) was used at 2mM as was the default substrate, 4-methyl catechol.

Figure 6.1: Structures of some phenolic substrates including d-I DOPA (centre), catechol (left) and chlorogenic acid (Caffeoyl quinic acid) - bottom left. Catechin (bottom right) is an example flavan-3-ol substrate type.
Chapter 6. The Effect of Substrate on Polyphenol Oxidase Activity

6.2 Results

Fruit were sampled for a period of 92 days at weekly intervals as described in chapter 3. PPO activity with D-L DOPA as a substrate over various apricot fruit developmental ages is shown in figure 6.2 below. This shows a significant reduction in PPO activity compared with the standard 4-methyl catechol substrate. The pattern of activity by age is similar to that described in Chapter 4.

![Graph showing PPO activity over 92 days]

Figure 6.2: Effect of the use of D-L DOPA as a substrate (2mM) on apricot (cv. Moorpark) PPO activity as a function of the maturity of the fruit from ‘day’ 1 (at capfall, approx. 5mm) to 92 days (over-ripe). PPO activity measured at 25°C in a Clark-type oxygen electrode, pH 6.8 buffer. PPO activity data are relative to the pH 6.8 standard using 4-methyl catechol as substrate, both at 2mM. Error bars are standard error of means (n=10).
Chapter 6. The Effect of Substrate on Polyphenol Oxidase Activity

Catechol as a substrate of the apricot PPO is shown in figure 6.3 below. Catechol as a substrate was not as effective as 4-methyl catechol in terms of apparent PPO activity and although perhaps relatively more so in the early maturing stages, day 36 to 71. However, the trend of rising PPO activity with development age is similar to the general trend described in Chapter 4. Some difference is evident. Again, days 29 and 57 show higher activity to catechol in a similar way that they do with 4-methyl catechol. Comparing days 85 and 92 for catechol substrate versus the standard 4-methyl catechol we find activity with catechol much higher for day 92. Day 8 activity appears more depressed, relative to the general trend.

![Graph showing PPO activity with catechol substrate]

Figure 6.3: The effect of catechol as a substrate (2mM) on apricot (cv. Moorpark) PPO activity as a function of the maturity of the fruit from 'day 1' (at capfall, approx. 5mm) to day 92 (over-ripe). PPO activity measured at 25°C in a Clark-type oxygen electrode, pH 6.8 buffer using catechol as substrate, at 2mM. Error bars are standard error of means (n=10).
Chapter 6. The Effect of Substrate on Polyphenol Oxidase Activity

The general response of the apricot PPO to chlorogenic acid as a substrate over various developmental ages is shown in figure 6.4 below. Chlorogenic acid as a substrate was nearly as effective as 4-methyl catechol in terms of apparent PPO activity. Examining the response by age in comparison with the general effect described in Chapter 4 we find a similar rise in PPO activity with age, however with chlorogenic acid there is greater activity for age at day 64, 71 and 92 and less at the younger ages. The variability is much less between fruits at older ages compared with catechol or 4-methyl catechol substrates.

Figure 6.4: Effect of the use of chlorogenic acid as a substrate (2mM) on apricot (cv. Moorpark) PPO activity as a function of the maturity of the fruit from ‘day’ 1 (at capfall, approx. 5mm) to 92 days (over-ripe). PPO activity measured at 25°C in a Clark-type oxygen electrode, pH 6.8 buffer. Figures of PPO activity are relative to the pH 6.8 standard using 4-methyl catechol as substrate, both at 2mM. Error bars are standard error of means (n=10).
6.3 Discussion

The most common phenols reported in apricots are chlorogenic acid and quercetin 3-o-rutinoside (Fernandez de Simon et al., 1992). However, Villanua-Marti et al., (1992) using HPLC and TLC reported only traces of chlorogenic acid in their apricots, claiming 3,4-dihydroxybenzoic (protocatechoic acid) and vanillic acid as the main phenols and the flavonols quercetin and its glycosides (rutin and hyperoside) also present. The affinity of polyphenoloxidases for both substrate and oxygen is relatively low making substrate comparisons difficult (Rouet-Mayer et al., 1993).

Dijkstra & Walker (1991), in New Zealand using the cultivar "Moorpark", found that Chlorogenic acid, (+)-catechin and p-coumaric derivatives were the main phenolics in healthy tissue with traces of caffeic acid. *Penicillium* and *Sclerotinia*-rotted tissue had caffeic acid present and approximately half the previous level of chlorogenic acid found in healthy tissue.

A general trend is evident (Figures 6.2, 6.3 and 6.4) whereby the activity of PPO shows an increase with age as suggested by Dilley et al. (1993). Some difference is evident. Again, days 29 and 57 show higher activity to catechol substrate in a similar way that they do with 4-methly catechol. Comparing days 85 and 92 for catechol substrate versus the standard 4-methly catechol we find activity with catechol much higher for day 92 while day 8 activity appears more depressed, relative to the general trend.
Chapter 6. The Effect of Substrate on Polyphenol Oxidase Activity

Catechol and 4-methyl catechol react with PPO with identical affinities, but are oxidised at significantly different maximum rates: the presence of a methyl group in the \textit{para} position increases the catalytic efficiency of the enzyme. This methyl group is an electron donor. Therefore, one can expect that electron-attracting substituents will result in a decrease of enzyme reaction rate.

Chlorogenic acid as a substrate was nearly as effective as 4-methyl catechol in terms of apparent PPO activity. Examining the response by age in comparison with the general effect described in Chapter 4 we find a similar rise in PPO activity with age, however with chlorogenic acid there is greater activity for age at day 64, 71 and 92 and less at the younger ages. Variability is much less between fruits at older ages compared with catechol or 4-methyl catechol substrates, remembering that the overall levels were much higher. This lower variability is consistent with the literature, however the relatively higher activity at higher ages has not been reported previously.

DOPA, when used as substrate, induces a reaction lag, seen here also, thought to be due to the need to form an enzyme-substrate complex where the phenolic -OH group facilitates a nucleophilic attack of the oxygen on the copper within the enzyme active site. There is considerable debate on the mechanism. It is known that the lag period increases with increasing pH and decreases with increasing hydrogen peroxide concentrations (Jimenez & Garciacarmona, 1993).
Chapter 6. The Effect of Substrate on Polyphenol Oxidase Activity

The lag period is a function of

i) enzyme concentration where higher DOPA concentrations extend the lag period,

ii) solution pH,

iii) substrate concentration and

iv) the presence of a catalytic concentration of ortho-diphenols (Lerner et al., 1972; Espin et al., 1997).

In various sub-samples the lag varied from approximately 30 seconds to 180 seconds although there appeared to be no consistent trend. Records made of fruit appearance did not suggest any correlation between appearance and the DOPA substrate lag period.

Note that DOPA is highly hydrophilic and the use of SDS, a detergent, may have had an effect on the result compared with non-hydrophilic substrates like catechol.

With the low affinity of PPO for its substrate and oxygen, the results indicate more work could be done on apricot fruit PPO with chlorogenic acid together with flavenol and flavenol glycosides as substrates. Examining interactions between these natural substrates may help account for the chlorogenic acid substrate response with age particularly as ripening occurs.
Chapter 7. Inhibition of Apricot Polyphenoloxidase Activity by Sulphite

At the present time, the use of sulfur dioxide or sulfites is the most effective method of inhibiting browning in commercial use. When dried apricots and potatoes were analysed (McBean et al., 1963), it was found that in most cases about 50 percent of the sulphur dioxide was lost before any significant amount of browning developed (Monzini & Gorini, 1991; Hauser et al., 1993). The temperature of storage and the moisture content of the product also influence the loss of SO$_2$ during storage (Monier-Williams, 1927; Cape, 1984; Karolewski, 1986; Dahlenburg et al., 1990a; Valero et al., 1992; Baritaux et al., 1991; Wedzicha & Herrera-Viloria, 1991; Diehl et al., 1992; Swales & Wedzicha, 1992; Edberg, 1993).

Sulphite ion acts as a reducing agent and is consumed in the process of reducing o-quinones produced by PPO back to the parent o-diphenols. Metabisulphite can also react with intermediate quinones to form sulpho-quinones. Finally, the metabisulphite may inhibit PPO. The inhibition of PPO by SO$_2$ is not reversible; so that after-sulphuring removal of excess SO$_2$ will not result in regeneration of the PPO which has been inactivated (Imberty & Goldberg, 1984; Gould, 1989; Valero et al., 1992).

SO$_2$ is added as sulphite solution or as SO$_2$ gas, and to be effective the SO$_2$ must penetrate the fruit tissue quickly before any oxygen inside the tissue reacts with quinones formed near cut surfaces; otherwise discoloration occurs. SO$_2$ as a gas
penetrates more quickly and evenly than as a sulphite solution (McBean et al., 1963; McBean & Wallace, 1967b; Embs & Markakis, 1965; Cape, 1984; Russell & Gould, 1991). Once in tissue or juice the SO\textsubscript{2} is generally in the sulphite ion form at the pH of fruits.

Both sulphite and bisulphite may be oxidised by oxidising agents to produce substances such as sulphates or dithionate anions. The sulphite anion may act as a Lewis base due to its lone pair of electrons. Conversely, since it may accept electrons in its 3\textit{d} shell, it may also act as a Lewis acid. Sulphite anions react with oxygen to form free radicals of the form SO\textsubscript{3}\textsuperscript{-} and it is thought that superoxide radicals are also formed as intermediates (Valero \textit{et al}., 1992; Du \textit{et al}., 1994). In short, sulphite is very reactive and may form many compounds (Petrucci, 1989).

Sulphite may also react with a number of other functional groupings and the following list is compiled from Wedzicha (1984, 1992) indicating why sulphite prevents PPO activity.

**TABLE 7.1:** Reactive organic groups with sulphite.

<table>
<thead>
<tr>
<th>Reactive group</th>
<th>Biochemical source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonyl</td>
<td>Aldehydes and ketones</td>
</tr>
<tr>
<td>Disulphide bond</td>
<td>Protein, glutathione</td>
</tr>
<tr>
<td>Schiff's bases</td>
<td>Enzymes and cofactors</td>
</tr>
<tr>
<td>Pyridines</td>
<td>NAD\textsuperscript{+}, NADP\textsuperscript{+}</td>
</tr>
<tr>
<td>Pyrimidines</td>
<td>DNA, RNA</td>
</tr>
<tr>
<td>Quinones</td>
<td>Electron carriers</td>
</tr>
<tr>
<td>C=C double bonds</td>
<td>Fatty acids, enzyme cofactors</td>
</tr>
</tbody>
</table>
Wedzicha suggests that carbonyls are amongst the most common targets for sulphite attack because they are found in many aldehydes and ketones in fruit tissues. Proteins and enzyme co-factors may contain disulphide links such as cysteine. These too, are often important in the stability of tertiary structure in proteins (Brandon & Tooze, 1991). These bonds react with sulphite to give a cysteine-thiosulphonate:

\[ R-S-S-R + SO_3^- \rightleftharpoons R-S-S-O_3^- + R'S^- \]

This reaction causes not only a disulphide link break and structural damage, but also produces a pair of negatively charged residues causing changes in hydration. Enzymes may thus react specifically with sulphite forming an inactive adduct with sulphite (Gould, 1989). The susceptibility of disulphide bonds to sulphite damage varies with the nature of the adjacent residues which have formed from the folding of the protein (Wever, 1987). Apricot polyphenoloxidase has a number of disulphide bonds that could be affected in this manner (Berke et al., 1999).

