



SUGARCANE POLYPHENOL OXIDASE

CAROLYN BUCHELI

Thesis submitted for the degree of Doctor of Philosophy

in

The University of Adelaide

(Faculty of Agricultural and Natural Resource Sciences)

TABLE OF CONTENTS

ABSTRACT	vi
DECLARATION	viii
ACKNOWLEDGEMENTS	x
ABBREVIATIONS	xi
CHAPTER 1. GENERAL INTRODUCTION	
Sugar industry	1
Sugarcane	1
Milling	3
Browning	3
Sugarcane colourants	6
Enzymic browning	7
Polyphenol oxidase	10
Latency	10
Cellular localisation	11
Structure of PPO	13
Properties	14
Inhibitors of PPO	15
PPO is nuclear encoded	16
PPO genes	16
Function of PPO	17
Sugarcane PPO	20
CHAPTER 2. THE CONTRIBUTION OF ENZYMIC BROWNING TO COLOUR IN CANE JUICE	
Introduction	22
Materials and Methods	23
Plant material	23
Tissue extraction and enzyme assays	23
Juice colour determination	24
Measurement of phenolics	25
Partial purification of PPO	25

Results	26
Heat inhibition of colour formation	26
Enzyme inhibition	29
Oxygen-dependency of the browning reaction	31
Correlation between PPO activity and juice colour	32
Effect of substrate on browning	34
Effect of added PPO on colour formation	38
Discussion	40

CHAPTER 3. PPO, PHENOLICS AND COLOUR IN SUGARCANE

Introduction	44
Materials and Methods	45
Plant material	45
Extraction and assay of PPO activity, phenolics and colour in different plant parts	47
Sectioning and staining	48
Heat inactivation of PPO	48
Clonal studies	49
Results	49
Localisation of tissue browning and phenolics	49
PPO activity throughout the cane plant	54
Heat inactivation of PPO	59
<i>Erianthus arundinaceus</i> - a high browning species	62
Discussion	63

CHAPTER 4. PROPERTIES OF THE PPO ENZYME IN SUGARCANE

Introduction	69
Materials and methods	70
Plant material	70
PPO solubility	70
Chlorophyll determination	70
Assay linearity	71
pH optimum of sugarcane PPO	71
Effect of substrate concentration	71
Heat inactivation	72

Results	72
Solubility of the PPO enzyme	72
Effect of enzyme concentration on reaction rate	75
pH optimum	76
Effect of substrate concentration on PPO reaction rate	78
Enzyme stability	80
Heat inactivation	81
Halide inhibition	82
Other inhibitors	85
Discussion	87

CHAPTER 5. PURIFICATION OF A 45 KD PROTEIN SHOWING PPO ACTIVITY

Introduction	91
Materials and Methods	93
Protein purification	93
Method 1-Purification of active PPO	93
Method 2-Purification for antibody production	96
Measurement of PPO activity and protein estimation	99
Electrophoresis	99
Protein sequencing	100
Results	101
Purification-Method 1	101
Purification-Method 2	109
Protein sequencing	116
Discussion	118

CHAPTER 6. SUGARCANE POLYPHENOL OXIDASE IS A 60 KILODALTON PROTEIN SUSCEPTIBLE TO *IN VITRO* CLEAVAGE

Introduction	122
Materials and Methods	122
Antibody preparation	122
Preparation of different sugarcane tissues for blotting	124
Acid-mediated cleavage of sugarcane PPO	124
Protease digestion	124
Cross-reactivity of plant PPOs	125

Immunoblotting of activity stained gel slices	125
Results	126
Detection of PPO in different tissue types	128
Protease digestion	131
Acid-mediated cleavage	133
Immunoblots of activity stained gel slices	135
Cross-reactivity of sugarcane PPO antibody	137
Discussion	139
CHAPTER 7. ISOLATION OF THE STRUCTURAL GENE ENCODING SUGARCANE POLYPHENOL OXIDASE	
Introduction	143
Materials and Methods	143
Plant material	143
Total RNA extraction and mRNA purification	144
cDNA library construction	145
<i>In vivo</i> excision of pBluescript	146
Plating and screening the cDNA library	146
Bacterial growth media	147
Extraction of plasmid DNA	148
Northern Blot analysis	148
Subcloning	149
DNA sequencing	149
Results	150
PCR	150
Screening the cDNA library	150
Characterisation of SUGPPO1	154
Expression of PPO mRNA in sugarcane	162
Discussion	165
CHAPTER 8. GENERAL DISCUSSION	170
REFERENCES	180

ABSTRACT

The contribution of polyphenol oxidase (PPO) and peroxidase (POD) to enzymic browning in sugarcane juice was investigated. Inactivation of these enzymes with heat resulted in juice of lower colour (absorbance measured at 420nm), but POD was found to be more heat stable than PPO. Salicylhydroxamic acid (SHAM) completely inhibited PPO activity and markedly reduced juice colour but had no effect on POD activity. Removal of oxygen in the presence of the substrate chlorogenic acid stopped colour formation. Upon subsequent addition of oxygen, browning continued, indicating that the process was oxygen dependent. This evidence suggested that enzymic browning contributes significantly to colour formation in sugarcane juice and that PPO is the major enzyme involved.

Differences were observed in levels of PPO, phenolics and colour in different sugarcane clones. There was a correlation between juice colour and phenolic content but not between juice colour and PPO activity. PPO activity throughout the sugarcane plant was measured and found to be highest on a fresh weight basis in the growing point. PPO activity was also higher in nodes than internodes and activity declined progressively down the stalk. PPO activity was high in young leaves, decreasing with leaf age. When extracts of sugarcane stem or leaf were subjected to differential centrifugation, most of the PPO activity remained in the supernatant even after ultracentrifugation, suggesting the enzyme was soluble or readily released from membranes. The PPO enzyme was most active with chlorogenic acid, not active with *para*-diphenols and was inhibited by SHAM suggesting it is a catechol oxidase-type enzyme and not a laccase. At temperatures above 65 °C the enzyme was rapidly inactivated.

A 45 kD protein with PPO activity was purified to homogeneity from young sugarcane stem using cation and anion exchange chromatography, gel exclusion and preparative

electrophoresis. A polyclonal antibody was raised against the purified 45 kD protein. Western blots identified a 60 kD PPO in stem and leaf extracts which is cleaved *in vitro* to the 45 kD protein in the presence of proteases and under acid conditions, without loss of activity. The antibody was used to screen a cDNA library constructed from immature sugarcane stem and a number of positive clones were identified. Mapping and cross-hybridization showed the clones to be closely related. A putative full length clone was sequenced and verified to encode PPO by comparison with other plant PPO sequences. Northern blots probed with this full length cDNA clone, identified a transcript of approximately 2.2 kb, which is highly expressed in young tissue with expression declining sharply in mature sugarcane. The complete cDNA encodes a 67 kD protein with a putative 8 kD transit peptide. The mature 59 kD PPO protein contains two copper-binding regions and a C-terminal extension believed to be 14 kD which is readily cleaved *in vitro* to give the 45 kD form.

The results suggest that PPO does contribute significantly to browning of sugarcane juice. Two possible approaches for decreasing this enzymic browning are suggested. The first, would involve inhibition of PPO activity during processing using either chemical inhibition or heat inactivation. The second approach would be to select for genotypes with a low potential to brown as there does seem to be a genetic basis to juice colour. Isolation of the gene encoding sugarcane PPO makes it feasible to use genetic manipulation to decrease expression of the PPO gene in existing commercial cultivars.

DECLARATION

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

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

SIGNED :

DATE : 24-2-95

Sections of this thesis have been published in the following paper and also presented as a conference seminar.

Publication

Bucheli, C.S. and Robinson, S.P. (1994). Contribution of enzymic browning to color in sugarcane juice. *Journal of Agricultural and Food Chemistry*. 42: 257-261

Conference seminar

Bucheli, C.S. and Robinson, S.P. (1994) Sugar polyphenol oxidase: purification of the protein and isolation of the structural gene. Abstracts of the Australian Society of Plant Physiologists, 34th Annual General Meeting, September 1994, Queensland

ACKNOWLEDGEMENTS

The work described in this thesis was supported by the Sugar Research and Development Corporation, whose financial assistance is gratefully acknowledged. There are many individuals whom I wish to acknowledge and thank for their contribution to this thesis. Firstly I would like to thank my supervisor Dr. Simon Robinson for his guidance, friendship and encouragement. I am deeply indebted, thanks Simon. Thanks also to my second supervisor Dr. Joe Wiskich whose dry wit was greatly appreciated. Many thanks to Dr. Ian Dry for his reading and criticising various aspects of this thesis, for his assistance with various techniques, his support and very importantly for being a great friend. Thanks also to Dr. Nils Berding for providing sugarcane material and access to his vast knowledge of the sugar industry. I gratefully acknowledge the RNA extraction method provided by Dr. Bob Bugos (USDA/HSPA). I would also like to acknowledge Dr Chris Davies for providing the invertase primers. Dr Anna Koltunow is thanked for her invaluable advice on the construction and screening of the cDNA expression library. I am very grateful for the facilities and warm friendly environment provided at the CSIRO Division of Horticulture. To my friends at CSIRO, many thanks for the good times, your encouragement and support. Special thanks to my lab Chris, Jude, Paul, Peter and Simon who have had to endure the last year of my Ph.D. Thanks Anne for proof-reading it was greatly appreciated. Lastly I thank my family who have supported me in everything I have ever set out to do and for their love and understanding despite the difficulties which distance imposes. Michael, thanks for putting up with me over the past year and for being so supportive.

ABBREVIATIONS

ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-1-phosphate
BSES	Bureau of Sugar Experiment Stations
BTP	bis tris propane
CAPs	3-(cyclohexylamino)-1-propanesulfonic acid
cDNA	complementary DNA
C _i	curie
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CTAB	cetyltrimethylammonium bromide
DIECA	diethyl dithiocarbamate
DNA	deoxyribonucleic acid
L-DOPA	L-3,4-dihydroxyphenylalanine
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
GCG	Genetics Computer Group (University of Wisconsin)
GSH	reduced glutathione
HSPA	Hawaiian Sugar Planters Association
IPTG	isopropyl- β -D-thiogalactoside
kb	kilobase pairs
kD	kilodalton
MES	4-morpholinethansulfonic acid
min	minutes
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NBT	4-nitro blue tetrazolium
OPC	oligonucleotide purification column
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
POD	peroxidase
PPO	polyphenol oxidase
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
SA-PMPS	streptavidin-paramagnetic particles
SDS	sodium dodecyl sulfate
SHAM	salicylhydroxamic acid

SRDC	Sugar Research and Development corporation
TBS	tris buffered saline
Tricine	N-Tris(hydroxymethyl)methylglycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
USDA	United States Department of Agriculture

CHAPTER 1. GENERAL INTRODUCTION

Sugar industry

Sugar is produced commercially from two plant sources - sugarcane and sugarbeet, with sugarcane producing 64% of the world's sugar supplies. World production since 1987 has exceeded 100 million tonnes of raw sugar per annum (SRDC, 1992).

In Australia sugarcane is grown from Mossman in North Queensland to Grafton in Northern New South Wales. In 1993 Australian sugar production was a record 4.37 million tonnes from 32 million tonnes of cane crushed. This was from a total of 340,000 ha harvested (SRDC, 1994). Australia exports approximately 80% of its raw sugar and is one of the world's largest exporters of raw sugar, with export sales worth more than \$1000 million annually.

Sugarcane

Sugarcane is a large grass of the genus *Saccharum* within the family Gramineae and is in the tribe Andropogoneae. Plants of the genus *Saccharum* have been selected and used as a source of sugar since the days of primitive man, however the origins of sugarcane continue to be debated. The term '*Saccharum* complex' has been used to describe a closely related interbreeding group believed to be progenitors of modern sugarcane (see review by Daniels and Roach, 1987).

Cultivated sugarcane are thought to have originated in New Guinea and other islands of the Malayan Archipelago. It is suggested that *Saccharum officinarum* L. may have been selected by man for its sweetness and low fibre and subsequently became dependent upon man for its propagation. Interspecific hybridisation of *S. officinarum* ('noble' canes) with the wild *S. spontaneum* L. or *S. robustum* Brandes and Jeswiet ex Grassl., and subsequent backcrossing gave significant yield increases, a process known as 'nobilisation'. In this process the female parent, *S. officinarum* contributes its somatic chromosome number to

the progeny whilst the male parent contributes vigour and disease resistance in a normally reduced chromosome complement. Commercial sugarcane cultivars are complex hybrids arising from this original 'nobilisation' and have high aneuploid chromosome numbers due to chromosomal increases accompanying certain crosses and backcrosses (see review by Daniels and Roach 1987).

Sugarcane is probably the most spectacular member of the grass family not simply for its sheer size, but also for its ability to synthesize, translocate and store sucrose in vast quantities. The basic structure of the sugarcane plant is closely related to other members of the Gramineae. Sugarcane reaches a height of 2-4 metres when mature and has a stem composed of a series of nodes and internodes which grow acropetally from a cone-shaped growing point. The increase in length of the stem, however, is due to an intercalary meristem at the base of each internode (Artschwager, 1925). Vascular bundles are scattered through the parenchymatous tissue of the internode, increasing in number, but decreasing in size, towards the periphery of the stem (see review by Moore, 1987). Leaves grow from the nodes on alternate sides of the stem. The leaf sheath envelopes the stem with the margins overlapping to protect the lateral buds present at each node. The leaf blade is linear lanceolate, up to two metres long. Inflorescence formation is known as arrowing, whereby a tassel of tiny flowers is formed. However, flowering in commercial sugarcane is an undesirable characteristic and is not often seen.

Sugarcane is propagated asexually, which involves the planting of sections of cane stem with one or more lateral buds, these stem sections are referred to as setts. The bud grows into a primary shoot and the roots develop under the influence of auxins. As the primary shoot grows it develops its own root system and a series of closely spaced joints, each with its own tiny bud and embryo roots, which give rise to the secondary shoots.