As a result of the complex chemistry we find sulphite function is different with type of product. For dried fruits or those products whose water activity has been reduced, the mechanism of action is perhaps mainly by directly inhibiting chemical changes, some of which are enzyme enhanced (Schroeter, 1966; Wever, 1987), compared with drinks and fresh foods which consist mainly of low pH, dilute solutions.
Chapter 7. Inhibition of Apricot Polyphenoloxidase by Sulphite

The level of sulphite required to prevent apricot PPO activity is high. The range for dried fruit is typically 100 to 3000ppm with the allowed concentrations varying greatly from country to country, although increasing pressure is being applied to reduce usage due to a small risk to asthmatics (Edlefsen & Brewer, 2001). Dried apricot may require 4000ppm, falling to 2500ppm after a month (Dahlenberg et al., 1989a; Hill, 1988; Edberg, 1993), whereas prevention of discoloration in dried potato may require only 250ppm (Kim & Kim, 1986; Moylan et al., 1986; Giannuzzi & Zaritzky, 1990).

Whilst Dahlenberg (1976) and McBean et al. (1950a, 1963, 1964, 1967a,b) had reported on the effects and retention of SO$_2$ on halved, fresh apricots, no PPO specific testing of sulphite inhibition in these has been reported. This experiment therefore examined the effect of five levels of sulphite on PPO activity through the 12 different development ages.
7.1 Materials & methods

The basic material and methodology is set out in Chapter 3 and was that used except for the conditions outlined below.

The standard method was adjusted to include the delivery of sulphite ion into the reaction cell prior to temperature stabilisation. All tests were conducted using 4-methyl catechol, as before, as PPO substrate. pH was maintained constant using pH 6.8 buffer in both the extraction phase and the reaction cell. Sodium metabisulphite (Na$_2$S$_2$O$_5$, SMS) 0.5mM was used as the source of sulphite ion (SO$_3^-$) for these experiments in line with commercial conditions, since the use of SO$_2$ gas was not practical with such small samples.

7.2 Results

A similar trend to previous results (c.f. Chapter 4) was found for the relationship between age and activity (Figure 7.2). There was a general increase in activity with age of development for the fruit. The added sulphite caused a depression in average activity levels to approximately 30% of non-inhibited levels (Figure 7.2).

The effect of sulphite addition to the PPO activity was not only on the rate of the reaction, but also on the initiation. A pronounced lag was evident in the reaction where higher concentrations of sulphite were used (Figure 7.1).
Chapter 7. Inhibition of Apricot Polyphenoloxidase by Sulphite

Figure 7.1: Reaction lag output from the Clark oxygen electrode. Normally, following addition of substrate, there is a rapid development of a linear slope (left), which degrades into a slower rate curve associated with auto-inhibition. The straight line was used to show the slope of the initial reaction. When a lag occurs, as shown by the right hand slope line, a distinct delay occurs between adding the substrate and the beginning of oxygen use indicated by the sloping line. Lourenco et al. (1992) associated this lag with complexing of quinones with sulphite.

Figure 7.2: Averaged PPO activity of apricot (cv. Moorpark) in the presence of 0.5mM sulphite by development ages. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Error bars are standard error of means (n=10).
Chapter 7. Inhibition of Apricot Polyphenoloxidase by Sulphite

Results (Figure 7.3) demonstrate that sulphite ion, under the experimental conditions, shows strong inhibition of PPO activity. As the concentration of sulphite is increased there is an approximately exponential increase in the inhibition.

Figure 7.3: Effect of different concentrations of sulphite addition as sodium metabisulphite on apricot (cv. Moorpark) PPO activity. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Means of 10 fruit sampled by 12 developmental ages. Error bars are standard error of means (n=118).

There was a high degree of variation in inhibition for individual samples tested at the two highest levels of sulphite in the more mature fruits and this is illustrated for these two higher concentrations (Figure 7.4). There appears to be an interaction between age and sulphite inhibition at the higher concentration of 5 mM sodium metabisulphite.
The inhibition is very effective up to day 71, but for day 85 and day 92 there are a number of individual fruit samples which were not completely inhibited. That is, there were fruit where the highest concentration did not knock out all activity.

Figure 7.4: PPO activity of apricot (cv. Moorpark) in the presence of sulphite by development ages. Effect of sulphite level for 12 development ages showing trend for ages for the two highest SO2 levels applied. Activity was assayed with 2 mM 4-methyl catechol and pH 6.8 buffered apricot suspension containing PPO enzyme. Error bars are standard error of means (n=10). Note that the Y axis scale is expanded here compared with Figure 7.2.
7.3 Discussion

Sulphite application to an apricot (cv. "Moorpark") solution has demonstrated inhibition of the PPO activity by a significant, but not complete, amount. It is clear that a small amount (0.2mM) has had a large, initial effect while more than doubling the amount does not produce a corresponding doubled inhibition (Figure 7.2). Why this might be so is considered below.

Sulphite uptake and inhibitory actions are complex. Sulphite inhibits the enzyme directly and by a variety of competitive actions on substrates. Sulphite action is also partly controlled by the solution pH (Figure 7.5). The energy status of the electrons in the outer orbitals of the sulphur atom mean that it may exist in a "hypervalent" state. The atom can form up to six covalent bonds by promoting electrons into the '3d' level and using pd-orbital hybridisation for bonding (Timberlake & Bridle, 1980). A great variety of salts may thus form (Wedzicha, 1984). The tetravalent state of sulphur produces the simpler oxoanions that are salts of sulphurous acid \( \text{H}_2\text{SO}_3 \) which should be formed when SO\(_2\) and water meet. There is evidence from spectral studies that this does not happen but rather a clathrate of SO\(_2\) inside a sphere of water molecules forms (Cotton & Wilkinson, 1966).

The postulated clathrate structure may be designated as \( \text{SO}_2\cdot7\text{H}_2\text{O} \) from which a first dissociation produces a bisulphite anion as \( \text{HSO}_3^- \).

\[
\text{SO}_2\cdot\text{H}_2\text{O} \rightleftharpoons \text{HSO}_3^- \text{ and } \text{H}^+ 
\]
The bisulphite anion may undergo further dissociation to form the sulphite anion in which we are interested.

\[ \text{HSO}_3^- \leftrightarrow \text{SO}_3^{2-} \text{ and } \text{H}^+ \]

Studies on these anions suggest that the first dissociation has a pK value of about 1.9 and the second at about 7.2. This implies a mixture of SO$_2$.7H$_2$O and HSO$_3^-$ anion may be present in acidic fruits, whilst neutral or slightly alkaline fruits might have a balance of HSO$_3^-$ and SO$_3^{2-}$ present (Figure 7.4).

In an acidic fruit, if there is SO$_2$ applied, then there will be all three forms present either within or between the cells. This should reduce the uptake of sulphite by the tissue. However, the dissociations are calculated for dilute solutions which occur in fresh fruit juice. For less dilute solutions, the bisulphite may condense to form metabisulphite, with the loss of water. This reaction mechanism, when reversed, allows the use of the alkali metal metabisulphites, which have good solubility at room temperatures, to introduce SO$_2$ into foods for preservation. That is;

\[ \text{S}_2\text{O}_5^{2-} \leftrightarrow \text{HSO}_3^- \leftrightarrow \text{SO}_3^{2-} \leftrightarrow \text{SO}_2 \cdot x\text{H}_2\text{O} \]

For example, when sodium metabisulphite is added to water, bisulphite anions are formed in equilibrium with sulphite and sulphur dioxide. The equilibrium is pH dependent. Vas (1949) and Braverman (1953) found the optimum pH for combined SO$_2$ in peach and apricots to be between pH 3.0 to 5.5, but the uptake of SO$_2$ was increased with increased soluble solids (mainly as sugars).
When the molecular SO$_2$ reaches the interior of the cell it would be transformed into the HSO$_3^-$ and SO$_3^{-2}$ species by dissociation at about pH 6.5 leading to the removal of SO$_2$ from the diffusion equilibrium with sulphite, being the active anion left. Sooner or later the internal pH will fall with continuous addition of SO$_2$ to the system (Wedzicha, 1984; Pilkington & Rose, 1989; Garbarino et al., 1992; Mukai et al., 1993).

As fruit develop the soluble solids increase, as they did with these samples (Figure 3.1). One would expect that, if the premise above is correct, the uptake of sulphite would increase causing increased inhibition with development age and increased TSS. The results (Figure 7.3) do not support this idea. One could speculate a number of reasons. One, that the test conditions, even though a buffered puree was used, may not represent the even higher sugar concentrations in actual fruit. Second, that the PPO enzyme level increased disproportionately to the added sulphite with development age, thus actually reducing the sulphite inhibition effect. Thirdly, the in vitro experimental conditions of buffer chemicals and SDS, a sulphate, albeit at very low levels, may have reduced the PPO activity.

At the highest sulphite level there was a little activity shown between day 22 and day 71 (Figure 7.4), however even though 10 replicates were done, the level of activity is so low that it may reflect an artifact in the oxygen electrode method used.
In summary, the results obtained show that apricot PPO is inhibited by the addition of sulphite, in the form of sodium metabisulphite, to the reaction solution. There are a number of interactions that could confound the results, chief among them is the pH of the solution and the complexity of sulphur chemistry, although the pH used should not have caused these effects here given that buffers were utilised. With respect to the maturity of the fruit (development age) the result shows an indication that PPO isozymes are expressed throughout ripening, as suggested in previous chapters and by some authors (Chevalier et al., 1999), and may be differentially sensitive to sulphite. Further work, using molecular techniques, is required to confirm the existence of isozymes in apricot PPO.
Chapter 8. Inhibition of Apricot Polyphenoloxidase by Sodium Chloride

Salt is ambivalent in that we need a small amount to stay alive, but too much will kill us. So it may also appear to be the case with PPO.

Inhibition of PPO by halides has been demonstrated by many sources (Mayer & Harel, 1979). Here PPO activity was assayed in the presence of various levels of sodium chloride at pH 6.8, as inhibition has been shown to be strongly pH dependent (Mayer et al. 1966).

Salt as Na\(^+\) and Cl\(^-\) ions is naturally present in apricot fruit (Wills, 1987; Senhaji et al., 1991). The question is whether cultural techniques such as the use of saline irrigation water and resulting increases in tissue Na\(^+\) and Cl\(^-\) (Walker et al., 1988) or the use of NaCl in solutions added to apricot tissue may have an effect on PPO activity in the tissues. Assuming that substrate is present, then changes in PPO activity might be associated with the changing level of NaCl applied to the fruit.

A second question is whether the PPO in developing apricot exhibits a differential response to increased solution NaCl over the development period. These questions are examined in this chapter.
8.1 Materials and methods

The basic material and methodology used is set out in chapter 3 and was that used except for the conditions outlined below. The standard method was adjusted to include the delivery of a saline solution (0.01, 0.05, 2 and 5 mM) into the reaction cell prior to temperature stabilisation. All tests were conducted using 4-methyl catechol as before as the substrate and the pH was maintained constant using pH 6.8 buffer in both the extraction phase and the reaction cell as before. Control levels were 0 mM added NaCl but fruit would contain low levels of Na\(^+\) and Cl\(^-\). Figure 8.1 shows data for a preliminary study using fruit at day 85 age, results suggesting the method and ranges were effective in inhibiting PPO activity.

![Graph showing PPO activity of apricot (cv. Moorpark) assayed for a range of sodium chloride concentrations at pH 6.8 for five replicates at each concentration using a bulk sample of ripe fruit (age 85 days). Note the lower range used here is 0.01 and 0.05 mM whereas the main experiment used 0.2 and 0.5 mM. Error bars represent standard error of means (n=5).]

Figure 8.1: PPO activity of apricot (cv. Moorpark) assayed for a range of sodium chloride concentrations at pH 6.8 for five replicates at each concentration using a bulk sample of ripe fruit (age 85 days). Note the lower range used here is 0.01 and 0.05 mM whereas the main experiment used 0.2 and 0.5 mM. Error bars represent standard error of means (n=5).
Chapter 8. Inhibition of Apricot Polyphenoloxidase by Sodium Chloride

8.2 Results

Results are variable, principally reflecting stage of development, as do results in previous chapters. There were some significant differences. One sample with a large proportion of skin in it showed a high rate of PPO activity, whilst another with a lower proportion of skin in the sample had a lower apparent activity than others in that salinity level group. No real pattern emerged; however, it was suspected that the presence and state (coloured or not) had an effect on the outcome of individual tests in some instances. Figure 8.2 showing the response to five levels of added NaCl does show a slight increase at the 0.2mM level than for the control.

![Figure 8.2: Effect of different concentrations of NaCl on PPO activity of apricot (cv. Moorpark). Activity was assayed with 2 mM 4-methyl catechol and pH6.8 buffered apricot suspension containing PPO enzyme. Means of 10 fruit sampled by 12 developmental ages. Error bars are standard error of means (n=118)](image)
During experimental work it became apparent that there may be an interaction between pH level of the reaction buffer and NaCl concentration (Whitaker & Lee, 1995). Figure 8.3 depicts the outcome of testing to determine if this interaction was the case for these apricots under the conditions used. It is apparent that there is an interaction at pH 4.8 with higher NaCl concentration causing a greater depression of PPO activity, but this was not found for other pH levels. Dropping the pH level below pH 6 also reduced PPO activity.

Figure 8.3 : Effect of two concentrations of NaCl (33 and 66mM) at a range of lower pH levels on apricot (cv. Moorpark) PPO activity for firm-ripe fruit. Activity was assayed with 2 mM 4-methyl catechol and pH buffered apricot suspension containing PPO enzyme at pH indicated. Means of 3 fruit at 71 days age. Error bars are standard error of means (n=3).
Chapter 8. Inhibition of Apricot Polyphenoloxidase by Sodium Chloride

When the data are considered from an age by response to NaCl level one sees not only more tolerance for NaCl in more developed fruit, but also a relative decrease in inhibition of PPO activity in more mature fruit with increasing concentration of NaCl (Figure 8.4).

Figure 8.4: Effect of 4 different concentrations of NaCl by 4 ages of fruit on apricot (cv. Moorpark) PPO activity. Activity was assayed with 2 mM 4-methyl catechol and pH6.8 buffered apricot suspension containing PPO enzyme and various NaCl levels. Early development indicated as 'day1', 'day8' and later development as 'day85' and 'day92'. Log means of 10 fruit sampled by 4 developmental ages. Error bars are standard error of means (n=10).
8.3 Discussion

Sodium chloride, when added to the apricot PPO solution restricted activity depending on the salt concentration as demonstrated in Figure 8.2. Higher concentrations caused greater inhibition until, at 5mM added NaCl, PPO activity was negligible.

Nilsson and Adler (1990) point out that NaCl (range approx. 3 to 160mM) is found to initially promote PPO activity then inhibit, it in salt-tolerant yeast, as the concentration increases beyond a certain point. A slightly higher PPO activity was also shown to occur for apricot fruit here at the lowest added NaCl level. The results obtained (Figure 8.2) support the conclusion of Nilsson and Adler (1990) in that, PPO activity initially rose with some NaCl (0.2mM), then fell away as the concentration increased. The combination of conditions and NaCl concentration used in this experiment with this PPO may not have been sufficient to demonstrate the full effect. A finer range of NaCl concentration, perhaps 0.1 to 0.5mM, may have shown more increase in PPO activity.

Subhashini and Reddy (1990) in rice callus cultures found that increasing salinity in the growing medium increased PPO and other enzyme activities suggesting an evolved protective role of increased PPO activity under higher salinity conditions. No NaCl levels were measured, but callus cultures and apricot fruit suspensions are likely to have similar osmotic conditions. In apple cubes also, Pizzocaro et al., (1993) found that dipping cubes into NaCl solutions (approx. 3 to 15mM range) resulted in an apparent increase in PPO activity. While it can be suggested that low
levels of NaCl promote PPO activity Lourenco *et al.*, in 1992 reported that the PPO from sweet potato appeared to be protected from thermal degradation by elevated NaCl levels. They suggested the effect depends on the concentration and nature of the salts.

A particular facet of salt effect on plants is that of the molar ratio of Na$^+$, Ca$^{++}$ and Mg$^{++}$. The relative levels of these ions were not tested, but it is known that increasing fruit Ca$^{++}$ results in reduction of PPO activity in plums (Kotze *et al.*, 1989). Commercially, calcium addition is used to improve fruit quality and reduce browning using organically complexed carriers (Wooldridge & Joubert, 1997). Assuming that the calcium levels in the apricots was constant during the development ages tested would allow comparisons of NaCl by age. However, whilst no reference could be found for support, it might be expected that calcium levels in the flesh would decrease through the pit hardening ages, and later again, when the pectins de-polymerise (Lu & Ouyang, 1990; Javeri *et al.*, 1991; Kailasapathy & Melton, 1995; Otoguro *et al.*, 1995; Taylor *et al.*, 1995; Alonso *et al.*, 1995; Alonso *et al.*, 1996; Krall & McFeeters, 1998).

Similarly, no measure or estimate of the level of water stress of the plant or fruits was taken although no stress was apparent during development. It is known that compatible solutes such as betaine, which substitute for water of hydration around proteins, increase in response to water stress and salinity levels in some plant tissues (Matoh *et al.*, 1987) although it is not known that compatible solute level affects PPO activity.
In general NaCl, when added to grapevine extracts results in inhibition of the PPO at levels in excess of 50mM (Walker, 1994). Walker did not speculate upon the mode of inhibition, although one notes that the NaCl level was an order of magnitude higher so the report may not apply here.

It is possible that both Na\(^+\) and Cl\(^-\) have independent PPO inhibition effects. Laser flash photolysis was reported by Sullivan et al. (1993) as a technique used on another oxidase, sulphite oxidase, to determine the effects of increasing salt concentration. In their paper Sullivan et al. (1993) explored the dependence of the rate constant and extent of intramolecular electron transfers on pH and NaCl concentration resulting in decreased enzyme activity. Their results suggest that salt greatly decreased intramolecular electron transfer rates (1500 s\(^{-1}\) to < 100 s\(^{-1}\)) due to the binding of anions such as Cl\(^-\) and SO\(_4\)\(^{2-}\) to the enzyme, inhibiting the rate of intramolecular electron transfer. There is no evidence that this mechanism also operates in the phenoloxidases, but a similar semiquinone based electron reduction to sulphite oxidase has been proposed for PPO (Robinson & Eskin, 1991; Chang et al., 1994) and a similar process seems likely.

Inhibition by NaCl was found by Pizzocaro et al. (1993) in Golden Delicious apples where they obtained a 90-100% inhibition if the dip contained 10g ascorbic acid plus 0.5 g/litre salt. This is consistent with a model of salt induced hydration of the enzyme. Again in apple, Janovitz-Klapp et al. (1990) found that sodium halides inhibited PPO and in decreasing order from NaF, NaCl, NaBr to NaI, and this was
Chapter 8. Inhibition of Apricot Polyphenoloxidase by Sodium Chloride

corroborated by Lourenco et al. (1992). Fluoride ions were found to be particularly inhibiting with the Ki for HF being just 4µM.

The more common chloride ion was found to be a non-competitive inhibitor showing an apparent variation in Ki (0.1 to 0.8mM) with a lower 4.5 pH solution by Janovitz-Klapp et al. (1990). NaCl is normally present in its ionised form in biological systems. The free [Cl\(^-\)] is thus available to react in its own right. Janovitz-Klapp et al. (1990) noted that [Cl\(^-\)] was a non-competitive inhibitor of PPO. No mechanism was proposed. Previously, Penafiel et al. (1984) proposed that [Cl\(^-\)] and copper formed a complex after displacement of a protonated histidine residue from copper. This is only thought to apply when the enzyme molecule is protonated so that again, Cl\(^-\) inhibition would depend on pH. The effect would not be expected at the pH 6.8 used, but rather at below pH 5.0 (Janovitz-Klapp et al., 1990).

Two papers point out that dilute NaCl disperses microgels of alkaline soluble peach pectin into rods, segmented rods and kinked rods which collectively comprise the internal gel network in microgels. Dilute NaCl was applied to peach purees from mature fruit by Fishman et al. (1992, 1993a) when examining pectin dissociation. They found increasing NaCl (5 to 50mM) caused a change in the structural organisation of pectin microgels leading to disaggregation under alkaline conditions. It is of interest that pectinesterases are thus activated by elevated [Na\(^+\)] in Prunus avium (Kabar et al., 1997) so that there may be an interaction between pectin status and salt treatment in fruit suspensions such as used in this experiment.
Experimental results in this study indicate little variation in response with age other than a general increase in activity with increasing maturity found in previous chapters. It is apparent from Figure 8.5 that there are three developmental stages where a lower PPO activity exists. That is, day 1, day 15 and day 44.

Rzepecki & Waite (1989), in developing a chromogenic assay for PPO using proline adducts noted that salt affected the response especially when the pH was less than 7.5. In particular, the background oxidation of 4-methyl catechol (the substrate) increased with higher salt concentrations. Whether the adducts extra O\textsubscript{2} use is likely to cause a significant rise in apparent PPO activity rate is uncertain.

Response to salt varies within a plant tissue. Uritani et al. (1990) reported that banana pulp and peel PPO activity is inhibited 40-50% by 0.13M NaCl (a very high level) whereas the male flower, peduncle and bract PPO activity was only inhibited to 20-30% of normal by the same level of NaCl. Oba et al. (1992), again with banana, also found differential response of PPO to salt by tissue type. As tissue within fruit varies also, one could speculate that different PPO activity between fruit samples might be due to differing proportions of epi-, meso- and endo-carp as fruit develops.