Typically in Australia, cane is harvested after 12-16 months. Following harvesting the cane is able to regrow, a process known as ratooning.

Milling

In Australia, sugarcane is mechanically harvested and delivered to the mill for crushing within about 16 hours. Currently sugarcane is both burnt and green harvested. The harvester removes the leafy tops of the cane stalks, cuts the stalks off at ground level and chops the stalk into short lengths known as billets. At the mill the billets are shredded and passed through a series of mills where the cane is crushed and the juice extracted. After crushing the juice is heated to approximately 77°C where the starch is broken down by heat activated enzymes. The juice is subsequently clarified by heating to 100°C in the presence of lime and then filtering the juice. The clear juice from the clarifiers is concentrated to a syrup by evaporating most of the water from it under vacuum. The syrup is then further concentrated by heating in a vacuum pan and is seeded with small sugar crystals. The sugar crystals are grown to the required size and the syrup is then separated from the crystals by centrifugation. Molasses, which contains about 35% sucrose, is the remaining syrup from which it is not economic to crystallise sugar.

Browning

Although sugarcane is one of the world's largest crops, it is not a high value crop and its economic importance comes from the sheer tonnage of cane produced. Because of the large volumes of sugarcane processed, and its inherent low value, the aim is to produce sugar of high quality while minimising the costs of processing.

The colour of raw sugar is an important aspect of its quality. Brown pigments are formed during the processing of sugarcane from the initial juice extraction through to crystallisation of the raw sugar. The presence of these coloured impurities is of considerable significance to the sugar industry as they impede crystallisation and result in lower sugar yields and poorer quality sugars (Jimenez and Samaniego, 1981). Removal of these colourants from raw sugar adds to the costs of refining, particularly where high molecular weight compounds are present in the raw sugar. These high molecular weight colourants have been found to be preferentially included in the sugar crystal during

crystallisation (Tu *et al.*, 1977; Smith *et al.*, 1981) and so are difficult to remove during refining. The total cost of colour removal in the Australian sugar industry has been estimated at \$5 M per annum, but even a partial reduction in raw sugar colour could have significant economic benefits. Many industrial consumers of sucrose, such as the beverage and certain confectionary industries, require very high quality white sugar.

The chemistry of cane sugar colourants is extremely complex as the colourants are a mixture of cane plant pigments, factory produced colourants and colour precursors. At least four different mechanisms are believed to contribute to colour formation during raw sugar production (Kort, 1979):

1. Melanoidins formed from sugar-amino acid reactions via the Maillard reaction. Melanoidin colours do not form until the mixture of reactants is submitted to heat.
2. Thermal degradation and condensation reactions of sugars (caramelisation). Caramels increase in molecular weight with time and temperature.
3. Alkaline degradation and condensation reactions of reducing sugars. The colourants formed from this process are relatively uncharged and of medium to high molecular weight.
4. Oxidative reactions of phenolic compounds.

The first three are non-enzymic reactions (colourants developed during processing by chemical reactions) whereas the oxidation of phenolic compounds to the chemically more reactive quinones is predominantly enzymic and occurs early in the extraction process, when the cane is first crushed.

Much of the investigation into raw sugar colour, thus far, has centred around the formation of colourants by the non-enzymic reactions discussed above. The problem of colourant identification has been approached from many directions resulting in a diversity of conclusions concerning the qualitative and quantitative significance of particular

colourants (Kort, 1979). Model systems have been used to simulate these various processes whilst colourants have been isolated and analysed from different stages of the milling process. Paton and McCowage (1987) investigated the effect of process conditions (e.g. temperature, pH and °Brix) on colourant formation during mill processing. They found temperature to be the most important factor governing colour formation due to alkaline degradation of reducing sugars and in the formation of melanoidins. Similar studies have looked at the different stages of milling and the colourants produced at each of these stages with a view to identifying the factors contributing to non-enzymic colour formation e.g. (Tu *et al.*, 1977; Smith *et al.*, 1981).

However, studies suggest that enzymic browning may contribute significantly to colour in cane juice. Smith (1976) found heating cane to 80-90°C prior to crushing resulted in a 47% reduction in average juice colour and a consequent 44% reduction in crystal colour. The heated cane juice had mostly low molecular weight colourants whereas the control juice had mainly high molecular weight colourants. In raw sugar made from heat treated juice, the colourants were mainly low molecular weight whilst sugar made from untreated juice contained a mixture of high and low molecular weight colourants. Tu (1977) observed a similar reduction in cane juice colour when the juice was made alkaline to inhibit enzyme activity. Liming raised the pH of the juice to more than 10.5, leading to a 44% reduction in juice colour and a 50% reduction in crystal colour.

Coombs and Baldry (1978) found addition of the compound thioglycollate at a concentration of 0.01% decreased cane juice colour by more than 50%. This decrease in juice colour was attributed to inhibition of the enzyme polyphenol oxidase (discussed in detail later). In various cane tissue extracts, high juice colour was found to be associated with higher levels of phenolics, amino acids and polyphenol oxidase (Goodacre and Coombs, 1978). The authors suggested this colour resulted from the interaction of enzyme-generated quinones and amino acids or soluble proteins.

From this early work it would appear that enzymic reactions contribute as much as 50% to cane juice colour, and that inhibition of enzyme activity reduces the proportion of high molecular weight colourants. Also, reduction in juice colour through the inhibition of enzyme activity results in raw sugar of lower colour.

Sugarcane colourants

The main colourants in sugarcane are chlorophylls and flavonoids. Chlorophylls are not soluble in water, are prone to chemical change and degrade easily (Harborne, 1984) and there is little evidence of them in cane juice. Flavonoids are phenolic compounds with two six-membered rings joined by a three member chain. Anthocyanins are a type of flavonoid, which are intensely coloured and responsible for the coloured rind of some sugarcane clones. They are, however, unstable in neutral or alkaline solutions, and are decomposed by heat (Harborne, 1984) and, thus, do not survive mill clarification. Flavones are yellow flavonoids, present in cane as glycosides, which are generally stable in mill clarification. More than 20 flavones have been identified in *Saccharum* and all are derivatives of tricetin, luteolin and apigenin (Smith and Paton, 1985).

Phenolic compounds are usually derivatives of cinnamic acids and they are present in the plant as esters. The most common ester in sugarcane is chlorogenic acid. Upon hydrolysis, chlorogenic acid yields both caffeic and quinic acids. Caffeic acid has also been detected in raw sugar (Farber and Carpenter, 1971). Phenolics are subject to enzymic oxidation forming quinones which can further react with other quinones, phenols or amino acids to form polymeric colourants. A reduction, not only in chlorogenic acid levels, but also the concentration of luteolin-based flavonoids, in cane extracts induced to brown, led Paton and Duong (1990) to suggest that these flavonoids were also involved in browning. The tops of stalk and the leafy trash contained a higher concentration of chlorogenic acid and flavonoids than the rest of the stalk (Paton *et al.*, 1991). Paton *et al.* (1991) suggested that a small percentage of this extraneous matter would increase significantly the high molecular weight colourants in juice.

The other significant colour precursors in sugarcane are reducing sugars and amino acids. When solutions of reducing sugars are heated under alkaline conditions coloured polymers are formed, known as alkaline degradation products. However, when high brix solutions of reducing sugars and amino acids are heated, coloured polymers known as melanoidins are formed (see above).

Enzymic browning

Enzymic browning in plants is initiated when cell integrity is disrupted, allowing the enzyme and its substrates to mix. Both phenol oxidases and peroxidases have been implicated in enzymic browning of plant tissues (Vámos-Vigyázó, 1981).

Peroxidases

Peroxidases are widely distributed in the plant and animal kingdoms. Plant peroxidases are heme-containing glycoproteins which utilise H_2O_2 to oxidise a wide range of phenolic substrates. The physiological role of peroxidase is not well understood but the general consensus holds that a major function of the extracellular peroxidase concerns lignin synthesis, regardless of whether in connection with cell differentiation or with host defences against pathogenic invasions (van Huystee, 1987). Peroxidase is considered to be a relatively heat stable enzyme and as such is widely used as an index of blanching in the canning of fruit and vegetables (Burnette, 1977).

Much of the wound-induced browning of plant tissues has previously been attributed to phenol oxidases (see below) with little evidence for the involvement of peroxidase. Takahama and Oniki (1991) however, suggest that a peroxidase- H_2O_2 system, and not phenol oxidase participates in the oxidation of the phenolic substrate DOPA in the leaves of *Vicia faba*. They found that in the presence of added H_2O_2 a melanin-like pigment accumulated. When tropolone, an inhibitor of polyphenol oxidase was added, DOPA oxidation was stimulated. Furthermore, transgenic tobacco plants overexpressing peroxidase exhibited rapid browning of stems following wounding (Lagrimini, 1991). The transgenic plants had levels of peroxidase activity 10-fold higher than control plants and

peroxidase was expressed in all tissues. Pith tissue from the peroxidase overproducer plants browned within 24 hours of wounding, while control tissue did not brown even after seven days. Thus when peroxidase levels in tobacco were elevated, increased browning occurred, but the role of peroxidase in normal enzymic browning is still not well established.

Phenol oxidases

Phenol oxidases are copper-containing enzymes which in the presence of oxygen catalyse the oxidation of phenolics to quinones (Mayer and Harel, 1979). The highly reactive quinones, thus formed, can undergo secondary reactions and polymerise to form the red, black and brown pigments associated with the browning of plant tissues.

Many different enzymes have been described as phenol oxidases and the relationship between these different enzymes is not clear. Nomenclature of these enzymes is somewhat arbitrary and has changed several times in the past 25 years (Mayer and Harel, 1991).

In animal tissues, the major enzyme catalysing phenol oxidase type reactions is tyrosinase, which is mainly involved in melanin synthesis and oxidises a range of *mono-* and *di-*phenols including DOPA and tyrosine. Phenol oxidases have been identified in a number of fungi and the terms laccase, polyphenol oxidase and tyrosinase have variously been used. Fungal tyrosinases which have been studied in detail include those from mushroom (Ingebrigtsen *et al.*, 1989) and *Neurospora crassa* (Lerch, 1987). The overall reaction mechanism of *N. crassa* tyrosinase has been described in a model by Lerch (1981). This model includes a binuclear centre and copper liganded in part by histidine. Monophenols bind to one of the Cu²⁺ atoms, while diphenols bind to both of them. Fungal tyrosinases are cytoplasmic, and histochemical and immunological localisation showed tyrosinase to be present throughout mushroom tissues (Moore *et al.*, 1988).

In higher plants at least two different types of phenol oxidase have been identified: laccase and polyphenol oxidase (PPO). Substrate specificity is diagnostic of the type of enzyme

present, laccases being able to oxidise both *para*- and *ortho*- diphenols while polyphenol oxidases oxidise only the *ortho*- diphenols (Mayer and Harel, 1979). PPOs and laccases can also be distinguished by their different sensitivities to various inhibitors. Salicylhydroxamic acid (SHAM) is a potent inhibitor of PPO, but it has little effect on laccase activity (Allan and Walker, 1988). Cinnamic acid derivatives have also been reported to inhibit PPO and not laccase, whilst laccases are inhibited by cationic detergents such as CTAB (Walker and McCallion, 1980).

While PPO is almost universally present in the plant kingdom, in possibly all angiosperms as well as numerous fungi, algae, bryophytes, pteridophytes and gymnosperms (Mayer and Harel, 1979), laccases appear to occur much less frequently. Laccases have been reported in many fungi and in certain higher plants, including peaches (Lehman, 1974), apricots (Dijkstra and Walker, 1991), sycamore cell cultures (Bligny and Douce, 1983), loblolly pine (Bao *et al.*, 1993) and all members of the Anacardiaceae (Joel, 1978; Robinson *et al.*, 1993). Although plant laccases show certain similarities to those described in fungi, they do differ in some respects and the relationship between the plant and fungal laccases is not yet clear.

Laccase is a blue glycoprotein that has been reported to contain 40% carbohydrate in sycamore (Bligny and Douce, 1983). Plant laccases are generally secreted into extracellular fluids being present in the sap of mangoes (Joel, 1978; Robinson *et al.*, 1993) and secreted by suspension-cultured sycamore cells (Bligny and Douce, 1983). Fungal laccases are thought to be involved in the degradation of lignin in plant cell walls, and although the function in plants is unknown, it has been suggested that they promote polymerisation of polyphenols in sap, causing it to harden and seal off wounds (Robinson *et al.*, 1993). They may also be involved in the lignification of wood tissues (Bao *et al.*, 1993).

PPO catalyses two separate reactions, the hydroxylation of *mono*-phenols to *ortho*-diphenols (cresolase, tyrosinase or monophenol oxidase activity) and the oxidation of

ortho-diphenols to their corresponding quinones (catecholase or diphenol oxygen oxidoreductase activity). PPO may exhibit either or both of these activities (Mayer and Harel, 1979). Whilst laccase is generally thought to be an extracellular enzyme, PPO is localised in plastids. PPO is believed to be the enzyme involved in the browning of sugarcane juice and is discussed in detail below.