Considering NaCl as a sodium ion we find sodium dodecyl sulphate (SDS) is routinely used to activate PPO. It has been suggested that SDS alters both the enzymatic and physical characteristics of the enzyme molecules via a limited conformational change (Angelton & Flurkey, 1984; Moore & Flurkey, 1990).
Chapter 8. Inhibition of Apricot Polyphenoloxidase by Sodium Chloride

Routinely the levels suggested are 0.1% of SDS to achieve activation which is the level of SDS used in these experiments. It raises the question of whether the sodium ion, per se, is having an effect on PPO rate and may be a reason why no significant increase in rate with low NaCl added was observed in the results reported here.

The experimental method (chapter 3) used here did not provide for preconditioning of the enzyme. Considering NaCl as an osmotic salt, Tremolieres and Bieth (1984) examined poplar leaf PPO which had been purified to “..almost complete homogeneity…” by them with a combination of ammonium sulphate precipitation, Sephadex G75 filtration and DEAE-cellulose chromatography. They made the claim that PPO activity is insensitive to NaCl up to at least 1M if tested immediately after addition of the NaCl using two levels of catechol as substrate. However, after one hour pre-incubation of PPO with only 0.3M NaCl the enzyme activity was decreased by 50%. No explanation was put forward.

In summary, the results obtained show that apricot PPO is inhibited by the addition of NaCl to the reaction solution. There are a number of interactions which could confound the results, chief among them is the pH of the solution, although the pH used should not have caused these effects. With respect to the maturity of the fruit (development age) the result shows no substantial indication that PPO isozymes expressed with increasing maturity, suggested by some authors, are differentially sensitive to NaCl.
Chapter 9.  Heat Inactivation of Apricot Polyphenoloxidase

Apricot fruit used for drying and fruit purees are prone to go brown during storage at ambient and coolroom temperatures. The browning is primarily thought to be due to the action of polyphenoloxidase (Dahlenburg et al., 1989a). One avenue of control is the possibility of heating the samples or product with the objective of preventing the enzyme(s) acting later. The heating can cause denaturation or inactivation.

Stauffer (1989) states that denaturation is the heat induced spontaneous, irreversible breakdown of the secondary and tertiary structure of the enzyme protein such that the enzyme will no longer function and cannot re-activate. If the enzyme is tested by sub-sampling at various times during heating at a number of set temperatures, a log plot of activity of the sub-sampled enzyme against time will provide a straight line with slope \(-k\) as the first-order rate constant. With further testing at different pH levels Stauffer points out that one may predict the rate of denaturation of the enzyme, at any temperature and pH, so long as it is within valid extrapolation range.

Confirmation of the result is required, since it may be confounded by substrate recycling by ascorbate in the case of PPO; or by the presence of two enzymes - here catechol oxidase and laccase - with appropriate substrate (Walker & McCallion, 1980). Finally, the solvent system under test may influence the rate (Kazandjian & Klibanov, 1985).
Inactivation in some cases infers the enzyme will function at normal conditions again after the high temperature stopped it (Robinson et al., 1993a). In other cases inactivation means the enzyme will not function at or beyond a certain specified (higher than ambient) temperature (Cosnier & Innocent, 1993, Palmieri et al., 1993, Matsumoto et al., 1993; Heimdal et al., 1994). "Inactivation" is used in this thesis to describe the condition of the enzyme-substrate system subsequent to raising sample temperature above ambient.

When inactivated the system has been subjected to temperature conditions which prevent the system from operating again under normal conditions and operating temperatures. An example is the heating of Mango fruit sap polyphenoloxidase (Robinson et al., 1993) where enzyme samples were heated in a water bath at 80°C and then tested for activity at 25°C after specified times of exposure to find whether the enzyme had been inactivated.

In this study the large number of samples throughout development limited the range of test condition choices. The objective was primarily to determine if there was a differential response in PPO activity to high temperature inactivation with development stage. A secondary objective was to determine the temperature at which significant inactivation was obtained, this having potential value to commercial operations.
Various options exist for examining the high temperature inactivation of an enzyme under study. One may heat a sample, heat an extract, or heat a purified enzyme - perhaps assuming that the extraction has not materially altered the enzyme's *in vivo* response. Compounded on these choices one may alter the time, ambient test temperature (e.g. 20°C or 25°C), substrate and pH conditions (Kristjansson & Kinsella, 1991).

Due to the large number of sample replicates in this experiment testing for decreased activity following exposure to various temperatures for a fixed time was used, instead of varying time at a fixed high temperature.
9.1 Material and methods

The basic material and methodology is set out in Chapter 3 and should be referred to for details. Whole fruit wedges for fruit of various ages (day 1 to day 92) were incubated, submerged in Eppendorf tubes, in agitated water baths maintained at constant temperatures of 25°C, 35°C, 45°C, 55°C and 65°C for a 30 minute period before cooling and testing for ambient temperature activity. Due to the thin walled, small tubes used and the very low weight of sample (less than 0.3g) compared with the volume of heated water (2 litres), the temperature of the sample equilibrated with the water bath temperature within 1 to 2 minutes of the 30 minute heating period. Tissue was subjected to these conditions whilst intact with a small amount of air surrounding the sample in the tube. No buffer or liquid was added to the tube prior to heating.

At the end of the heating time the samples were immediately cooled to 10°C with running water over the test tube. Samples generally required somewhat more liquid nitrogen to freeze them prior to grinding than did those samples taken directly from the freezer, since they needed to be cooled from +10°C rather than -18°C. Samples were tested immediately for PPO activity using the basic method as described in Chapter 3.

The potential effect of heat on the phenolic substrates per se that might have been present in the tissue extract is discussed later. It was not provided for in the method used when determining high temperature inactivation.
A non-replicated pre-test indicated no activity following 30 minutes holding at 75°C. Temperatures were chosen for the range 25°C to 65°C similar to Baur et al. (1997). In the event that the results showed a decisive effect that could be recommended to industry, the above fixed temperature by time condition could be applied without change.
9.2 Results

The overall effect of high temperature treatment on activity is shown in Figure 9.1. In general there was a decline in activity with increasing temperature. Temperatures at, and above 45°C, caused significant reduction in apparent activity. Whilst the mean values for 55°C (446) and 65°C (4.3) are low, two development ages (day 22 and day 85) showed quite high residual activity, up to 10.6 µmol O$_2$ gFwt$^{-1}$ min$^{-1}$ for the higher 65°C as shown in Figure 9.3.

When age of fruit as days of development is considered by activity level at different temperatures we find a relationship as depicted in Figure 9.2. The overall trend to increasing activity with age exists while activity level is depressed with increasing heating temperature.
Figure 9.1: PPO activity for tissue intact apricot (cv. Moorpark) fruit wedges incubated at the heating temperature indicated prior to testing. Eppendorf tubed samples were held submerged in water baths at the indicated temperatures for 30 minutes prior to normal extract preparation. Bars are standard error of means (n=120).
Figure 9.2: PPO activity by age for tissue intact apricot (cv. Moorpark) fruit wedge samples incubated at the heating temperature indicated prior to testing. Tubed samples were held submerged in water baths at indicated temperatures for 30 minutes prior to normal extract preparation. Bars are standard error of means (n=10).
It is of interest to note that, as shown in Figure 9.3, there is still some small amount of PPO activity apparent at 65°C. There is greater variation shown with the later development ages. Whilst these trends here are means of 10 samples, it should be pointed out that the lower figures represent the limits of detection of the oxygen electrode, and need to be interpreted with caution.

Figure 9.3: PPO activity for tissue intact apricot (cv. Moorpark) fruit wedge samples incubated at the heating temperature of 65°C prior to testing. Tubed samples were held submerged in water baths at 65°C for 30 minutes prior to normal extract preparation. Columns represent means for 10 fruit samples from the specified age of fruit. Note the figure has the Y-axis scale expanded compared with Figure 9.1. Bars are standard error of means (n=10).
9.3 Discussion

This chapter examines the effect of sample heating on PPO activity. Temperature affects enzyme rate firstly in the kinetic sense as defined by the Arrhenius equation. Thermodynamics suggests that the activation energy, $\Delta G$, for the oxidation of phenols is a function of absolute temperature, T. The enzyme catalysed reaction could also therefore be expected to be a function of temperature, within limits, and increase in rate with T (Lehninger et al., 1993).

Heating temperatures above 65$^\circ$C were not tested since temperatures in excess of 65$^\circ$C lead to Maillard reactions and non-enzymic browning will begin to occur at high rates; and it would therefore be difficult to separate this Maillard reaction from enzymic browning (Maga, 1990, Richard-Forget et al., 1992a, Amiot et al., 1992).

Activity of the heated PPO in the apricot flesh was tested at 25$^\circ$C as an ambient temperature since it was the most commonly reported temperature for PPO activity tests (Amiot et al., 1997).

This experiment involved activation of the enzyme using SDS to effect release of the enzyme from the membrane bound condition. SDS added is presumed to act by releasing membrane-bound PPO (Moore & Flurkey, 1990; Mayer & Harel, 1994), although it has been speculated that Japanese apricot PPO (Prunus mume L.), which is naturally membrane bound, is likely to be more resistant to high temperature
proteolysis than non-membrane bound enzymes (Goto et al., 1988). Use of SDS limits the possible interpretation of this experiment's results compared with those reported where purified, or partially purified, enzyme extracts have been used to obtain temperature tolerance data using more control over the substrate and enzyme environment. On the other hand, the whole flesh samples used here are more akin to the commercial situation where the cut, but generally unruptured fruit is dried followed by browning.

In some fruit, such as mango, it was found that at least two isozymes of PPO operate with different temperature optima. In mango, a temperature optima difference of 40°C (30°C and 70°C) is suggested to be due to both a laccase and catechol-oxidase type enzyme (Robinson et al., 1993a). Just what the biological significance of such a temperature optima difference might be is unclear.

The general effect of heating temperature above 25°C on PPO activity shown (Figure 9.1) is to reduce activity. For this apricot cultivar (cv. "Moorpark") at pH 6.8 the thermal inactivation would appear to be almost complete at 65°C following 30 minutes of heating. This result is consistent with a number of findings for PPO from various fruit and vegetables (Lourenco et al., 1992). Of the temperatures tested the optimum in this experiment is 25°C with a significant decline at 45°C and effective inactivation and possible denaturation at 55°C and 65°C. Some residual activity appears to exist as shown in Figure 9.2, which may be an artefact of the equipment or method as mentioned. It is possible that the apparent PPO activity at
65°C is due to a rate of oxygen consumption from another thermostable enzyme system or oxygen consuming reaction.

A number of enzymes have been found to exist in apricot purees or slurries. Polygalacturonase, pectinase, pectinesterases and cellulases may be present (Deshpande & Salunke, 1964; Souty et al., 1981; Goto et al., 1988; Renard et al., 1991; Knee et al., 1994). How these other enzymes react within the tissue structure, releasing or binding PPO substrates during or after the heating, was not investigated.

The effect of heat on the phenolic substrates per se, that might have been present in the tissue extract, is suggested as a source of error (Hernandez et al., 1997) and may have applied in this experiment. Certainly in the case of potato it is documented (Friedman, 1997) that the major substrate, chlorogenic acid that is also the main apricot PPO substrate, is reduced by high soil temperatures during growth and later high temperature during tuber processing.