Polyphenol oxidase

Latency

In many plants the enzymic activity of PPO is latent. The latency and activation of plant polyphenol oxidases were first studied by Kenten (1957; 1958) in broad bean, where activation was achieved by exposure to acid or alkali or by incubation with ammonium sulphate. The activation was attributed to removal of an inhibitory protein, assumed to be attached to the enzyme. The activation of spinach PPO has been extensively studied and has been found to be initiated by the onset of senescence (Meyer and Biehl, 1981), with the freezing and thawing of chloroplasts (Lieberei and Biehl, 1978), by fatty acids (Golbeck and Cammarata, 1981), by trypsin (Tolbert, 1973) and by sodium dodecyl sulfate (SDS) (Satô and Hasegawa, 1976). SDS also activates PPO extracted from green olives (Kahn, 1977), avocado (van Lelyveld and Bower, 1984) and potato leaf (Sánchez-Ferrer *et al.*, 1993). Broad bean PPO can also be activated by denaturing agents such as urea (Robb *et al.*, 1965), proteolysis with trypsin, thermolysin and subtilisin (King and Flurkey, 1987) or by treatment with fatty acids (Hutcheson *et al.*, 1980). As in spinach, activation of broad bean PPO has been suggested to be the result of release from the thylakoid membranes, although a conformational change in the enzyme brought about by these agents has also been postulated. Lerner *et al.*, (1972) found that irreversible activation of the PPO enzyme from grape berries following long exposure to acid pH was accompanied by a change in the Stokes' radius of the protein, indicating the involvement of a conformational change (Lerner and Mayer, 1975). Valero and García-Carmona (1992) suggested the pH response of latent PPO may represent a mechanism of regulation of its activity *in vivo*. Broad bean PPO is extracted as a latent 60 kD protein which can be

activated by addition of SDS (Moore and Flurkey, 1990; Robinson and Dry, 1992). The 60 kD PPO can be cleaved by the protease thermolysin to a 42 kD form with no loss of activity. In Bruce's Sport, a variegated sultana grape mutant with abnormal plastid development and low levels of PPO activity, the majority of the PPO is in an inactive 60 kD form. It is suggested that the specific protease necessary for the cleavage to the active 40 kD form is absent or dysfunctional allowing accumulation of the 60 kD form (Rathjen and Robinson, 1992a). In carrot cell cultures, PPO activity was increased three- to 15-fold in the presence of 2 mM CaCl₂, and a 58 kD lectin which enhances the activation of carrot prophenoloxidase by calcium chloride has been identified (Söderhäll *et al.*, 1990).

Cellular localisation

In non-senescent plant tissues PPO appears to be localised in plastids. This is supported by the evidence accumulated from fractionation studies, cytochemical and immunolocalisation studies, and chemical and genetic modifications of the plastid (Vaughn and Duke, 1984a).

In cellular fractionation studies (using either differential or gradient centrifugation) PPO activity is found to be associated with pellets enriched in chlorophyll or other plastid markers (Tolbert, 1973; Vaughn and Duke, 1984a). Reports of PPO associated with other cellular fractions may indicate solubilisation of the enzyme during senescence, aggregation of protein and membranes and cross-membrane contamination.

Polyphenol oxidase reacts with DL-dihydroxyphenylalanine (DOPA) to form an electron dense product. Following incubation of the active PPO with DOPA the highly osmiophilic compounds produced can be viewed in fixed plant tissues with an electron microscope. Much of the work concerning localisation of PPO has involved this cytochemical technique. PPO has been localised in a diverse number of plastid types including carrot tissue culture plastids (Olah and Mueller, 1981), leucoplasts of *Aegopodium podagraria* (Vaughn *et al.*, 1981), epidermal plastids (Henry, 1976; Vaughn *et al.*, 1981) and potato tuber amyloplasts (Czaninski and Catesson, 1974) as well as chloroplasts of many different

species (Henry, 1976; Vaughn and Duke, 1981a; Vaughn and Duke, 1981b; Vaughn *et al.*, 1981).

Although PPO appears to be restricted to thylakoids in most tissues, not all plastid types contain PPO. The plastids in guard cells in *Sorghum bicolor*, *Aegopodium podagraria* and *Solanum berthaultii* have been shown to lack active PPO (Steffens *et al.*, 1994; Vaughn *et al.*, 1981; Vaughn and Duke, 1981b). Using cytochemical and fractionation techniques, PPO was found to be present in mesophyll plastids yet absent from the bundle sheath plastids in the C4 plant *Sorghum*. Bundle sheath plastids virtually lack grana stacking however these membrane structures are not a requirement for PPO, as mutants of *Aegopodium* lacking 70S ribosomes (with only thylakoid rudiments) do have PPO (Vaughn *et al.*, 1981).

Modification of the plastid can result in the complete loss of PPO activity. Tentoxin is a fungal toxin which affects the internal membrane structure of plastids, inducing severe chlorosis. PPO activity is completely absent in all plastid types from tentoxin sensitive plants grown in a solution of the toxin (Vaughn and Duke, 1981a; Vaughn and Duke, 1982; Vaughn and Duke, 1984b). Using immunolocalisation techniques, PPO was found to accumulate at the plastid envelope in tentoxin-treated plants suggesting that it is not processed to an active protein (Vaughn and Duke, 1984b). In the white regions of the variegated grape mutant Bruce's Sport, which have low PPO activity, the plastids were not fully developed and lacked internal structure (Rathjen and Robinson, 1992a). It is suggested that a mutation affecting chloroplast development prevented PPO from being correctly processed and activated.

Despite the reports of cytosolic localisation of some PPOs (Mayer and Harel, 1979; Mayer and Harel, 1981), to date no PPO genes encoding a non-plastidic enzyme have been isolated. All of the PPO genes isolated so far possess a putative plastidic transit peptide typical of lumen proteins (Newman *et al.*, 1993). Steffens *et al.* (1994) suggest that although some of the reports of soluble PPOs may be authentic, others may be due to the

distintegration of plastids during senescence or fruit ripening or luminal PPOs released on grinding.

The most compelling work reported thus far on the subcellular localisation of PPO has been in a recent paper from Sommer *et al.* (1994). They showed the import, targeting and processing of a [³⁵S]methionine-labelled PPO precursor protein in isolated chloroplasts. The protein was routed to the thylakoid lumen in two steps. It was first imported into the stroma in an ATP-dependent step, processed to a 62 kD intermediate and then translocated into the lumen in a light-dependent step where the intermediate was processed to the 59 kD mature form. Tentoxin severely inhibited PPO import in this system. Consistent with the earlier observations of Vaughn and Duke (1984b), a significant amount of the unprocessed PPO remained bound to the plastid envelope in the presence of tentoxin.

In summary, the recent literature is consistent with PPO being localised only in the plastids of plant cells. Furthermore, it appears to be within the thylakoid lumen of chloroplasts, but its precise localisation within other plastid types has yet to be established.

Structure of PPO

Molecular weight estimates for PPO vary widely with multiple forms frequently reported (Mayer and Harel, 1979; Vámos-Vigyázó, 1981). Harel and Mayer (1968) reported three forms of PPO from apple fruit with MWs of 30-40, 60-70 and 120-130 kD. These forms were shown to undergo interconversions. Different forms have also been reported from within the same species. In banana, Thomas and Janave (1986) found fourteen forms of PPO in the pulp fruit of unripe Dwarf Cavendish fruit whilst Galeazzi *et al.* (1981) reported the presence after electrophoresis of only four bands in the pulp tissue of an unknown variety of mature dwarf bananas. Few reports, however, describe these apparent isozymes or multiple forms purified to homogeneity and many of the estimates were made under non-denaturing or partially denaturing conditions. Many PPO studies report isoforms of 40-45 kD. These include the polyphenol oxidases purified from spinach, olive, grape and sago palm (Vaughan *et al.*, 1975; Ben-Shalom *et al.*, 1977; Nakamura *et*

al., 1983; Okamoto *et al.*, 1988). Western blots probed with a polyclonal antibody raised against purified broad bean PPO indicated a 43 to 45 kD band was present in broad bean, lettuce, mung bean, soybean, spinach and tobacco (Lanker *et al.*, 1988). Whilst a 40-45 kD major form of the enzyme has been observed in a range of plants, all the genes recently isolated encode proteins with a molecular weight of around 60 kD (Cary *et al.*, 1992; Hunt *et al.*, 1993; Newman *et al.*, 1993; Dry and Robinson, 1994; Boss *et al.*, 1995). The work by Robinson and Dry (1992) suggests that this discrepancy may in part be attributed to C-terminal proteolysis during isolation, cleavage of the 60 kD protein giving rise to the 40-45 kD form. The other isoforms are thought to be artefacts of isolation and purification generated either by quinone alkylation, partial denaturation, proteolysis and carbohydrate attachment. Harel *et al.* (1973) reported that upon exposure to acid pH or urea, the predominant 55-59 kD form of PPO could be converted to 31-33 and 20-21 kD subunits. This dissociation could be mimicked by short exposure to commercial proteases. Failure to completely inhibit quinone formation and prevent cross-linking in one study led to a molecular weight estimate in excess of 10^6 for a trichome-localised PPO later shown to be a 59 kD protein (Bouthyette *et al.*, 1987; Kowalski *et al.*, 1992).

Properties

Polyphenol oxidase has generally been found to have an acidic pH optimum, ranging from pH 4.0 in eggplant (Fujita and Tono, 1988) to pH 6.0 in Koshu grapes and artichoke (Nakamura *et al.*, 1983; Zawistowski *et al.*, 1988). However, higher pH optima have been reported for PPO in a number of other plant tissues including the leaves of the *Aranda* orchid and from sunflower seeds with pH optima of 7 and 7.9 respectively (Lam and Ho, 1990; Raymond *et al.*, 1993). There are also conflicting reports on the pH optimum from the same plant. The purity of the enzyme source and the type of phenolic substrate being oxidised may affect the pH optimum (Mayer and Harel, 1979).

The substrate specificities of plant polyphenol oxidases have also been reported to vary widely, with many able to oxidise an extensive range of both *mono*- and *ortho*-diphenols. However, the affinity of PPOs for their phenolic substrates is relatively low. The K_M is

high, usually around 1 mM and higher (Mayer and Harel, 1979). Frequently it appears the phenolic compound for which a PPO has the highest affinity is the most prevalent in that tissue. The affinity of PPO for oxygen is also relatively low, with an estimated K_M of 0.1 mM or 8% O_2 (Radler and Torokfalvy, 1973), so that PPO is saturated with oxygen at the normal partial pressure of air, and with excess substrate the reaction velocity is close to its maximum.

Most of the reports on the thermostability of PPO have been from fruit and vegetables. PPO is not an extremely heat stable enzyme, with short exposures to temperatures of 70-90°C sufficient in most cases to inactivate the enzyme (Vámos-Vigyázó, 1981). There are, however, reports of more tolerant PPO enzymes, for example in mango, 35 minutes at 80°C was required for a 50% loss in activity (Robinson *et al.*, 1993). Heat inactivation tends to follow first order kinetics (Lee *et al.*, 1983; Zawistowski *et al.*, 1988; Robinson *et al.*, 1993) but there are also reports of biphasic heat inactivation e.g. (Batistuti and Lourenço, 1985).

Inhibitors of PPO

As a copper-containing enzyme, PPO is inhibited by agents which chelate copper and prevent its activity. Copper chelators known to inhibit PPO activity include cyanide, diethyldithiocarbamate (DIECA), citric acid and carbon monoxide. In olives, 50% inhibition of PPO activity was achieved with 5 μ M DIECA (Ben-Shalom *et al.*, 1977) with varying degrees of inhibition reported by others (Interesse *et al.*, 1983; Lam and Ho, 1990). SHAM is also a potent inhibitor of PPO (Allan and Walker, 1988). Tropolone is a slow-binding competitive inhibitor of PPO, structurally analogous to the *o*-diphenolic substrates of PPO as well as an effective copper chelator (Bryant *et al.*, 1953; Valero *et al.*, 1991). In the presence of hydrogen peroxide, tropolone acts as a substrate rather than an inhibitor of peroxidase and so can also be used to distinguish between PPO and peroxidase reactions (Kahn and Andrawis, 1985). Inhibition of PPO activity by NaCl and other halide salts has been observed (Ben-Shalom *et al.*, 1977; Rouet-Mayer and Philippon, 1986; Janovitz-Klapp *et al.*, 1990) but the inhibition is strongly pH dependent. Reducing agents

such as sodium metabisulfite, dithiothreitol, β -mercaptoethanol and ascorbate can reduce the quinones formed by PPO back to *o*-diphenols. These reductants are oxidised in the process such that their inhibition of PPO is often temporary. It is, however, generally accepted that these compounds also inhibit the enzyme to some extent (Mayer and Harel, 1979).

PPO is nuclear encoded

It appears that while PPO is localised in plastids it is encoded in the nucleus. A study of PPO isozyme patterns of species of *Nicotiana* and their hybrids was used to determine the mode of inheritance of PPO (Lax *et al.*, 1984). The plastids of *Nicotiana* are strictly maternally inherited, and the presence of PPO isozymes from the paternal parent and absence from the maternal parent indicated the nuclear inheritance of PPO. The Mendelian segregation of PPO allozymes in the glandular trichomes of *Solanum berthaultii* also demonstrated the nuclear inheritance of this enzyme (Kowalski *et al.*, 1990). Further, a mutant of *Aegopodium podagraria* which lacks 70S ribosomes in its leucoplasts still contains PPO. Therefore translation in the plastids was not required for PPO protein synthesis (Vaughn *et al.*, 1981).

PPO genes

As mentioned earlier, genes encoding plant polyphenol oxidases have now been cloned from broad bean (Cary *et al.*, 1992), potato (Hunt *et al.*, 1993; P.Thygesen pers. comm.), tomato (Shahar *et al.*, 1992; Shahar *et al.*, 1992), grape (Dry and Robinson, 1994) and apple (Boss *et al.*, 1995). They all encode 56-62 kD mature polypeptides with 8-12 kD putative N-terminal transit peptides.

The PPO gene family in tomato was characterised by Newman *et al.* (1993). All seven genes are clustered on a 165 kb fragment on chromosome 8, they all lack introns and encode CuA and CuB putative copper sites characteristic of bacterial, fungal and mammalian tyrosinases. In another Solanaceae member, potato, there is evidence for at

least six PPO genes (Thygesen *et al.*, 1994). In grape, however, Southern analysis suggested the presence of only one PPO gene (Dry and Robinson, 1994).

In tomato and potato leaves and flowers, and grape berries, PPO transcripts of 2-2.2 kb were present predominantly at early developmental stages (Shahar *et al.*, 1992; Hunt *et al.*, 1993; Dry and Robinson, 1994). Potato leaf PPO mRNA was developmentally regulated and only detectable in young foliage, however, the protein profile remained fairly constant with leaf age (Hunt *et al.*, 1993). While grape berry PPO mRNA expression was significantly curtailed during berry development (Dry and Robinson, 1994), PPO activity on a per berry basis appeared to increase (Rathjen and Robinson, 1992b) indicating the relatively slow turnover of this protein. *Vicia faba* was found to have a different expression pattern to that of grape, tomato, and potato, with PPO transcripts abundant in mature leaves (Cary *et al.*, 1992). Generally however, PPO mRNA appears to be expressed early in development whilst PPO activity remains fairly constant throughout development, indicating a low turnover of PPO.

Thygesen *et al.* (1994) found differential expression of the PPO genes in different potato tissues whilst Newman *et al.* (1993) suggest that divergent DNA sequences in the 5' promoter regions in five of the seven tomato PPO genes may regulate the differential expression of the genes.

Function of PPO

Despite intense study, biochemical and physiological studies have provided few answers to the question of PPO function in plants. The function of PPO has been reviewed by a number of authors (Mayer and Harel, 1979; Vámos-Vigyázó, 1981; Vaughn and Duke, 1984a; Vaughn *et al.*, 1988; Steffens *et al.*, 1994).

Polyphenol oxidase has been implicated in the synthesis of phenolic compounds as a consequence of its ability to convert monophenols to *o*-diphenols *in vitro*. This is supported by correlations between hydroxyphenolic compound content and extractable

PPO activity, co-induction of PPO activity with activities of other enzymes synthesising phenolic compounds and inhibition of phenolic hydroxylation by DIECA, an inhibitor of PPO (Vaughn *et al.*, 1988). However, when mung bean seedlings were treated with tentoxin and PPO activity was eliminated, the accumulation of the ortho-hydroxylated flavonoids (rutin and delphinidin) and caffeic acid was unaffected (Duke and Vaughn, 1982). Further argument against the involvement of PPO in phenolic synthesis comes from the observation that PPO is localised in plastids whereas most phenolic compounds are found in the vacuole (Vaughn and Duke, 1984a).

Because of its thylakoid membrane association and high K_M for oxygen it has been suggested that PPO has some role in photosynthesis. Mayer and Harel (1979) speculated that PPO may be involved in regulation of plastidic oxygen levels, despite its low affinity for oxygen. A role in pseudocyclic photophosphorylation (ATP production with O_2 as a terminal electron acceptor) was suggested by Tolbert (1973). From this Vaughn and Duke (1984a) suggest a mechanism by which PPO could regulate pseudocyclic phosphorylation by mediating photoreduction of molecular oxygen (the Mehler reaction) via photosystem I. Support for this hypothesis included the knowledge that quinones can mediate the Mehler reaction and that PPO exists only in chloroplasts that generate high oxygen levels (Vaughn *et al.*, 1988). However, more recent evidence which argues against this proposal includes the co-localisation of PPO with the proteins of photosystem II rather than photosystem I (Lax and Vaughn, 1991) and the demonstration of import, targeting and processing of a precursor PPO to the thylakoid lumen (Sommer *et al.*, 1994). The Mehler reaction occurs in the stroma.

A long suggested role of PPO is in electron transport. As quinones are powerful oxidising agents, the quinone produced from the oxidation of *o*-diphenols by PPO could then reduce another cell constituent non-enzymically (Mayer and Harel, 1979). However there is no real evidence that the reduction of quinones is a normal physiological process, active in electron transport. There is also little evidence to support localisation of PPO in mitochondria (Mayer and Harel, 1979; Mayer, 1987) and although there are claims of the

mediation of cyanide-insensitive respiration in plants by PPO (Mayer and Harel, 1979) it seems unlikely given the sensitivity of PPO to cyanide.

PPO has also been implicated in the formation of hard seed coats (Marbach and Mayer, 1975). However, Egley *et al.* (1983; 1985) have shown in several species, that this process is associated with peroxidase and not PPO. No PPO activity was detected in developing seed coats either by spectrophotometric assay, immunochemical or cytochemical techniques (Egley *et al.*, 1985). It seems likely therefore that the correlation with PPO activity reported by Marbach and Mayer (1975) was an artefact, and that the lignification of seed coats is due to the action of peroxidase.

Many authors view the primary role of PPO to be one of plant defence. PPO activity is highest early in fruit development and Mayer and Harel (1981) suggest that a high level of PPO and its substrates protects the seeds from predation prior to maturity. The oxidation of phenolics and tanning of protein lowers the digestibility and therefore the palatability of the fruit. As the seed matures and the fruit ripens, PPO activity on a whole fruit basis decreases so that seed dispersal by consumption and scattering of the residual seeds becomes more desirable. Sequestration of PPO within the thylakoid prevents its interaction with phenolic substrates until the cell is disrupted by herbivores, pathogens, senescence or injury and the latent enzyme is activated. The bacteriocidal and fungicidal hydroxyphenolics and quinones formed as a result of this cellular disruption may then polymerise to seal off infected tissues (Vaughn *et al.*, 1988). Attempts to correlate PPO activity with disease resistance have, so far, proved inconclusive (see review by Vaughn *et al.*, 1988). Reports of a stimulation of PPO activity following infection are also somewhat inconclusive, with peroxidase activity often increasing in combination with PPO activity (Abbattista Gentile *et al.*, 1988). Vaughn *et al.* (1988) suggest that although PPO activity usually increases in infected tissues it may only be a function of cell damage.

In contrast, the role of PPO in polymerisation of trichome exudate and entrapment of small-bodied insects is well accepted. Foliar glandular trichomes of the wild potato

(*Solanum berthaultii*) entrap insects by rapidly polymerising the trichome contents after breakage by insect contact. Polymerisation of trichome exudate appears to be driven by a polyphenol oxidase which constitutes up to 70% of the protein in individually collected trichomes (Kowalski *et al.*, 1992).

In experiments with tomato foliage, the relative growth rate of tomato fruit worm (*Heliothis zea*) was found to be negatively correlated with PPO activity (Felton *et al.*, 1989). When tomato fruitworm feeds on tomato foliage much of the ingested chlorogenic acid is converted to chlorogenoquinone by PPO in the insect gut. The reduction in growth rate is proposed to result from the alkylation of amino acids and proteins by the reactive *o*-quinone and a subsequent reduction in the digestibility of dietary protein. Felton *et al.* (1989) suggest that this mechanism of digestibility reduction may be able to be extrapolated to other plant-insect systems.

In a recent review Steffens *et al.* (1994) cites unpublished work examining the growth and development of tomato-adapted Colorado potato beetle (*Leptinotarsa decelneata*) on transgenic PPO-null and PPO-overexpressing transgenic tomato plants. Preliminary results indicate that mortality of the beetle was lowest and weight gain and developmental rate was highest on the PPO-null plants, conversely mortality was highest and developmental rate and weight gain lowest on the PPO-overexpressing plants.

Recent work by Boss *et al.* (1995) shows PPO mRNA in apple is induced in wounded and damaged tissues and in fruit showing symptoms of superficial scald, providing the first evidence of a wound-inducible form of PPO and further evidence for a potential role in plant defence.

Sugarcane PPO

Sugarcane PPO was first studied by Alexander (1966b) who extracted what he referred to as a 'tyrosinase' from various sugarcane tissues and investigated some of its properties. He found by far the greatest enzyme activity in the meristem tissue with relatively high

activity also present in young leaves. The enzyme oxidised a range of phenolics including tyrosine, catechol and DOPA and had a sharp pH optimum of 7.5.

Since this early work, an active PPO with a high specificity for chlorogenic acid has also been isolated from sugarcane leaf tissue (Coombs *et al.*, 1974). The enzyme was readily soluble, with the authors suggesting that it was cytoplasmic. Gel filtration produced two PPO fractions of 32 and 130 kD respectively. It was therefore suggested that PPO existed as a tetramer of the 32 kD subunit. The higher molecular weight form could be quantitatively converted to the 32 kD form by eluting the columns with buffer of high ionic strength or eluting with SDS or urea. The two forms of PPO identified in extracts of leaf tissue were subsequently identified in stem extracts (Gross and Coombs, 1976a). Contrary to the work by Alexander (1966b) a broad pH optimum was observed using both fractions with little change in activity between pH 4.5 and 7.5 and the specific substrate was found to be chlorogenic acid. Both forms were denatured fairly rapidly at 55°C. PPO activity was found to be high in the growing point and immature stem and higher in the nodes than the internodes (Goodacre and Coombs, 1978).

In summary, there is evidence to suggest that enzymic browning does contribute to colour formation in sugarcane. The literature on browning suggests that both PPO and peroxidase may be involved but this has not yet been determined and neither enzyme has been well characterised in sugarcane tissues.

The aims of the research described in the following chapters were:

- To determine the contribution of enzymic browning to colour in sugarcane juice.
- To determine which enzymes are involved.
- To determine the properties of these enzymes in sugarcane.
- To characterise, and if possible purify the enzyme from sugarcane.

CHAPTER 2. THE CONTRIBUTION OF ENZYMIC BROWNING TO COLOUR IN CANE JUICE

Introduction

Brown pigments are formed during the processing of sugarcane from the initial juice extraction through to crystallisation of the raw sugar. The presence of these coloured impurities is of considerable importance as their presence impedes crystallisation and results in lower sugar yields and poorer quality sugars (Jimenez and Samaniego, 1981). The removal of these colorants in the refining process, particularly the high molecular weight colourants increases the cost of refining.

The brown colour of raw sugar results from a complex interaction of substances derived from sugarcane and formed during processing. As discussed in Chapter 1, at least four different mechanisms are believed to contribute to colour formation during raw sugar production: (1) melanoidins formed from sugar-amino acid reactions via the Maillard reaction; (2) thermal degradation and condensation reactions of sugars (caramelisation); (3) alkaline degradation and condensation reactions of reducing sugars and (4) oxidative reactions of phenolic compounds (Kort, 1979). The first three processes are nonenzymic reactions, whereas the oxidation of phenolic compounds to the chemically more reactive quinones is enzymic and occurs when the cane is first crushed and its cellular contents are disrupted.

Previous studies suggest that enzymic browning may contribute significantly to colour in cane juice. Smith (1976) found that heating cane to 80-90°C for 10-15 minutes prior to crushing resulted in a 47% reduction in average juice colour, and a 44% reduction in the washed crystal colour. In the heated cane juice, the colourants were predominantly low molecular weight whereas the control juice contained mostly high molecular weight colourants. Tu (1977) observed a similar reduction in the colour of both cane juice and raw sugar by raising the pH of the juice above 10.5, thereby inhibiting enzyme activity.

Both polyphenol oxidase (PPO) and peroxidase (POD) have been implicated in the enzymic browning of plant tissues (Vámos-Vigyázó, 1981) by catalysing the oxidation of phenolics to the highly reactive quinones. An active PPO with a high specificity for chlorogenic acid has previously been isolated from sugarcane leaf tissue (Coombs *et al.*, 1974). Subsequent inhibitor and heat inactivation studies suggested PPO contributed significantly to colour formation in sugarcane juice (Gross and Coombs, 1976a; Gross and Coombs, 1976b; Coombs and Baldry, 1978; Goodacre *et al.*, 1980). The presence of POD has been reported in sugarcane and its properties have been studied (Alexander, 1966a). However, its contribution to colour formation in cane juice has not been investigated.

The work presented in this chapter aimed to establish the contribution of enzymic browning to colour formation in sugarcane juice and to determine the relative contributions of both polyphenol oxidase and peroxidase. Subsequent experiments were designed to determine the influence of substrate on the extent of colour formation.

Materials and Methods

Plant material

Sugarcane cultivars Q87 and Q96 were propagated from clonal setts obtained from the Bureau of Sugar Experiment Station (BSES), Mackay, Queensland and grown in a heated glasshouse with an average day temperature of 30°C and a night temperature of 17°C. The clones H56-752, Q87, Q96, 81C236, 81C337, 81C497, 81C509, 81C542 and 81C558 (Figures 2.4 and 2.5) were grown at BSES, Mackay, Queensland, under field conditions and harvested September 5, 1991.

Tissue extraction and enzyme assays

All experiments were repeated several times and unless otherwise stated, the data shown are representative of a typical situation. Individual measurements were replicated two to three

times. Lengths of stalk were sliced transversely into 2-3 mm thick discs using a Berkel food processor and then ground with a Polytron blender in four volumes of ice-cold 100 mM NaH_2PO_4 pH 5.0 containing 1 mM MgCl_2 . For the inhibition studies, specific inhibitors were added to the extraction buffer and the tissues homogenised in the presence of the inhibitors. Where tissue was heat treated, the discs were added directly to buffer of the appropriate temperature and immediately homogenised. The homogenate was filtered through Miracloth and used directly for enzyme assays. PPO activity was measured as substrate dependent oxygen uptake using a Hansatech oxygen electrode at 25°C. The reaction chamber contained 50 mM NaH_2PO_4 pH 6.0 and extract in a total volume of 1 mL, and the reaction was initiated by the addition of chlorogenic acid to a final concentration of 2 mM (unless otherwise stated). A pH of 6.0 was found to be optimal for the reaction (see Chapter 4). The units (U) of PPO activity are measured as $\mu\text{mol O}_2$ consumed per minute. Peroxidase activity was measured spectrophotometrically at 470 nm and 25°C. The reaction cell contained 0.56% (v/v) guaiacol, 50 mM NaH_2PO_4 pH 6.0 and extract in a total volume of 1 mL. Hydrogen peroxide was added to a final concentration of 0.3% (v/v) to initiate the reaction. No change in colour was detected in the absence of added hydrogen peroxide. The units (U) of POD activity are measured as the change in absorbance at 470 nm min^{-1} .