Morphological changes during the development of the apricot fruit include pit hardening. Pit hardening uses phenolics that might otherwise be available as PPO substrates. Thus one might expect a dip in PPO activity during pit hardening ages in the mesocarp and epicarp. According to Jackson (1965), endocarp growth peaks at 20 days after anthesis whilst pit hardening appears in the period 50 to 80 days after anthesis. With respect to “age” in this experiment these were ages 60 to 90 days. Figure 9.2 showing results by age does have a dip in PPO activity around 60 to 70
days, but any association made would presume more than could be justified from the experimental conditions used here.

Nearly complete thermal inactivation is evident at 65°C. The response by development age suggests that thermal inactivation is less with increasing age over all heating temperatures. More variation in inactivation appears to occur with more mature fruit (Figure 9.3), however these responses are at the measurement limit of the equipment used. If true, however, it would support the contention of Femenia et al. (1998) that ripening results in cationic species changes that, in turn, have an effect on enzyme stability in the matrix of pectic polysaccharides present in crude extracts leading to protection of the enzyme from heat induced inactivation.

This study shows that there was not a differential response in PPO activity with development age of any consequence. Further, it was determined that PPO activity is effectively stopped in samples held at 55°C for 30 minutes. PPO activity in the apricot flesh held at 35 and 45°C was reduced to 82% and 31%, respectively, of the non-heated controls. These results confirm that heat treatment could be used to prevent PPO activity in fresh apricot fruits, however other processes, such as Maillard reactions may limit its usefulness in practice.
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Chapter 10. Other Inhibitors of Apricot Polyphenoloxidase

Various approaches to the control of browning in fruit have been investigated (Martinez & Whitaker, 1995) with the principal aim of inhibiting PPO activity. Sulphite is an effective inhibitor, however, the American Food and Drug Authority and other regulatory bodies have restricted its use prompting a search for alternative inhibitors. Effective inhibitors might be found by differentiating the response of PPO isozymes and types to inhibitors (Walker & McCallion, 1980). Three potential inhibitors were applied to the apricot fruit extract in this experiment. SHAM (Salicylhydroxamic acid) is reported (Allan & Walker, 1988) to inhibit catechol oxidases but not laccases (Dawley & Flurkey, 1993) or peroxidases (Bucheli & Robinson, 1994). Since laccase activity has been reported in apricot fruit as well as the normal catechol oxidase behaviour of PPO (Dijkstra & Walker, 1991) the degree of response to SHAM could indicate the importance of the laccase component.

Cinnamic acid is also reported to inhibit catechol oxidases (Hernandez et al., 1997; Iyengar & McEvily, 1992; McEvily et al., 1992; Son et al., 2001), but not laccases, and further is reported to be a competitive inhibitor as an example of the class of aromatic carboxylic acids (Hernandez et al., 1997). Finally, tropolone was used to examine the potential for this reportedly very powerful inhibitor of grape PPO (Valero et al., 1991) on apricot PPO, where it could possibly be approved for use due to the reportedly low levels required for inhibition.
10.1 Materials and methods

The basic material and methodology is set out in chapter 3 and was that used except for the conditions outlined below.

The standard method was adjusted to include the delivery of various inhibitors into the reaction cell prior to temperature stabilisation at 25°C. All tests were conducted using 4-methyl catechol as before as the substrate and the pH was maintained constant using pH6.8 buffer in both the extraction phase and the reaction cell. Inhibitors were added immediately after the fruit sample was loaded into the reaction cell. Following temperature stabilisation the phenolic substrate 4-methyl catechol was added as per the normal protocol outline in chapter 3. Due to the single concentration of inhibitor used, these results do not provide an indication of the effects that variations in inhibitor levels may have had on apricot PPO activity.

SHAM (Salicylhydroxamic acid or N,2-dihydroxybenzamide) Sigma (S 7504, C₇ H₇ NO₃, FW 153.1) was used at a fixed rate of 0.2 mM in the reaction cell. Levels as low as 0.1mM have been reported to give significant inhibition (Dawley & Flurkey, 1992; Bucheli & Robinson, 1994). Levels of SHAM inhibitor used by various workers ranged from 0.1 mM to a high 10 mM. The 0.2 mM level used was based on various reports in the literature; however it was a relatively high level to ensure that some response would occur.
Cinnamic acid sourced from Sigma (C 6004, beta-Phenylacrylic acid, 3-Phenylpropenoic acid, FW 148.2) was added to the reaction at a fixed rate of 2.5 mM. The rate used was based on a number of PPO inhibition studies in various fruit juices, fruit purees and vegetables (Ju et al., 1988; Spanos & Wrolstad, 1992; Lourence et al., 1992; Vamos-Vigyazo, 1995).

Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), Sigma (T7387, C₇H₆O₂, FW 122.1), was used at an addition rate of 0.5mM. The level used was based on reports (Khan & Andrawis, 1985; Valero et al., 1991) with the object of obtaining reasonable levels of inhibition without obscuring the effect of development age.
10.2 Results

Results principally reflect stage of development effects, as do those of previous chapters (Figure 10.1). The common theme is reproduced of increasing activity with the developmental age as discussed in Chapter 4. There were some significant differences in the reaction of the enzyme extract to the inhibitors. When added to the reaction vessel with the fruit sample SHAM depressed the PPO activity by approximately 70% (Fig. 10.1) compared with control (see Chapter 4). The depression did appear to be age specific at this single level of addition. SHAM did, overall, inhibit the activity of the PPO extract relative to the control and indeed apparent activity was more reduced relative to control in the most ripe fruit and those of age 57 days (Figure 10.1). From these results there appeared to be only a specific age by inhibitor effect for SHAM for ages 8 and 57 days, as a heightened inhibition of PPO of the three compounds tested under these conditions.

The second PPO inhibitor used was cinnamic acid. The literature had suggested that cinnamic acid acted as a competitive inhibitor of substrate, in this case 4-methyl catechol. A level of 2.5mM cinnamic acid was chosen. Again, this was on the basis of previous reports and a preliminary test on the standard sample which showed that inhibition of at least 50% occurred on a fruit sample equivalent to day 85. The result is presented in Figure 10.2 which shows a similar pattern of inhibition as SHAM. There is not a special age where the inhibition was more or less effective relative to control values (Chapter 4).
Figure 10.1: Effect of development age on response of apricot (cv. Moorpark) PPO to a fixed level of inhibitor - SHAM (Salicylhydroxamic acid), 0.2mM. Control is pH 6.8 buffered apricot suspension using 20mM 4-methyl catechol as substrate in a Clark oxygen electrode. Error bars are standard error of means (n=10).

Figure 10.2: Effect of development age on response of apricot (cv. Moorpark) PPO to a fixed level of inhibitor - Cinnamic acid, 2.5mM. Control is pH 6.8 buffered apricot suspension using 20mM 4-methyl catechol as substrate in a Clark oxygen electrode. Error bars are standard error of means (n=10).
The final inhibitor used was tropolone. Figure 10.3 shows that, under the conditions used in this experiment, tropolone was quite effective in inhibiting PPO activity in the apricots at all the ages tested. The inhibition was not absolute, being approximately 5 to 8% of the control samples, somewhat higher than the literature (Chapter 2) would suggest. The inhibition with age followed a similar pattern to that of other inhibitors and substrates used in these experiments with no age showing a particular tolerance or sensitivity to tropolone.

Figure 10.3: Effect of development age on response of apricot (cv. Moorpark) PPO to a fixed level of inhibitor - Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), 0.5mM. Control is pH 6.8 buffered apricot suspension using 20mM 4-methyl catechol as substrate in a Clark oxygen electrode. Error bars are standard error of means (n=10).
10.3 Discussion

The main findings are that, as tested, SHAM does act as a significant inhibitor of apricot fruit PPO as might be expected from the literature (Allan & Walker, 1988). Cinnamic acid, as a representative aromatic carboxylic acid did inhibit the apricot PPO to a level of approximately 30% of normal activity and inhibition is presumed to be of the competitive inhibition type as reported (Walker & McCallion, 1980; Iyengar & McEvily, 1992). Tropolone was the most effective inhibitor of apricot PPO activity with a 90 to 95% reduction achieved, although this is somewhat less than might be expected from inhibition of other fruit PPO (Khan, 1985).

Salicylhydroxamic acid (N,2-dihydroxybenzamide, SHAM) is known to be an inhibitor of polyphenoloxidase (Allan & Walker, 1988; Bucheli & Robinson, 1994). The latter authors suggested that, for sugar cane PPO, a catechol oxidase type of PPO in sugarcane was indicated since SHAM only inhibits para-phenolic oxidase action. From other Prunus PPO reports and from P. armeniaca reports (Fraignier et al., 1995) it was expected that a catechol oxidase type of PPO was present in apricot fruit and thus there would be inhibition at some level.

In general, SHAM when added to apricot extract at the level used (0.2mM) caused an inhibition of PPO activity. The resultant activity was in the order of 25% of non-treated extract. This is somewhat contrary to expectation from the literature where SHAM has been described as a "powerful and selective inhibitor of catechol oxidases" (Allan & Walker, 1988). They were using purified mushroom, spruce and
potato enzyme, rather than fruit extracts which may point up to different types of PPO in fruit and/or *Prunus* spp. Laccases have been reported in apricots (Dijkstra & Walker, 1991) where SHAM at 0.02mM showed "mixed type inhibition" although no data were presented. As SHAM does not inhibit laccases this may be the cause of the apparent residual activity in Figure 10.1, particularly at the high level of SHAM used. No other evidence was found in the literature to support the laccase speculation for apricots.

In general, cinnamic acid when added to apricot extract, appeared to cause inhibition of PPO activity at a constant level relative to the levels observed for the different development ages without the inhibitor. Residual activity increased with age with peaks at age 57 days and full ripeness. Both Lourenco *et al.* (1992) and Walker & McCallion (1980) point out that cinnamic acid and substituted cinnamic acids inhibit catechol oxidases, whereas laccases are not inhibited. Again this inhibitor may have been only partly active for the reason that laccases were present in the fruit extract. Billaud *et al.* (1996) suggest that cinnamic acid operated as a mixed inhibitor of gum arabic PPO, which is a catechol-type PPO, but inhibition increased with lowered pH.

Tropolone is a slow binding competitive inhibitor, whose structure is analogous to the o-diphenolic substrates of PPO (Valero et al., 1991). It is also able to chelate copper ions and, in the presence of hydrogen peroxide will act as a substrate for peroxidases present rather than as a PPO inhibitor (Kahn, 1985). Tropolone showed very complete inhibition of the PPO extract with perhaps 5-8% of the non-inhibited activity remaining. Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) is very
interesting since it was reported to inhibit grape PPO at very low levels. This characteristic ensures its consideration as a possible commercial inhibitor with respect to the low level of residues in foodstuffs so treated. Tropolone has previously been used to prevent browning in canned peaches.