Juice colour determination

Unless otherwise stated, extracts were allowed to stand for at least 40-60 minutes, by which time browning was complete, and then centrifuged at 40,000g at 4°C for ten minutes. The supernatant was filtered through a 0.45 μM Millipore filter and colour assayed spectrophotometrically at 420 and 720 nm. The absorbance at 720 nm reflects the degree of turbidity and was subtracted from the absorbance measured at 420 nm. Where the change in colour was measured over time (Figures 2.3 and 2.6), samples were taken and salicylhydroxamic acid (SHAM) added to a final concentration of 2.5 mM to prevent any further oxidation. This inhibitor stop method was found to prevent any further colour development in the juice.

Measurement of phenolics

Phenolics were measured in aqueous extracts (Table 2.1) prepared as described for the measurement of colour. For the clonal studies (Figure 2.5) total phenolics were measured in methanolic extracts. Stems were sliced and ground with a Polytron blender in four volumes of 80% methanol and then centrifuged at 40,000g for ten minutes. The supernatant was collected and the pellet re-extracted in methanol and centrifuged again. The supernatants were combined and filtered through a 0.45 μ M Millipore filter. Total phenolics were measured following the method of Rathjen and Robinson (1992b), using the Folin-Ciocalteu reagent. Phenolic levels are expressed in gallic acid equivalents per gram of tissue. All measurements were made in duplicate.

Partial purification of PPO

Stems of sugarcane cultivar Q87 were sliced and homogenised using a Polytron blender in 20 mM MES, 10 mM L-ascorbic acid (pH 5.0), 2 μ M leupeptin and 1 mM phenylmethylsulfonylfluoride (PMSF). The extract was then filtered through Miracloth and centrifuged at 15,000g for 40 min at 4°C. The supernatant was loaded onto a S-Sepharose Fast Flow column (2.5x15 cm) equilibrated with 20 mM MES, 1 mM L-ascorbic acid (pH 5.0) at a flow rate of 10 mL min⁻¹ and subsequently washed with 200 mL of buffer. PPO was eluted with a gradient of 0-300 mM NaCl in the above buffer and the active fractions were pooled. The pooled extract was desalted on 4x40 cm column of Sephadex G-25 equilibrated with 20 mM Mes pH 6.0 at a flow rate of 10 mL min⁻¹. The active fractions were pooled, made 20% (v/v) in glycerol and frozen at -70°C.

Partially purified PPO was used to examine the effect of various substrates on colour formation (Table 2.4). Specific phenolics and flavonoids at the appropriate concentration were added to paired cuvettes, where one cuvette of the pair had been heat treated. The cuvettes were left shaking for 24 hours and the colour measured as described previously.

Stems of sugarcane cultivar Q96 were sliced into discs using a Berkel food processor and immediately dropped into boiling sterile water and reheated to boiling. The tissue was homogenised using a Polytron blender and the extract again heated to boiling to ensure no PPO activity remained. The extract was filtered through Miracloth and centrifuged at 40,000g for ten minutes at 4°C. The supernatant was collected and separated into two vessels; to one partially purified PPO was added to a concentration of 500 mU mL⁻¹. Both vessels were then shaken at room temperature for 90 minutes. The extracts were then filtered through a 0.45 µM filter and the colour at 420 and 720 nm was measured.

Results

Heat inhibition of colour formation

Preliminary experiments aimed to establish the contribution of enzymic browning to colour formation in sugarcane stems. Transverse sections through internodes were left for 3-4 hours to brown at room temperature or heated in a microwave for a minute to inhibit enzyme activity. There was an obvious inhibition of colour formation in the heat treated discs compared to the controls (Figure 2.1) suggesting that browning was an enzyme dependent process.

To investigate further this enzymic browning, the contribution of polyphenol oxidase and peroxidase to the browning of sugarcane juice was examined. Sugarcane stem was extracted at various temperatures and juice colour, total phenolics, and PPO and POD activities determined (Table 2.1). As there was some heat loss upon addition of plant material to the extraction buffer, and also during homogenisation, both the initial temperature of the buffer and the final temperature of the extract were measured.

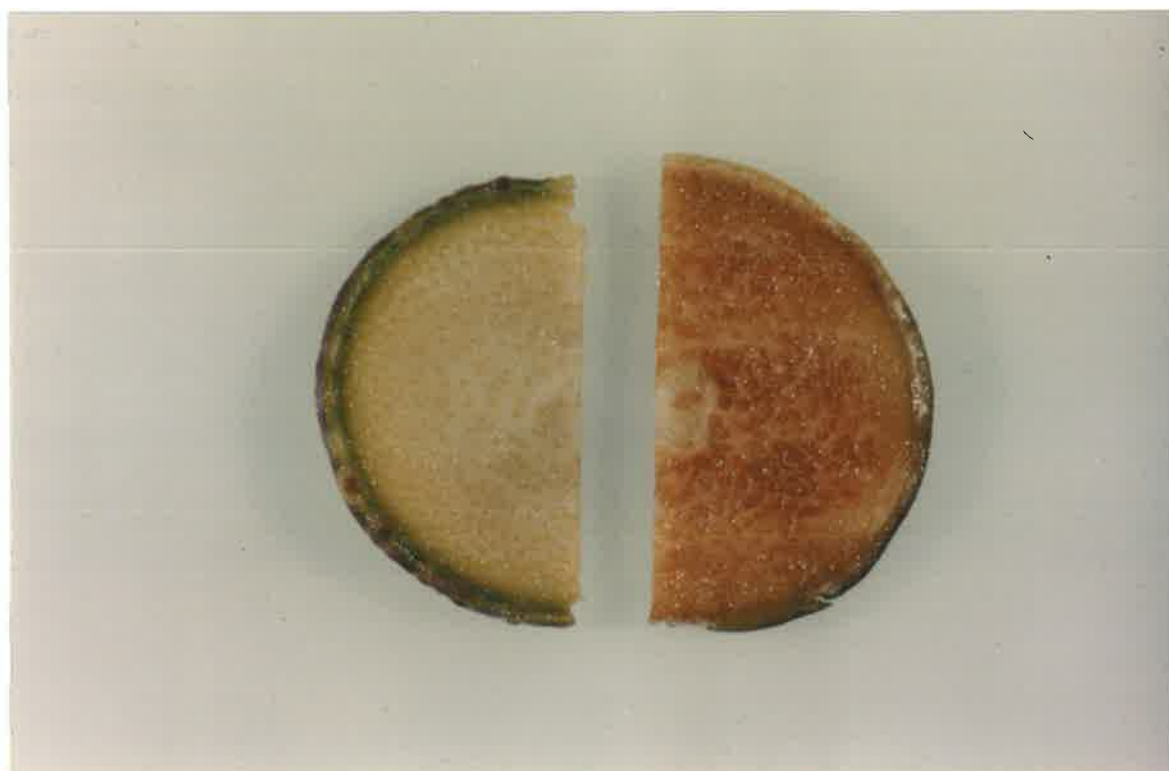


Figure 2.1. Transverse sections of internode sections which were either heat treated or left to brown at room temperature. The two halves are representative examples. The left half was heat treated, the right half was kept at room temperature.

Table 2.1. Effect of temperature on PPO and POD activities, colour and total phenolics in sugarcane juice. Stems of sugarcane clone 81C337 were homogenised in buffer at the temperatures indicated and the final temperature of the extract was determined after homogenisation.

Buffer temp. (°C)	Final temp. (°C)	Colour (A420 nm)	PPO activity (U gFW ⁻¹)	POD activity (U gFW ⁻¹)	Phenolics (mg gFW ⁻¹)
25	25	0.792	2.1	76	1.16
50	41	0.830	2.0	97	1.19
65	48	0.715	1.1	104	1.24
80	58	0.467	0.2	63	1.36
100	72	0.230	0.0	47	1.75

The colour of the sugarcane juice initially increased then progressively decreased with increasing extraction temperature. In extracts at 100°C, colour was reduced by 71% compared to those extracted at 25°C. There was a corresponding increase in the level of phenolics remaining in the juice following extraction at higher temperatures, which is consistent with phenolics acting as substrates for the browning reaction in cane juice. Polyphenol oxidase activity decreased with increasing temperature, with no PPO activity detected in juice extracted at 100°C. Peroxidase was much less sensitive to heat treatment, and more than half of the activity still remained in juice extracted at 100°C compared to that extracted at 25°C.

Enzyme inhibition

The relative contribution of PPO and POD to colour formation was further characterised using enzyme inhibitors. SHAM is a powerful specific noncompetitive inhibitor of polyphenol oxidase (Allan and Walker, 1988). Diethyl dithiocarbamate (DIECA) is also an effective inhibitor of PPO and acts by chelating copper in the active site of the enzyme (Anderson, 1968). Tropolone is a slow binding competitive inhibitor, structurally analogous to the *o*-diphenolic substrates of PPO, as well as an effective copper chelator (Valero *et al.*, 1991). In the presence of hydrogen peroxide, tropolone acts as a substrate for peroxidase rather than an inhibitor and so can be used to distinguish between the reactions of polyphenol oxidase and peroxidase (Kahn, 1985).

The effect of the addition of these inhibitors on juice colour and the activities of PPO and POD is shown in Table 2.2. A heat treatment where sugarcane stem was extracted in grinding medium heated to 100°C was also included for comparison. All of the treatments tested were found to cause a concomitant decrease in PPO activity and juice colour. In contrast, POD activity was not inhibited in all cases and the extent of inhibition was always less than for PPO. PPO activity was completely inhibited by SHAM and there was a marked decrease in juice colour yet there was no effect on POD activity (Table 2.2). Juice colour in the sample with tropolone was lower than might be expected from the measured PPO activity which was 23% of the control. An example of the colour of juice samples treated to inhibit enzymic browning is shown in Figure 2.2. Untreated juice was yellow brown to dark brown depending on the age of the tissue and the sugarcane clone, whereas juice treated with heat or SHAM was much lighter in colour and was clear to a very pale yellow.

Table 2.2. Effect of enzyme inhibitors on PPO and POD activities and colour in sugarcane extracts.

All values are expressed relative to the control i.e. PPO activity, $3.0 \mu\text{mol O}_2 \text{ min}^{-1} \text{ gFW}^{-1}$, POD activity, $98 \Delta\text{A}470 \text{ min}^{-1} \text{ gFW}^{-1}$ and the A420 was 0.6 absorbance units. SHAM and tropolone were present at a final concentration of 1.7 mM, DIECA was 8 mM. For the heat treatment stem portions were extracted at 100°C . The sugarcane clone was 81C337.

Inhibitor	PPO activity (%)	POD activity (%)	Colour A420nm (%)
Control	100	100	100
SHAM	0	103	30
Heat	0	55	35
Tropolone	23	79	36
DIECA	7	62	51

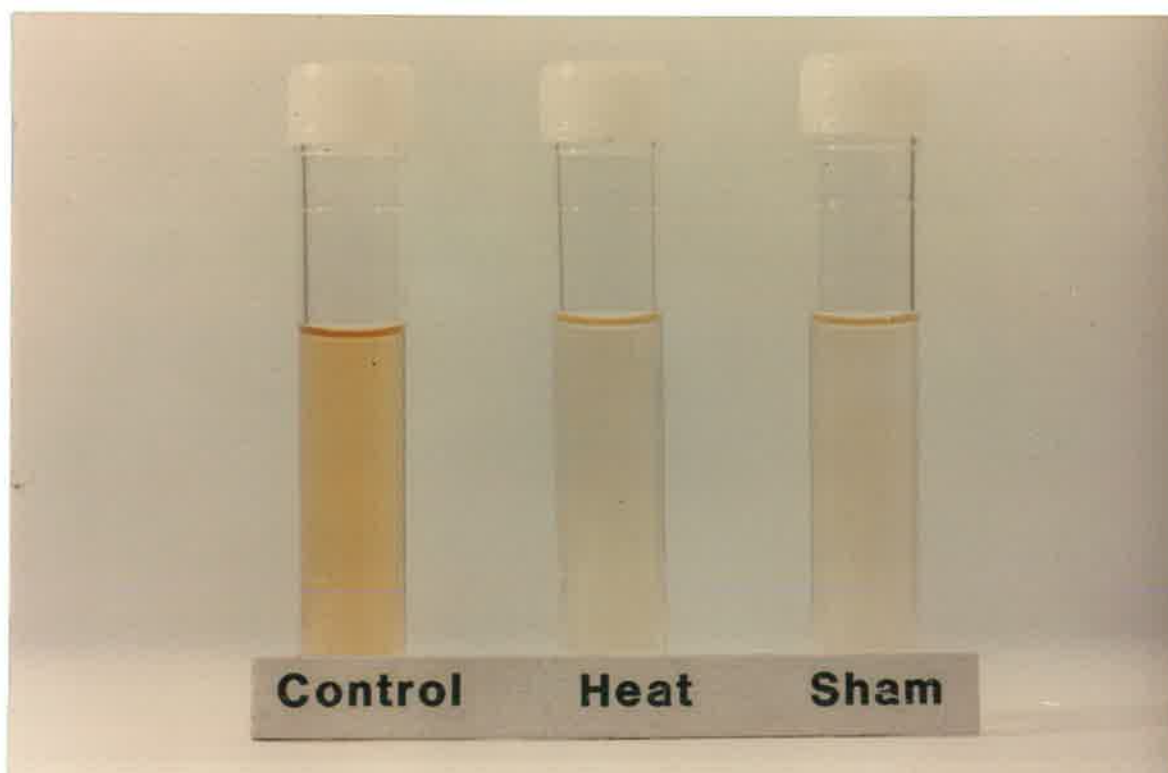


Figure 2.2. Inhibition of colour formation in sugarcane extracts. The extracts were made as described in Table 2.2

Oxygen-dependency of the browning reaction

Enzymic browning catalyzed by PPO is an oxygen-dependent reaction, unlike the oxidation reaction catalysed by POD. If PPO is a major contributor to browning, removal of oxygen from the extraction system should inhibit colour formation. Stems of cultivar Q96 were ground in the cold to minimise PPO activity, the extract degassed to remove oxygen and then placed in a sealed chamber. Nitrogen was blown over the extract, and the oxygen concentration was monitored with an oxygen electrode. When the oxygen tension was near zero, substrate (chlorogenic acid) was added and half the extract was exposed to air while the other half remained under nitrogen.

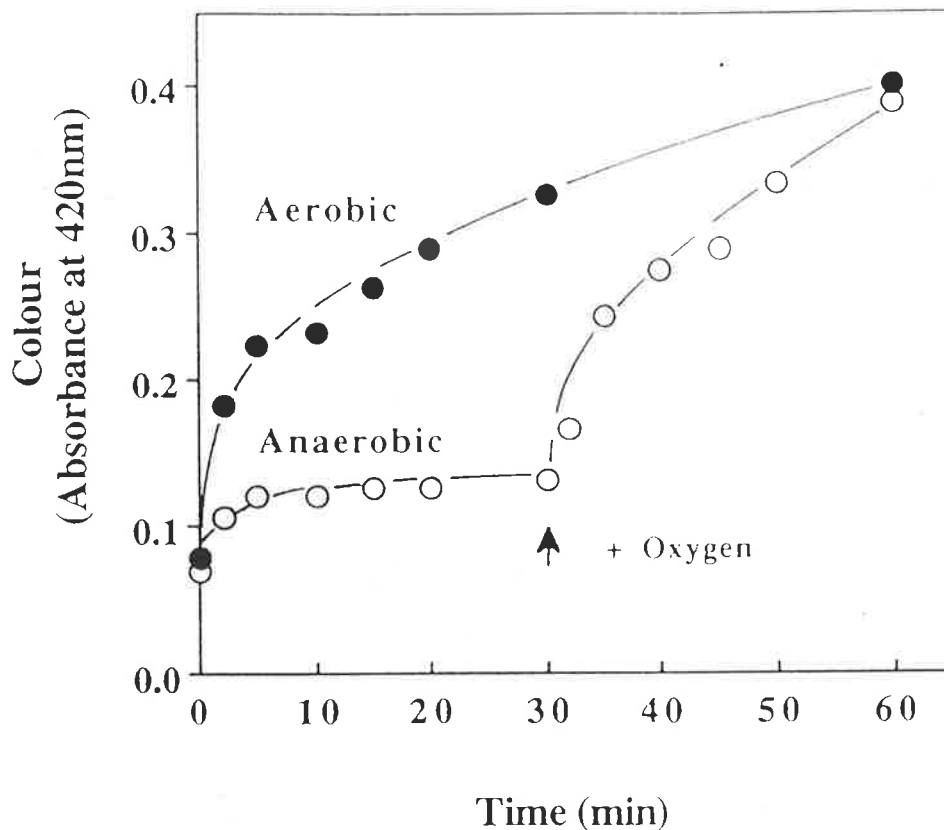


Figure 2.3. Colour development in cane in the presence and absence of oxygen. Oxygen was added back to the anaerobic sample after 30 minutes.

There was an initial increase in colour in the anaerobic sample which may have resulted from residual oxygen in the sample, but there was no further increase in colour after the first five minutes, whereas colour of the extract exposed to air increased (Figure 2.3). After 30 minutes the extract under nitrogen was exposed to air, resulting in a sharp increase in A420, and after 1 hour the two extracts had achieved the same A420 (Figure 2.3). Thus, colour development was severely inhibited in the absence of oxygen and was restarted upon addition of oxygen suggesting that under normal extraction conditions enzymic browning is predominantly an oxygen-dependent reaction in sugarcane juice.

Correlation between PPO activity and juice colour

A survey of a number of different sugarcane clones previously shown to produce juice of varying colour (Clarke *et al.*, 1990) was undertaken to determine the variation in juice colour and PPO activity. These nine clones grown at Mackay, Queensland were assayed for PPO activity, total phenolics and absorbance at 420 nm. The results for three separate stalks from each clone are presented in Figures 2.4 and 2.5. It can be seen that there was wide variation in all three parameters. PPO activity ranged from 1-6 Units gFW⁻¹ and there was an almost four-fold range in juice colour but only a two-fold difference in total phenolics measured. A least-squares regression showed only a weak correlation ($R^2=0.17$; p value=0.037) between colour and PPO activity (Figure 2.4). There was however a strong correlation ($R^2=0.76$; p value <0.0001) between total phenolics extracted and A420 measured at pH 5.0 (Figure 2.5).

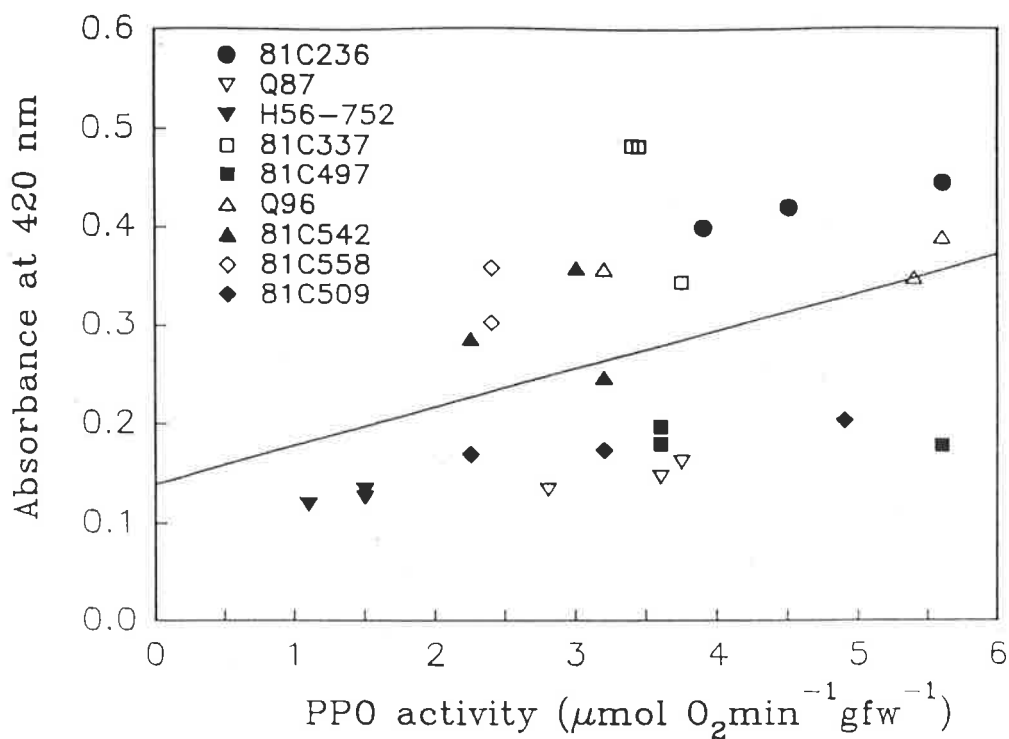


Figure 2.4. Relationship between PPO activity and colour in nine different sugarcane clones. Each point represents the PPO activity and colour determined in an extract from a single stalk. Extracts from three individual stalks were made for each clone. The line indicated had a correlation coefficient (R^2) of 0.17.

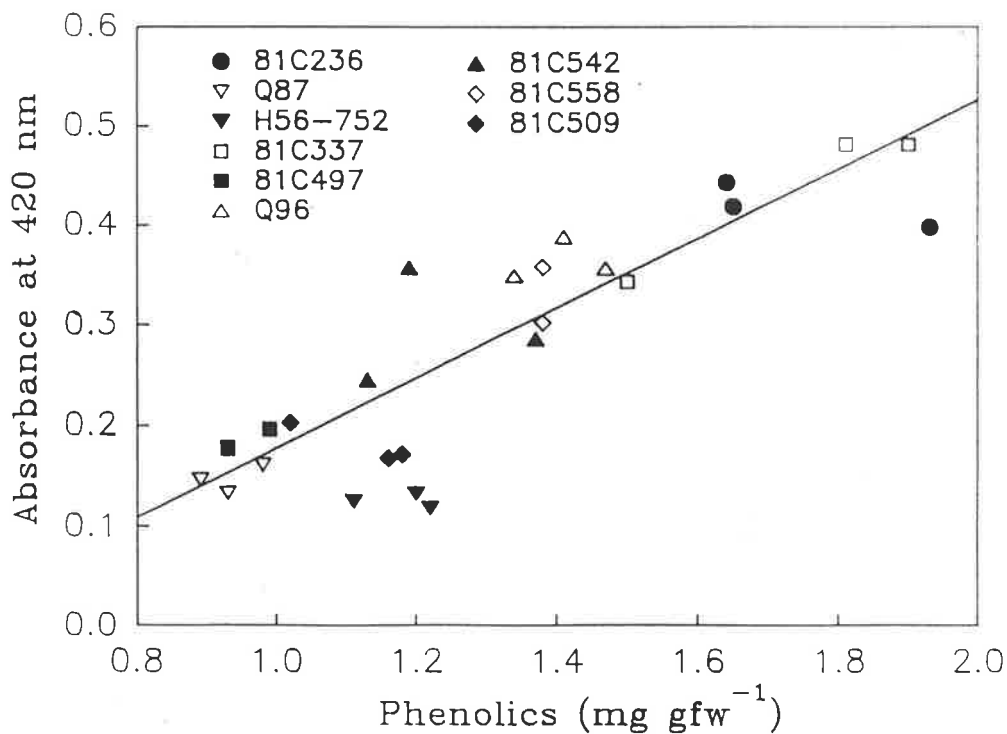


Figure 2.5. Relationship between phenolics and colour in nine different sugarcane clones. Each point represents phenolic content and colour determined in an extract from a single stalk. Extracts from three individual stalks were made for each clone. The line indicated had a correlation coefficient (R^2) of 0.76.

Effect of substrate on browning

Given the strong correlation between total phenolics and juice colour shown for the nine sugarcane clones, the involvement of substrate in the browning reaction was further investigated. Internodes of Q96 were extracted at 4°C, warmed to 25°C, and colour development (A420) monitored over time. Colour development was rapid initially but reached a plateau after 30 minutes (Figure 2.6) even though 75% of the initial PPO activity remained. Addition of 1 mM chlorogenic acid at this point resulted in a rapid increase in A420, suggesting that a lack of appropriate substrate was limiting the colour reaction. After 90 minutes, colour development of the extract with added chlorogenic acid had slowed considerably. PPO activity declined substantially over time, suggesting it was being inhibited by product formation or by other compounds in the extract. Walker (1964) observed an inhibition of apple PPO by its reaction products after only 20 minutes.

The substrate specificity of PPO extracted from sugarcane is shown in Table 2.3. PPO utilised a limited range of phenolic compounds with the *o*-diphenol, chlorogenic acid, by far the preferred substrate. The enzyme had a low activity with monophenols (ferulic acid, tyrosine, and *p*-cresol), and oxidation of the *p*-diphenols, toluquinol, and *p*-phenylenediamine was less than 3% of the activity with chlorogenic acid. The nature and occurrence of phenolics and flavonoids in sugarcane have been extensively studied in the sugar industry (Williams *et al.*, 1974; Smith and Paton, 1985). Flavonoid compounds are principally extracted from the cane plant in the maceration step of the mill process and are mainly located in the tops and rind of the cane plant (Smith and Paton, 1985). The involvement of specific sugarcane flavonoids in the browning process is shown in Table 2.4. It would appear that PPO cannot oxidise flavonoids themselves, however the reactive quinone produced from the oxidation of chlorogenic acid can oxidise specific sugarcane flavonoids and other *o*-diphenols resulting in an increase in extract colour above that when only chlorogenic acid is added. When the phenolics caffeic acid and chlorogenic acid were added in combination, the A420 nm

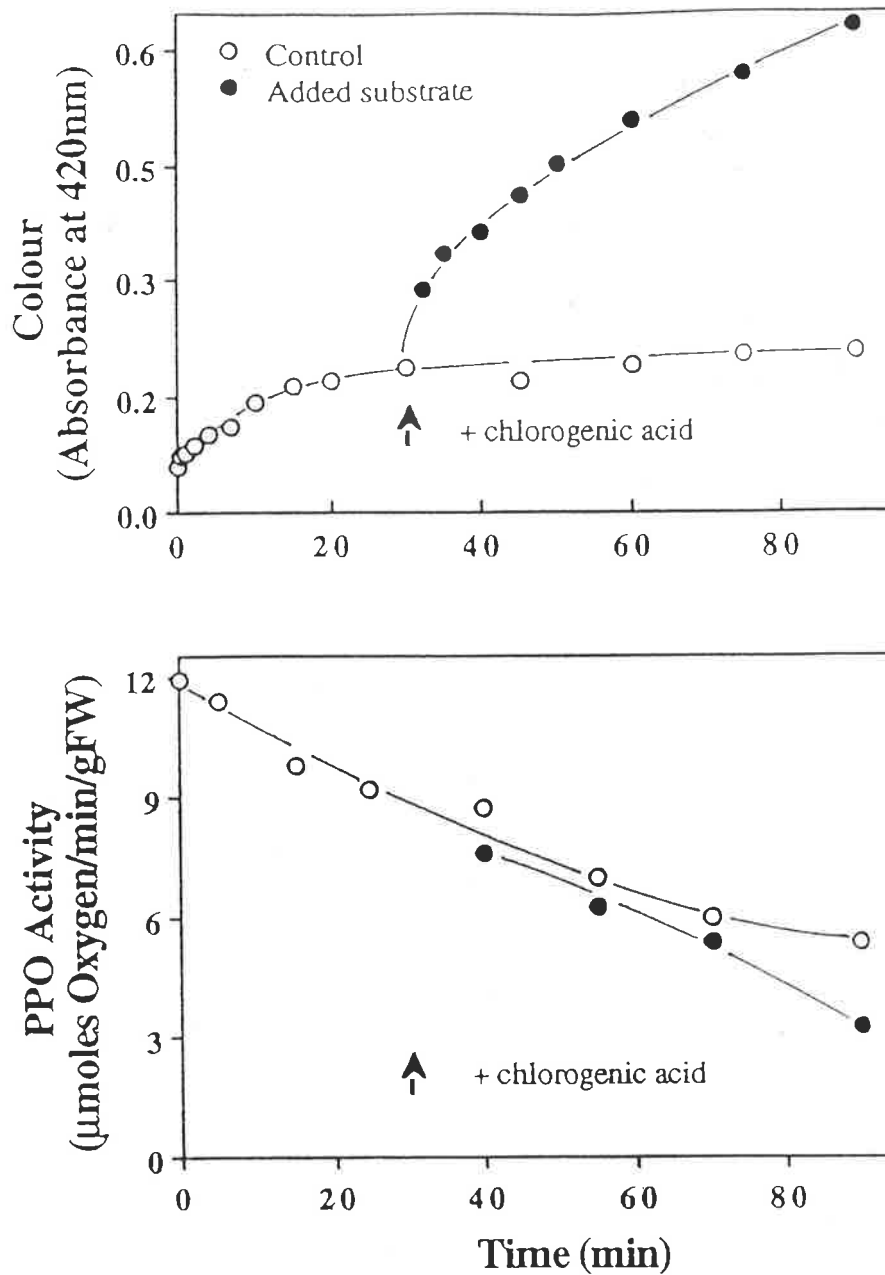


Figure 2.6. Colour development and PPO activity in sugarcane juice (cultivar Q96) over time. Chlorogenic acid was added to half the extract to a final concentration of 1 mM after 30 minutes, as indicated by the arrow. Samples were taken over time and immediately assayed for PPO activity and colour as described under Material and Methods.