There are a number of interactions which could confound the results. Banana PPO inhibition by tropolone was reportedly reduced by SDS use during preparation of the PPO (Sojo et al., 1998), although the SDS level used, 0.1 percent, should not have caused these effects here. In other interactions, Marques et al. (1995) in apple, Moore & Flurkey (1990) with broad bean both refer to activation of PPO suggesting the presence of SDS altered pH optimum, possibly via a pH controlled regulatory domain. Marques et al. (1995) also mention interaction of SDS with tyrosine residues thus altering the PPO physical characteristics and conformation.

In summary, the results obtained show that apricot PPO is inhibited by the addition of SHAM, cinnamic acid and tropolone to the reaction solution to different degrees. The results also indicated that the inhibition observed was reasonably common to all developmental ages which suggests a common structure by development age for the PPO, or PPO isozymes if present, allowing this inhibition.

With respect to the maturity of the fruit (development age) the result shows no substantial indication that PPO isozymes were expressed with increasing maturity, as suggested in previous chapters and by some authors, as no differential sensitivity to the inhibitors tested was observed.
Chapter 11. General Discussion

Chapter 1 introduced apricot fruit development, the problem of browning, and apricot polyphenoloxidase as the main culprit, which led to a consideration of the timing and level of this enzyme's activity. A review of the literature available (Chapter 2) provided indications as to the likely PPO activity in other fruit, other Prunus and in apricot and this led to the aims of this work. The principal aim was to determine PPO activity during fruit development. A number of reaction variables for the enzyme were considered important. These included pH, response to added sulphur dioxide, reaction to inhibitors and substrate changes. These are discussed below and lead to conclusions from the work. Firstly, however, some explanation of the relationship between fruit development and the methodology used is in order.

11.1 Apricot fruit development

Developmental expression of PPO has been studied in a number of fruits, particularly in peach (Cary et al., 1992, Shahar et al., 1992, Hunt et al., 1993, Steffens et al., 1993). Most studies were initiated with a view to commercially reducing browning in the fruit or vegetable, caused by PPO activity. As a consequence very often only mature or ripe fruit were reported on.

The major thrust for browning reduction has been to examine the conditions under which SO₂ addition (1500 to 5000ppm) is applied and also the condition of the fruit
at the time of application (Ponting, 1960; McBean, 1985; Dahlenburg, 1990; Rettke, 1993). Factors such as ripeness, sugar content, pH, flesh characteristics and sulphur uptake have been considered in relation to PPO activity, but were often not examined for less developed fruit. Characterisation of the PPO enzymes in apricot is also important from a processing viewpoint. Manipulation of the harvest and drying conditions could reduce the activity of the enzyme or its substrate levels, and so inhibit browning in dried fruit or enable use of lower sulphur dioxide levels.

The increase in PPO activity with age is not linear in any experiment. The effect may be due to a number of things - possibly just cell wall/vacuolar state of development and relative surface area (Dilley et al., 1993). However, the low PPO activity shown very early from day 1 to day 15 may be associated with the degree of activation and/or release from membrane attachment as supposed by some authors (Zhang et al., 1994). That is, the PPO may not be available to the substrate and so the apparent rate of its activity is lower. "Apparent" since tests were invitro on blended extracts, where the enzyme was activated with SDS which should have ensured physical availability.

In apricot, Dijkstra & Walker (1991) reported that there are two types of PPO present, a laccase and a catechol oxidase. A number of workers, notably Kumar (1986, 1987); Torres, (1989); Byrne (1989) and Fourie et al. (1992), have reported multiple PPO enzymes in Prunus spp. The evidence for apricots is not conclusive since there are insufficient confirmed reports, whilst a number of authors contend there is only one PPO isozyme (Boss et al., 1995; Fraignier et al., 1995). There is
strong evidence in other *Prunus* spp. (Fraignier et al., 1995) where multiple forms of PPO (active and inactive) have been detected by SDS-PAGE and by Western blot, revealing multiple active forms from proteolysis of one major form.

The results obtained suggest that three isozymes may exist in apricot (cv. Moorpark) during development. Thus there is often a peak at ages 22 and 29 days, one at 57 days together with a high peak for fully ripe fruit at 85 plus days. There is sufficient variation in the pH data to question these trends, particularly for day 1, but in most of the other results the trend is evident. This raises the question as to whether these are artifacts, for example associated with some other cause correlated to a development event, or the normal physiological response as seen for a number of enzymes.

The apparent peaks in PPO activity were found under conditions that do not allow a direct inference on whether isozymes are present. Purified PPO may be more suitable for this purpose (Thomas & Janave, 1986; Fraignier et al., 1995), but ultimately electrophoresis or Western blotting would need to be done to distinguish isozymes present (Durham et al., 1987). However, an *in vitro* system that contains all the elements naturally present, such as pectins, phenolic substrates and co-substrates, sulphhydryl compounds such as cysteine and the various anti-oxidants, will give a more realistic indication of actual activities in the fruit (Lourenco et al., 1990; Fragnier et al., 1995). Some cultural actions may still cause PPO activity measurement errors, one example being the increased PPO activity associated with boron sprays (Lee & Kim, 1991a,b) and skin contamination with iron (Arakawa & Ogawa, 1994).
The results obtained here do not support, but equally do not refute, a claim for multiple isozymes of PPO in apricot. Clearly the highest activity is associated with maximum ripeness of fruit in the apricot fruit. That would not mean that a molecular approach should target this development stage since there is usually a lag between expression and activation of PPO in fruits, for example apples (Boss et al., 1995). The possibility of pseudo-isozymes, due to partly folded or refolded protein domains related to the extraction techniques variously used (Marques et al., 1995), suggests there may be fewer apricot fruit isozymes present than apparent from previous experiments.

11.2 Methodology

Methods available to examine PPO activity in apricot are reviewed in Chapter 2. They can be divided into qualitative ones using crude extracts or quantitative ones using purified PPO protein-enzyme and DNA or cDNA methods. Sufficient evidence exists to suggest that purified PPO reactions are not representative of in vivo outcomes (Loomis, 1974; Mayer & Harel, 1979; Fraignier et al., 1995). In apricot fruit there is some evidence also for the interaction of two enzyme functions, a major catechol oxidase (o-diphenol oxidase) and a less significant laccase (p-diphenol oxidase) (Dijkstra & Walker, 1991).
Since highly purified PPO does not necessarily reflect likely activity in vivo, crude puree of apricot fruit was utilised in these experiments. Here the objective was to maintain at least the principal balance of constituent chemicals found in the fruit flesh. The use of a Clark-type oxygen electrode suggested itself due to its ability to handle the particulate nature of a puree whilst exhibiting exceptional sensitivity to solution oxygen levels (Delieu & Walker, 1981).

Purees of fruit lack the organisation of a cell and the specificity of normal enzyme environments. The less controlled reactions that result may not be fully representative of the normal state of cut, but otherwise intact, drying fruit halves. Here less activity might be expected with intact vacuoles which separate substrate and enzymes, although some stability is afforded with the use of buffers in a puree.

The basic material and methodology is set out in Chapter 3. Two buffers were used - an extraction buffer and a reaction buffer. The polarographic half-wave potentials of certain types of organic compounds depend strongly on the ionic strength of the solution. For the pH experiment the reaction buffers used were thus altered from the ‘standard’ pH 6.8 with the use of various ratios of citric acid and sodium hydrogen phosphate. Perria and Dempsey (1974) suggest the use of constant ionic strength McIlvaine citrate/phosphate buffers covering the range of pH 2.2 to 8.0 at 25°C noting also that surfactants, such as SDS, do not interfere with citrate-phosphate buffer function.
Chapter 11. General Discussion

Whether one should be measuring the "natural" state of the apricot puree with any ascorbic already present or attempting to remove it is not clear. A decision was made that normal PPO activity is associated with the natural background level of ascorbic acid and, since the natural levels are what is being measured, the ascorbic acid should not be compensated for, notwithstanding any variation during ripening.

11.3 Pectins, sugars and calcium may affect apricot PPO activity

Changes in cell wall polysaccharides are thought to play a role in altering fruit texture during fruit development. Most reported changes (Souty et al., 1981; Asami et al., 1988; Deveau et al., 1993; Huber & Odonoghue, 1993; Batisse et al., 1994; Taylor et al., 1995; Redgwell et al., 1997) involve an increase in water-soluble pectic polysaccharides accompanied by a loss of sugars into solution. The pectins undergo depolymerisation with a decrease in average molecular weight. The extent of the changes depends on cultivar, the capacity of the fruit to maintain enzyme synthesis over developmental stages and the ionic/pH conditions in the apoplast (Fischer & Bennet, 1991).

An apricot, when fully ripe may have a TSS level of more than 18%, mainly sugars (Witherspoon & Jackson, 1996). The fruit tested ranged from very low sugar content on day 8 (unmeasurable with the refractometer) up to more than 20% for the overripe fruit (Anet & Reynolds, 1955). When purees were diluted as tested, such sugar levels may still have had an effect on the enzyme-substrate reaction (Fennema, 1996).
The results obtained suggest that PPO activity increased with development. This may be an artifact reflecting the increase in sugar, and possible Ca\(^{++}\) level changes, in solution. Some evidence to support this contention is presented below. The enzymes, polygalacturonase and pectin methylesterase, appear to regulate cation binding and post-harvest ripening in apricot with a large proportion of water-insoluble pectins solubilised by a weak base, possibly by hydrolysis of weak ester cross-links (Cardarelli et al., 2002). Van Buren (1970) suggested that increasing Ca\(^{++}\) and Mg\(^{++}\) increases fruit firmness. Further, while Na\(^{+}\) and K\(^{+}\) increases improve texture by reducing the electrostatic repulsion of acidic groups, they also reduce firmness by competing with Ca\(^{++}\). Castaldo et al. (1997), examined changes in the free Ca\(^{++}\) available in solution due to depolymerisation of the high pectic content characteristic of apricots. They associated a decreased Ca\(^{++}\) with residual pectin methyl esterase activity which removes methyl groups from the pectin chain allowing the site to be replaced with the divalent Ca\(^{++}\), and further, that the ratio of ionic species initially present may have an effect on the depolymerisation. Cardarelli et al. (2002) found a decrease in pectin methyl esterase post-harvest (after day 85 here) that lends support to the idea of free Ca\(^{++}\) increasing with ripening.

Calcium ions in solution appear to suppress PPO activity. Partially purified palmito PPO activity, with chlorogenic acid as a substrate, was not affected by 10\(^{-2}\)M Na\(^{+}\) or K\(^{+}\) addition (Lourenco et al., 1990). However, when Ca\(^{++}\) was added to the reaction mixture PPO activity was depressed some 17%. This is consistent with the pectin binding and causing steric hindrance to both substrate and enzyme in the gel formed
when calcium is added to low methylated pectins (Fennema, 1996). Unfortunately, the "partially purified" polyphenoloxidases Laurenco et al. (1990) used may have contained pectins.