Table 2.3. Substrate specificity of sugarcane PPO.

All substrates were present at a final concentration of 2 mM, except for dihydroxyphenylalanine, which was 1 mM. Activity was measured in extracts of cultivar Q87 and expressed relative to that obtained with chlorogenic acid .

Substrate	Relative PPO activity
Chlorogenic acid	100
Catechol	9
Caffeic acid	19
4-methyl catechol	22
Catechin	5
Dihydroxyphenylalanine	0
Ferulic acid	4
Tyrosine	0
p-cresol	1
p-phenylenediamine	2
Toluquinone	1

measurement was almost twice that when chlorogenic acid alone was added, which may suggest substrate was limiting when the two phenolics were added in isolation. The situation with the flavonoids is more complex however as they could not be directly oxidised by PPO. Of the flavonoids, luteolin was the most readily oxidised, the A420 measured when chlorogenic acid and luteolin were added in combination was again almost twice that when only chlorogenic acid was added (Table 2.4).

Table 2.4. Effect of different PPO substrates on colour formation in a partially purified sugarcane PPO preparation.

The phenolics chlorogenic acid (CA) and caffeic acid were added to a concentration of 1 mM and *L*-Dopa to 0.2 mM. The flavonoids luteolin and protocatechuic acid were added to 0.5 mM and apigenin to 0.25 mM.

Substrate	Extract + substrate (A420 nm) (A)	Heated extract + substrate (A420 nm) (B)	A - B (A420 nm)
CA	1.22	0.41	0.81
Luteolin	0.18	0.28	-0.1
CA+Luteolin	2.06	0.35	1.71
Apigenin	0.21	0.24	-0.03
CA+Apigenin	1.46	0.43	1.03
Protocatechuic acid	0.17	0.15	0.02
CA+Protocatechuic acid	1.55	0.44	1.11
<i>L</i> -Dopa	0.37	0.19	0.18
CA+ <i>L</i> -Dopa	1.80	0.52	1.28
Caffeic acid	0.87	0.28	0.59
CA+Caffeic acid	2.07	0.470	1.6

Effect of added PPO on colour formation

Partially purified PPO was added to heat treated extracts made from different internodes of cultivar Q96 (Figures 2.7 and 2.8). Each of the extracts had the same amount of PPO activity added to them, the different final A420 measurements reflecting the availability of substrate in each of the extracts. This experiment demonstrates that browning can be stopped by heat inactivation, and restarted with the addition of partially purified sugarcane PPO enzyme.

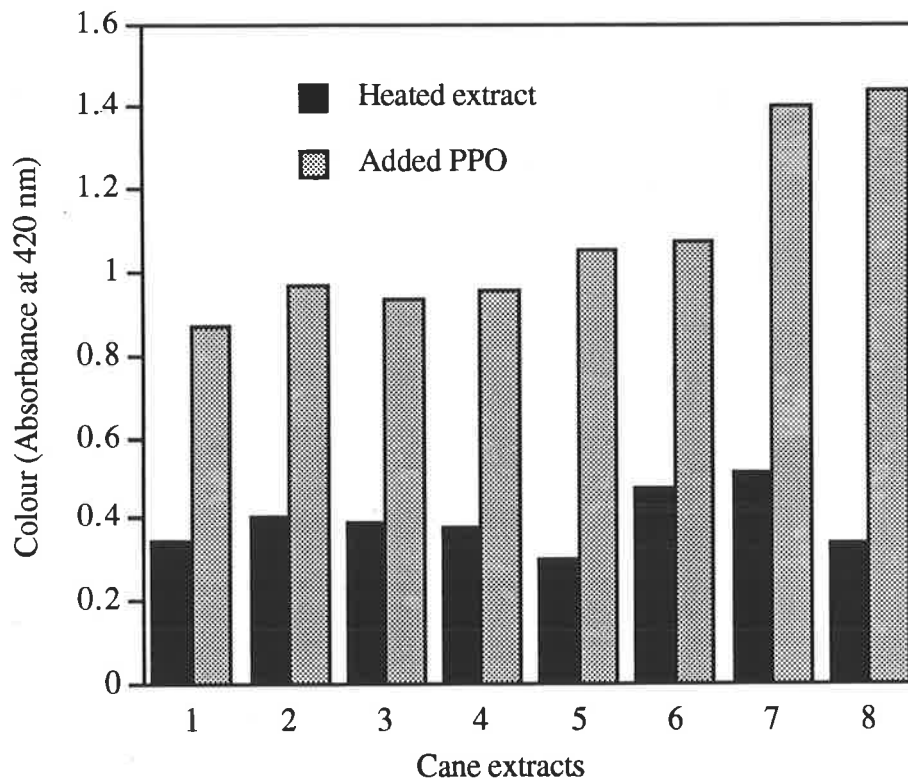


Figure 2.7. The effect of added PPO on colour formation in heated sugarcane extracts. The numbers indicate different extracts.

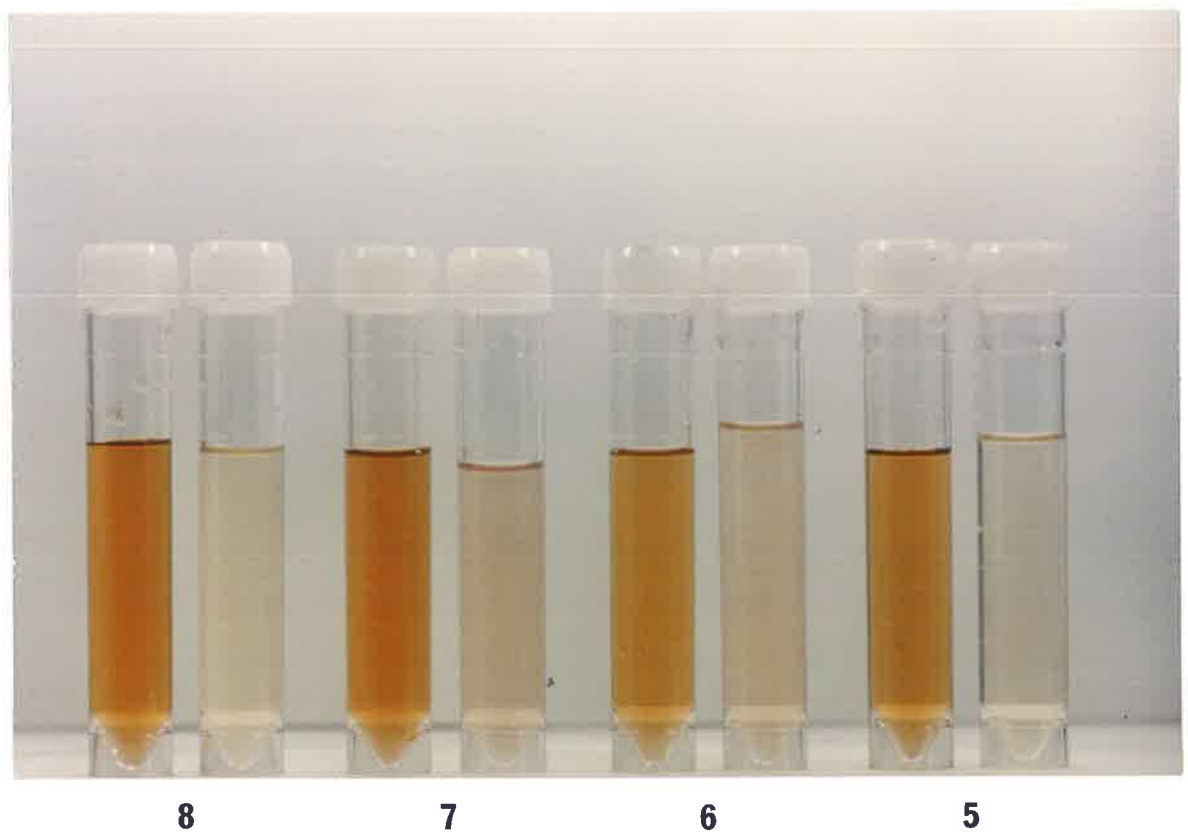


Figure 2.8. The change in colour observed in heated sugarcane extracts with the addition of partially purified PPO. Four representative extracts seen in Figure 2.7 are shown 5, 6, 7 and 8. The extract with added PPO is the lefthand tube of the pair.

Discussion

The inhibition of colour formation in the internode sections when heated suggests that enzymic browning does contribute to colour formation. This is reinforced by the progressive decrease in colour measured at 420 nm with increasing temperature of extraction of the dilute stem extracts (Table 2.1). The data suggests that more than 70% of the colour formed in cane extracts could be attributed to enzymic browning reactions. This is in agreement with previous studies where heat treatments and the addition of lime inhibited colour formation (Smith, 1976; Tu, 1977; Goodacre and Coombs, 1978) and decreased the level of high molecular weight colourants present in cane juice. The degree of colour inhibition reported is dependent on both the time taken to inhibit enzymic activity from the point of cellular disruption and the extent of inhibition. Thus, differences in the degree of colour inhibition may reflect inadequate inhibition or a delay in inhibition from when the sugarcane is first crushed. Furthermore, PPO in sugarcane juice is more sensitive than peroxidase to inhibition by heat. This is consistent with previous reports on peroxidase, which indicate that it is a relatively heat-stable enzyme and is thus widely used as an index of blanching and other heat treatments (Burnette, 1977).

The inhibition of colour formation with specific inhibitors of PPO activity again suggests that PPO is the predominant contributor to enzymic browning in sugarcane extracts. SHAM had the most dramatic effect on browning with a 70% reduction in colour and total inactivation of PPO yet it had no effect on POD activity. With both DIECA and tropolone there was also a significant decrease in colour with a reduction in both PPO and POD activities, however PPO activity was most severely affected. In earlier work, Gross and Coombs (1976b) showed more than a 50% reduction in cane juice colour using DIECA to inhibit colour formation and achieved a similar reduction with thioglycollate, another inhibitor of PPO. The inhibition of juice colour with tropolone appears to be greater than would be expected if PPO activity was the major contributor to browning. This may indicate that tropolone inhibition of PPO in the juice, where the level of phenolic substrates may be limiting, was greater than that determined

in the PPO assay with saturating levels of substrate. As a slow binding competitive inhibitor (Valero *et al.*, 1991) tropolone would be competing with the substrate to bind to PPO.

The different sugarcane clones may have a lowered potential for enzymic browning as a result of either low levels of endogenous phenolic substrates, lower levels of the enzymes involved, or the presence of endogenous inhibitors. The nine clones used in the clonal studies (Figures 2.4 and 2.5) are commercial hybrids and have been bred to have desirable traits such as high yield, high sucrose, and disease resistance. The poor correlation between PPO activity and colour (Figure 2.4) may well be due to tissue heterogeneity and the difficulty in sampling uniform plant material. The strong correlation between colour and total phenolics (Figure 2.5), however suggests that this is not the case and that availability of phenolic substrates limits the browning reaction in sugarcane juice. Similarly in apples, Harel *et al.*, (1966) found a good correlation between browning and *o*-diphenol content, but the correlation of browning with catechol oxidase activity was poor.

There are conflicting reports in the literature relating to the factors involved in the browning reaction. Sciancalepore and Longone (1984) found a direct correlation between PPO activity and the rate of browning of crude olive fruit homogenates. In contrast, Golan *et al.* (1977) showed the rate of browning of different avocado cultivars was positively correlated with the total phenol content. Addition of exogenous substrate (Figure 2.6) led to an increase in the rate of browning, suggesting that the reaction was not limited by PPO activity but by the presence of the appropriate substrate. Sapis *et al.* (1983) reported a similar finding for grapes, showing the potential for browning to be substrate limited. Substrate concentration also governed the degree of actual browning of different apple varieties (Walker, 1964). This is not unexpected as PPO is a catalyst and the endpoint (juice colour) should be proportional to the concentration of substrate, provided the reaction goes to completion.