Water soluble polysaccharides in apricot increased from 7.1% (day 40) to 16% (day 70) during development according to Femenia et al. (1998), who also reported a change in the Ca\(^{++}\)/Na\(^{+}\) ratio towards more Na\(^{+}\). Presumably the change in Ca\(^{++}\)/Na\(^{+}\) ratio reflects the use of the Ca\(^{++}\) in binding pectin molecules where the methyl group was removed by pectin methyl esterase during development to day 70. Femenia et al. (1998), suggest the increasing Na\(^{+}\) would prevent formation of new pectin cross-links by competing with Ca\(^{++}\). Taken as a whole these findings indicate that pectin methyl esterase increases at maturity, resulting in pectin breakdown and possible release of Ca\(^{++}\) to be re-bound to shorter pectin molecules, but that pectin methyl esterase does not have a role after ripening at perhaps day 85 and beyond in this experiment. The result obtained by adding modest amounts of Na\(^{+}\) as NaCl (Chapter 8) appear to support this, however since Ca\(^{++}\) was not measured by development age, it cannot be said that the increase in activity with development age is due to Ca\(^{++}\)-related inhibition of PPO activity.

The expected activity of PPO may be reduced if it is being inactivated by phenolics within the crude extract (Piffaut & Metche, 1991; Fu et al., 1992). Apricots have low levels of flavan-3-ols (Errea et al., 1992; Radi et al., 1997), these are mainly (+)-catechin and epicatechin. In some fruits loss of astringency has been attributed to insolubilisation of tannins due to reaction with acetaldehyde (Fulcrand et al., 1996).
Apparently acetaldehyde induces polymerisation of tannins with flavan-3-ols leading to coloured complexes. Radi et al. (1997) particularly noted a correlation with browning potential and the initial concentration of flavan-3-ols, with PPO activity in 9 apricot cultivars tested. There was no correlation between chlorogenic acid content, the main substrate, and PPO activity. However, the apricot cultivars tested did not include cv. 'Moorpark'. Bakker & Bridle (1992), examining strawberry PPO and sulphite make a similar comment that "the puree itself in the presence of SO₂ seems to have a protective effect on the anthocyanins, inhibiting the formation of polymeric pigments...". Whilst various end point colour changes were observed in the apricot PPO reaction, there appeared to be no correlation between PPO activity and a particular colour development (Piffaut & Metche, 1991; Nicolas & Potus, 1994).

11.4 Optimum pH

Chapter 5 results confirm the general finding that a broad pH optimum exists for apricot PPO activity. The pH optimum was found to increase with development age. This could be due to different isozymes of PPO being dominant for different development ages - although the results do not provide support for this possibility. The increase could be due to a reduction in steric hindrance associated with pectin breakdown as the fruit matures and ripens. The increase could also be associated with a change in the ratio or level of ion species in the reaction buffer with development age as outlined in Chapter 5.
11.5 Effect of substrate concentration and type

Chapter 6 examined types of substrate. The commonly used experimental substrate is 4-methyl catechol and is the basis for comparison with other substrates and other experimenters. Tremolieres & Bieth (1984) point out that the presence of a methyl group in the para position of the aromatic ring increases the catalytic efficiency of the PPO and this is one reason why 4-methyl catechol is the substrate of choice in PPO activity studies.

D-L DOPA as a substrate demonstrated a PPO activity of approximately 15% that of 4-methyl catechol at 2mM concentration. This result is generally consistent with other reports (Siddiq et al., 1992; Arslan, 1998). Additionally, a lag period was observed between initiating the reaction and the beginning of activity, again consistent with reports in other fruits (Haslam, 1994). Lerner (1972), suggested the variation could be due to enzyme concentration, pH, substrate concentration or catalytic ortho-diphenols present. The initial level of catalytic ortho-diphenols in the puree used, to initiate the second stage of the reaction, is unknown.

Catechol showed a restricted activity for PPO compared with 4-methyl catechol (ca. 40%). Allowing for this 40% difference, when developmental age is considered, the relative response to catechol was high for day 29 and day 92, compared with 4-methyl catechol. In addition, there was a more variable result for the day 92
samples, which might suggest that there are interfering substances from ripening/cell breakdown present in some fruit samples at this age for this substrate.

Caffeoyl-quinic acid (chlorogenic acid) is a natural substrate for apricot PPO. Results obtained indicate that PPO activity against chlorogenic acid was similar (ca. 90%) to 4-methyl catechol but with a higher (relative) activity in fruit that were beyond stone hardening stage. Also there was a more consistent response at these ages. Finally, chlorogenic acid increased in activity at day 92 compared with a fall-off for the 4-methyl catechol at that age. These results are difficult to explain, as the evidence is conflicting. Vamos-Vigyazo et al. (1984) reported on changes in polyphenol oxidase activity and browning rate during storage (20 days at 5°C, 85%RH) with initial, 5 day and 20 day levels of polyphenol oxidase activity, o-dihydroxyphenol concentration and rate of enzymatic browning recorded for apricot. They found a poor correlation between polyphenol oxidase activity and o-dihydroxyphenol concentrations, but noted that trends in variation differed with the cultivars examined. In peach and nectarine, on the other hand, Cheng & Crisosto (1995) found that only chlorogenic acid had a positive correlation with browning potential (presumed to reflect PPO activity), with epicatechin having a negative correlation.

Chlorogenic acid as a substrate appeared to show low PPO activity with immature apricots. This is somewhat confusing, since other substrates used indicate that PPO enzyme is present and active. Chlorogenic acid may be made unavailable due to competition with other substances, not being the preferred substrate for the PPO
enzyme examined, or for a number of other reasons such as young trees (Vierling et al., 1992), cultivar (McRae et al., 1990), insect damage to fruit (Felton et al., 1989), acetone soluble fraction of the fruit (Komiyama et al., 1991), cysteine level (Richard-Forget et al., 1992) and competition with other phenolic acids (Kermasha et al., 1993).

Dijkstra & Walker (1991) identified laccase-type and catecholase-type PPO by substrate specificity tests and the effects of selective inhibitors. The enzymes were tightly bound to the fruit tissue. Both enzymes acted on catechin and chlorogenic acid as the natural substrates. It is possible that the extraction method used may have been inadequate for these young fruit, i.e. the PPO was strongly bound, causing an enzyme concentration limit to give a falsely low activity level (Moore & Flurkey, 1990; Fraignier et al., 1995). It is of interest to speculate what effect the glycosylation of chlorogenic acid and other phenolic substrates in young versus more mature fruit might have on PPO activity.

With chlorogenic acid substrate, palmito PPO was not affected by $10^{-2}$M Na$^+$, K$^+$ or sulphydryl-blocking reagents (Lourenco et al., 1990). These researchers suggested that this indicated that the PPO-chlorogenic acid reaction was not affected by -SH groups. One presumes the inference is that other substrates, such as 4-methyl catechol, are inhibited as (Mayer & Harel, 1994) confirm for other fruits. A chlorogenic acid substrate used in the sulphur dioxide level experiment may have presented a different result from the 4-methyl catechol used here.
Chapter 11. General Discussion

One of the reasons for variation of individual samples could be bruising and/or pathogenic damage. Dijkstra & Walker (1991) found the levels of phenolic compounds in rotted fruit were lower than in healthy tissue. If this were the case in individual fruit sampled here, then a phenolic substrate deficit may have resulted, compared with other fruit tested. It should be noted that the fruit tested here were carefully selected for uniformity and lack of bruising, although there is the possibility that fruit were bruised without visible sign. Evidence is mounting that plant growth regulator activity, particularly ethylene and methyl jasmonate, has a direct effect on PPO activity (Knapp et al., 1970; Crane, 1969; Paulson et al., 1979; Cutting et al. 1990, 1992; Selvarajah et al., 2001). This effect is often regardless of the concentration of actual enzyme present. If there is interaction between phenolic substrates and PPO products also, then a fruit phenolic deficit could change the rate not withstanding the ample supply of chlorogenic acid itself.

Subsequent to the conclusion of the experimental work, de Rigal et al. (2000) reported high levels of trans-β-carotene isomerisation (20%) induced by PPO in bruised apricots. Trans-β-carotene was found to be a potent inhibitor of phenol degradation associated with PPO activity. The large fruit-to-fruit variation here may have been associated with the level of trans-β-carotene also.

Substrate-enzyme-product relations in PPO are complex. Goodenough et al. (1983) reported that apple polyphenol oxidase oxidised p-coumaric acid with co-factors present or phloridzin alone in a complex reaction which finally results in a dimeric quinone. They discuss the difficulty in establishing the Km of the polyphenol
oxidase due to the complex cross-oxidation and also substrate inhibition by oxygen. Cheynier & Da Silva (1991) used model solutions containing trans-cafféoyl tartaric acid and polyphenol oxidase to study grape procyanins suggesting that although the procyanins were not oxidised by the polyphenol oxidase they were oxidised by the quinones produced by the activity of the polyphenol oxidase. Rouet-Mayer et al., (1993) make the point that different fruit PPO's can have quite different apparent Km's citing chlorogenic acid as substrate for apple PPO (4.2mM), pear PPO (16.1mM) and apricot PPO (1.2mM) as examples.

The double action of the PPO enzyme and substrate system has led to a considerable confusion in not only the literature but also to the naming conventions used on compounds. It is only with the ready availability of NMR (Pope et al. 1991) and HPLC that some of the picture is becoming clearer. A further report by Fulcrand et al. (1994) records the characterisation of compounds obtained by the oxidation of caffeic acid over a range of acid pH. The products formed by the quinone evolved were being monitored by HPLC.

The principal factors which influence the reversible association of polyphenols with other substrates are:- (i) solubility, solvation and desolvation; (ii) molecular size and conformational flexibility; (iii) general salt and specific metal ion effects (e.g. Ca++, Al+++); (iv) hydrophobic effects; (v) hydrogen bonding via phenolic groups and (vi) the presence of tertiary amide groups in the co-substrate (Haslam, 1994). The results outlined in Chapter 6 do not indicate that there was any inhibition in general or from specific samples.
One could conclude that cleaner extraction of the sample is required, perhaps with acetone. Lee et al. (1991) recorded a seven-fold increase in PPO activity with acetone extraction of cocoa PPO while a seven-fold increase in phenols extracted with cold acetone is reported by Villanua-Marti et al. (1992). As indicated above, however, this may be at the expense of substrate and product interference that reflects the 'natural' state of the apricot PPO activity.

11.6 Heat inactivation

As outlined in Chapter 9, PPO enzyme may have a temperature optimum, heating tolerance (inactivation followed by recovery) and denaturation above a certain temperature, perhaps 55°C. The latter two effects are usually conditional on the time of treatment. Amiot et al. (1997) have suggested that “Polyphenol Oxidase is not a very heat stable enzyme”. However, the results obtained and those of others for Prunus spp. appear to be at odds with that claim (Mayer & Harel, 1994) when PPO activity can occur to at least 10 degrees beyond the normal range of physiological activity of apricot.

PPO as an enzyme is quite stable when stored at low temperatures (Richard-Forget et al., 1992b). Temperature optima for activity vary considerably with purified PPO from different fruits. Kumar (1987), for example, reported the temperature optimum for peach polyphenoloxidase was 37°C and Km was 2.5 x
10\(^2\) when catechol was used as substrate. Temperature optima for PPO vary with fruit and tissue. Fruit skin catechol-oxidase PPO had a relatively low maximum temperature optimum of 30°C compared to the sap laccase PPO that had a high optimum of 75°C reported by Robinson et al. (1993) for sap burn on mango skin, a 45°C difference! In apricot, Dijkstra & Walker (1991) identified both laccase and catechol-oxidase by substrate specificity tests and the effects of selective inhibitors. The enzymes were tightly bound to the fruit tissue. Both enzymes were reported inactivated by holding at 80°C for 10 minutes. It is not clear from their paper that both individual types required 80°C for inactivation.

Several researchers have reported a reduction in thermal tolerance with lowered pH (Walker, 1964, Park & Ko, 1986, Hadziyev, 1987, Cilliers & Singleton, 1989, Miller et al. 1990). For example, Cruess & Sugihara in 1948 reporting on olive PPO, showed a reduction from the maximum PPO activity at pH 5.0, 32°C for either increased pH (6.0) or lowered temperature. The effect on PPO activity of temperature variation with pH is not thought to have influenced results obtained here because a constant temperature water bath was used in the Clark electrode operation.

Nearly complete thermal inactivation is evident at 65°C. It was determined that PPO activity is effectively stopped in samples held at 55°C for 30 minutes and was reduced to 82% and 31%, respectively, for 35 and 45°C. These results confirm the expectation from the literature that temperatures above 60°C would limit PPO activity. A number of factors were considered such as pit-hardening, pH changes with age and cell development types which may have influenced the results.
obtained. More tolerance to higher temperature exposure may occur in later development ages.

This study shows that there was not a differential response in PPO activity for heat inactivation with development age of any consequence for this apricot cultivar and these experimental conditions.

11.7 Inhibition by the halide, sodium chloride

Salt is a very common inhibitor of PPO used incidentally for microbial spoilage control (Ponting, 1960).

Inhibition by chloride was found to be pH dependent. Inhibition increases as the pH decreases (Janovitz-Klapp et al., 1990). The suggestion is that halide ions bind to only protonated enzyme (EH) or protonated enzyme-substrate complex (EHS) to yield inactive EH-halide or EHS-halide forms (Rouch & Cadet, 1998).

The likelihood is that the sensitivity shown very early in development at days 1, 8 and 15 may be associated with the degree of activation and/or release from membrane attachment supposed by some authors (Zhang et al., 1994). That is, the PPO may not be physically available to the substrate and so the apparent rate of its activity is lowered as outlined in 11.3. These limitations might be overcome with purification but purification, does not necessarily produce accurate results (Marques
At the later development age of 44 days, showing a dip in activity, there is no corresponding dip in other tests. Sensitivity to salt at this development age may be due to an association with pit-hardening. Assume, for the moment, that there is an isozyme for PPO that is specific for the pit-hardening stage. During pit-hardening lignins are formed using phenolic substances. PPO may be "designed" to shut down with the release of sodium ions and other cations into the cytosol, thus allowing lignin formation to begin (Tyerman, 2002). There is no evidence in the literature to support this speculation for apricot PPO as yet.

In practice the use of salt as a sole inhibitor of PPO is prohibitive as the concentration required makes the fruit unpalatable (Janovitz-Klapp et al., 1990, Pizzocaro et al., 1993, Rouch & Cadet, 1998, Rouet-Mayer & Pilippon, 1999). With inhibition of this apricot cultivar PPO activity by salt there is a tendency for PPO activity to be more sensitive at the younger development ages and also at development age 44 days. To conclude that the activity of PPO is more sensitive to salt at these two periods in the development of the fruit may be premature.
Chapter 11. General Discussion

11.8 Sulphite inhibition

Sulphite is the traditional inhibitor of polyphenoloxidases in fruit and vegetable products. Application as gaseous SO\textsubscript{2} or solid/solution of sodium or potassium metabisulphite results in sulphite being available, to inhibit PPO activity, in the tissue or fruit to which it has been added (Ponting, 1960; Schroeter, 1966; Wever, 1987).

Results discussed in Chapter 7 indicate that increasing inhibition does occur with increased levels of sulphite added to apricot puree as tested. A number of factors influence this inhibition. Dahlenburg et al. (1990b) have shown an interaction with fruit sugar content. Higher sugar levels in "Moorpark" apricots, measured as Total Soluble Solids (TSS), result in greater uptake of SO\textsubscript{2} in cut fresh fruit for drying (Table 11.1). The authors suggested that this increase could be due to increased binding sites associated with higher soluble solids content, or it could be due to increased rate of binding reaction associated with the higher pH of more mature fruit.

Table 11.1: Fresh fruit pH, soluble solids content, titratable acidity (TA) and initial SO\textsubscript{2} uptake levels for "Moorpark" apricots at three maturity levels (Dahlenburg et al., 1990b)

<table>
<thead>
<tr>
<th>Maturity</th>
<th>pH</th>
<th>TSS (°Brix)</th>
<th>TA (as ml 0.1NaOH)</th>
<th>Initial SO\textsubscript{2} (uptake ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>4.28</td>
<td>12.2</td>
<td>12.9</td>
<td>1706</td>
</tr>
<tr>
<td>Medium ripe</td>
<td>4.45</td>
<td>14.0</td>
<td>10.9</td>
<td>1948</td>
</tr>
<tr>
<td>Fully ripe</td>
<td>4.59</td>
<td>16.8</td>
<td>9.6</td>
<td>1953</td>
</tr>
</tbody>
</table>
This interaction of maturity and applied SO\textsubscript{2} may have shown up in the results obtained (Chapter 7). "May" because, on the one hand if the SO\textsubscript{2} was absorbed and held by compounds other than PPO, phenolics and quinones, then one would expect to see increased PPO activity in fully mature (high sugar) fruit and this was the case. On the other hand, one could postulate that higher SO\textsubscript{2} retained should result in more PPO inhibition. This appears not to have been the case, although at high SO\textsubscript{2} levels there is certainly a definite lag prior to PPO activity.

A number of compounds and reactions have been put forward to explain the fate of SO\textsubscript{2}. It is noted that SO\textsubscript{2} bleaches some anthocyanins reversibly (Bakker & Bridle, 1992) so it is possible that apricot quercetin or rutin may be binding with the SO\textsubscript{2} initially, then releasing SO\textsubscript{2} as the concentration decreases, although it is unlikely due to the short reaction time involved in the Clark electrode. If so, since the quercetin and rutin levels are higher in the more mature fruit (Villanua-Martí \textit{et al.}, 1992), this leads to less inhibition in those fruit, as was the case (Figure 7.3).

The many reactions of sulphite in fruit are discussed in Chapter 7. How significant these reactions are to PPO activity in developing apricot fruit is difficult to determine from the results. Indeed, whether further work could be suggested which provides unambiguous results devoid of uncontrolled interaction is doubtful.
11.9 Other inhibitors - SHAM, tropolone, cinnamic acid

Resource constraints prevented an in-depth study of these inhibitors. It was possible, however, to examine the effects of these three inhibitors on a developmental age basis. The results are in general agreement with others, insofar as the inhibitory action described (Andrawis & Kahn, 1985; Rzepecki & Waite, 1989; Janovitz-Klapp et al., 1990; Lopez-Serrano & Barcelo, 2001).

SHAM (salicylhydroxamic acid) was previously reported by Allan & Walker (1988) to be selective in inhibiting catechol oxidases, thought to be the main apricot PPO, but not the laccases which are the more common PPO's found in fungi. Catechol oxidases are ortho-diphenol oxidases, whereas laccases are para-diphenol oxidases. Apricot PPO activity in the presence of SHAM as reported in Chapter 10 suggests that SHAM is not acting as a complete inhibitor. This may be due to the method employed, particularly the use of a puree causing steric hindrance, or the existence of enzymes not sensitive to SHAM. It is unlikely that apricot PPO is unique in being insensitive to SHAM inhibition as there are a number of references relative to SHAM effectiveness with other polyphenoloxidases (Allan & Walker, 1988; Robinson et al., 1993). This suggests it is of limited use as a PPO inhibitor.

Results presented in Chapter 10 confirm earlier findings (Hernandez et al., 1997) that cinnamic acid is an inhibitor of PPO activity. Although every effort was made to obtain fruit with uniform colouring, on the assumption that this reflected state of ripeness (Brown & Walker, 1990), it was found that some fruit halves
were more colored than others of the same age group. Variation in individual fruit response to cinnamic acid was observed. One could speculate that this may have been due to the differences in pigment content observed in the fruit tested. Turner et al. (1993) reported that light caused isomerization and cyclodimerization of the cinnamic acid content of plant cell walls which reduces cinnamic acid availability as an inhibitor. That cinnamic acid is present in relatively high levels in the fruit of *Prunus spp.* has been recorded by Kubota and Kudo (1992) in peach and Radi et al. (1997) in apricots.

Tropolone was shown to be a potent inhibitor of the apricot PPO activity at very low concentration. Additionally, tropolone has low toxicity to humans at the level used (Khan, 1985; Sholberg & Shimizu, 1991). Tropolone therefore has potential to replace SO$_2$ use in preventing apricot browning.

If peroxidase is present as well as PPO, then tropolone is a preferential substrate for the peroxidase (Valero et al., 1991). Thus if large quantities of peroxidase were present in the fruit extract one would expect an incomplete inhibition. That this has not happened in these tests suggest that peroxidase is not present in the fruit at high levels.
11.10 Concluding remarks

Determining the expression and activity of PPO during apricot fruit development together with the biochemical characteristics of the enzyme extract at different development stages was the aim of this work. Biochemical factors considered included pH, salinity, sulphur dioxide level and other inhibitors such as salicylic acid derivatives, tropolone and aromatic organic acids.

One firm conclusion is that PPO activity varies with development age in apricot fruit. Another is that prior work regarding inhibitors and substrate responses has been generally supported by the results reported here. Where there is variation it may be due to the combination of methodology and non-purified enzyme extract used to probe the activity response under conditions more representative of the field.

More tentatively, one can suggest that PPO activity in apricot is different to other fruit due to higher levels of pectins, possible ortho-diphenolase activity, the presence of fruit carotenoids and the balance of phenolic substrates available. All of these suggestions indicate a tantalizing array of findings from future work to confirm or deny them. For industry, these results indicate a need to analyse the apricot fruit composition more carefully with a view to applying a combination of directed inhibitors, possibly including tropolone, specific to the varietal response shown by testing.
Future PPO work in apricots should consider some measures of calcium and pectin levels and the use of EDTA (100mg L\textsuperscript{−1}) as suggested by Bakker & Bridle, (1992) to chelate and remove trace metals such as calcium to clarify the effect on PPO activity of calcium and pectins.

Experimental work as is described here therefore still suffers from a lack of reaction control normally present in intact fruit tissues. Imaging nuclear magnetic resonance techniques may be used in future, but are currently inadequate (Maas & Line, 1994), similarly, near infra-red techniques could be of value in determining the levels of organic acids and sugars as the reaction progresses both \textit{in vivo} and \textit{in vitro}.

The experiments conducted, with the possible exception of Fraignier \textit{et al.} (1995), appear to be exceptional in examining fruit of such a young age and thus the work needs to be repeated using fruit from various developmental ages. This would complement work from France (Chevalier \textit{et al}, 1999) on the molecular basis of browning in apricot.
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