It is apparent that the formation of colourants in sugarcane juice is the result of a complex interaction between the enzyme, the type of phenolic substrate present and its concentration, and possibly other reactive compounds present in the juice. The fundamental step in browning

is the transformation of *o*-diphenols to the corresponding *o*-quinones (Pierpoint, 1966). Once formed, the *o*-quinones are very reactive species which may polymerise (Pierpoint, 1966; Rouet-Mayer *et al.*, 1990), oxidise other substrates, and, in the process, be reduced to the original phenolic (Pierpoint, 1966). Substrate specificity is diagnostic of the type of PPO enzyme involved. Laccases can oxidise both *p*- and *o*-diphenols while catechol oxidases only oxidise the *o*-diphenols (Mayer and Harel, 1979). Sugarcane PPO had a much higher affinity for the *o*-diphenols than the *p*-diphenols. It was in fact highly specific for chlorogenic acid (Table 2.3), but would oxidise other *o*-diphenols such as caffeic acid, 4-methylcatechol and catechol to a lesser extent. The enzyme did not readily oxidise any of the *p*-diphenols suggesting it is not a laccase type enzyme. Both chlorogenic acid and its hydrolysis product, caffeic acid, have been identified in sugarcane tissues (Gross and Coombs, 1971). In cane leaf extracts chlorogenic acid and caffeic acid were the major *o*-diphenols identified (Baldry *et al.*, 1970). When the *o*-diphenols, chlorogenic acid and caffeic acid were added to sugarcane PPO there was a large increase in colour as measured at 420 nm (Table 2.4). When chlorogenic acid was added in combination with the other *o*-diphenols (caffeic acid or *L*-Dopa) or the flavonoids (luteolin, apigenin or protocatechuic acid) there was increased colour formation even though the flavonoids were themselves not readily oxidised by sugarcane PPO. This suggests that the primary catalysed oxidation of chlorogenic acid is followed by the chemical oxidation of a secondary phenolic by the resulting quinone. Coombs *et al.* (1974) using sugarcane leaf brei found a similar occurrence in mixed phenol reactions, where enzyme-catalysed oxygen uptake was only observed with a number of phenolics when catalytic amounts of chlorogenic acid were added.

Addition of partially purified PPO to heat treated stem extracts led to a dramatic increase in colour measured at 420 nm compared to the heated extracts without added PPO (Figure 2.7). The same level of PPO activity was added to all extracts so that differences in the final colour measurements must reflect the abundance of oxidisable phenolics and presence of other components in the extracts, with which the reactive quinones are able to interact. The inhibition of colour formation in juice by heat (Table 2.1), inhibitors of PPO (Table 2.2), and anaerobic conditions (Figure 2.4) indicates that enzymic reactions make a major contribution

to colour formation in sugarcane juice. The oxygen requirement for colour formation (Figure 2.4) and the observation that SHAM, which totally inhibited PPO without affecting POD, reduced colour formation to the same extent as boiling (Table 2.2), are strong evidence for the dominant role of PPO in enzymic browning in cane juice. Finally, addition of partially purified PPO to heat treated stem extracts caused them to brown (Figures 2.7 and 2.8). Thus, it appears, that although cane juice exhibits significant peroxidase activity which can cause enzymic browning, peroxidase does not contribute to colour formation in cane juice under normal extraction conditions, possibly because of insufficient levels of hydrogen peroxide required by the enzyme to catalyse the reaction. Whilst Lagrimini (1991) was able to produce transgenic tobacco plants overexpressing peroxidase which showed rapid browning in response to wounding, more evidence for the involvement of peroxidase in normal enzymic browning is required.

The results of this chapter suggest that inactivating the PPO enzyme would result in a significant decrease in cane juice colour. Selection of low browning phenotypes on the basis of PPO activity does not appear feasible due to the weak correlation between juice colour and PPO activity (Figure 2.4). However, if the level of PPO activity were reduced sufficiently, browning would be decreased in the commercial mill situation where the reaction can only proceed for a limited time, initiated by crushing of the cane and ending when the juice is sufficiently heated to inactivate the enzyme. Chemical inhibition of PPO is possible, but may not be an economic or desirable option for a food product. Thermal inactivation of the PPO enzyme is also possible as it does not appear to be a very heat tolerant enzyme. But white sugar produced from steamed cane has been reported to have a strong and persistent taste (Coombs *et al.*, 1980).

Further studies were undertaken to characterise the sugarcane PPO enzyme with the aim of decreasing its activity during processing or producing cultivars with low levels of PPO.

CHAPTER 3 . PPO, PHENOLICS AND COLOUR IN SUGARCANE

Introduction

The results presented in Chapter 2 suggest that polyphenol oxidase contributes significantly to colour in cane juice. The sugarcane plant, when harvested for crushing, is cut at the ground level and as much of the top leafy material is removed as possible. Given the size and complexity of the sugarcane plant, more information on the tissues contributing to colour formation in cane juice would appear necessary. PPO in higher plants has been found in a variety of organs and tissues. It is often abundant in leaves, tubers, storage roots, floral parts and fruits. Its abundance in tubers and fruits in the early stages of development has led to speculation that it has a role in protection against predation (Mayer and Harel, 1981). PPO is localised in plastids, although there are reports, particularly in fruit tissues, of its presence in other cell compartments (Mayer and Harel, 1979; Mayer, 1987). Earlier work on sugarcane by Goodacre and Coombs (1978) showed levels of colour to be higher in juice from nodes than from internodes, and higher in extracts of rind than from the storage parenchyma. Maximum absorbance at 420 nm was observed in extracts made from growing point tissue. PPO activity was found to be highest in the growing point and in tissue immediately below it. PPO was also reported to be higher in the nodes than the internodes and higher in the outer layers of stem tissue than internal pith tissue. Further, based on a measurement of absorbance at 320 nm of ethanolic extracts, the authors suggested that the concentration of phenolics was highest in the growing point and higher in the nodes than internodes.

The aim of the following experiments was to measure PPO activity throughout the sugarcane plant, and to determine the location of the phenolic substrates and the tissues contributing to colour in juice extracts. From initial studies (Chapter 2) and the observations by Clarke *et al.* (1990), the commercial cultivars Q87 and Q96 are distinctly

different in their potential to brown, Q96 producing much darker juice. The differences between these clones were further investigated.

Furthermore, clones of the genus *Erianthus*, reported to be involved in the origin of sugarcane (Mukherjee, 1957) have been observed to brown very quickly when cut or crushed. The levels of phenolics, PPO and colour were investigated in a number of *Erianthus* clones and compared with a number of commercial breeding clones grown at the same site.

Materials and Methods

Plant material

The cultivars Q87 and Q96 were grown under glasshouse conditions at the CSIRO Division of Horticulture, Adelaide. All other material including the *Erianthus arundinaceus* clones were sent overnight from BSES Meringa, Queensland courtesy of Dr Nils Berding. The *Erianthus arundinaceus* clones were IK76-20, IK76-22, IK76-62, IK76-103 and IK76-124, and the *Saccharum* spp. hybrid canes were 60N1853, 61N1232, 62N1659, 63N1700, 66N2008, 67N1691, 67N2254, 72N424 and 88N1797.

Plants were harvested and leaf, node and internode position assigned based on the TVD (top visible dewlap) counting system (Moore, 1987). The dewlap is the transitional tissue between the leaf blade and the leaf sheath which differs in colour from the lamina and is readily identifiable. For the purposes of this thesis, the youngest leaf whose dewlap is fully exposed (e.g., not enclosed in older leaves) has been designated as leaf D1, the node at which it is attached to the stalk as node 1 and the internode subtending it as internode 1. Older internodes and leaves were numbered consecutively down the stalk. For the purposes of this study, the growing point tissue is defined as the 0.5 cm of tissue below and including

the apical meristem. The leaves were numbered relative to the first exposed dewlap (D1). These designations are shown in a diagrammatic form in Figure 3.1.

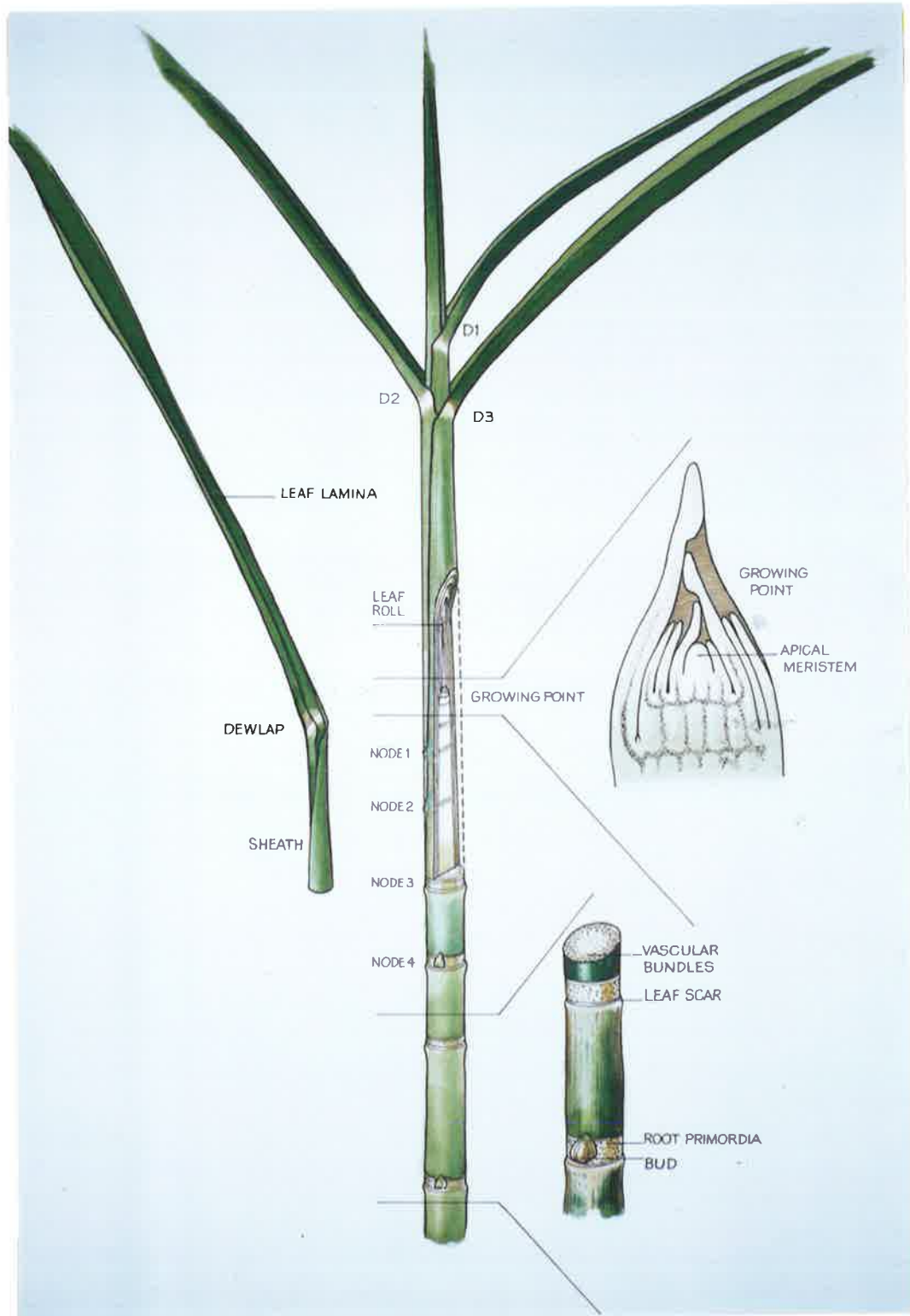


Figure 3.1. Diagrammatic presentation of the designations given to the various parts of the sugarcane plant. (After Artschwager (1925) and van Dillewijn (1952)).

Root tissue was from germinating setts of both cultivars Q87 and Q96. Where measurements were made on leaves, the whole leaf blade was homogenised to avoid any variation along the leaf blade.

The distribution of PPO activity, phenolics and colour within the sugarcane stem was investigated. The 8th and 10th internodes and the 9th and 11th nodes of glasshouse grown Q87 were used. Sugarcane stem was separated into internodes and nodes and the internode tissue further separated into parenchyma and rind (2-3 mm in from the epidermis).

Extraction and assay of PPO activity, phenolics and colour in different plant parts

Each of the various tissues was dissected out and frozen immediately in liquid nitrogen. The tissue was ground to a fine powder in a pre-cooled coffee grinder and the powder added immediately to three volumes of stirring extraction buffer (PPO assay) containing 50 mM NaH₂PO₄ pH 6.0 or four volumes of methanol (total phenolics assay). For the measurement of PPO activity the homogenate was filtered through Miracloth and then assayed for PPO activity as described in Chapter 2.

For measurement of phenolics, the extracts were vigorously shaken and centrifuged to pellet the insoluble material. The supernatant was retained and the pellet re-extracted with 80% methanol to a volume equivalent to the original tissue volume. The supernatants were pooled, 1 mL taken and dried down to 200 μ L in a Speedivac and made up to the original volume with deionised water and then filtered through a 0.45 μ M Millipore filter. The filtrate was assayed as described in Chapter 2.

Where colour was measured, 4 mL of the extracts prepared to measure PPO activity were shaken for 12 hours at room temperature. The extracts were then centrifuged in at 14,000g for 10 minutes, filtered through a 0.45 μ M Millipore filter and absorbance at 420 and 720 nm measured.

When young stem was assayed for PPO activity, 10 mM ascorbate was included in the extraction buffer to prevent tanning of protein, as these tissues tended to brown rapidly once cut. Tissues were dissected and immediately placed in the extraction buffer containing ascorbate and the tissue homogenised with a Polytron Blender and filtered through Miracloth. The tissue:buffer ratio varied depending on the potential of the tissue to brown. Protein concentrations were determined using the Biorad Protein Assay, based on the method of Bradford (1976) using bovine gamma globulin as a standard.

Sectioning and staining

Both transverse and longitudinal hand sections through the internodes and nodes of sugarcane cultivar Q90 were cut and left to brown undisturbed at room temperature. The longitudinal section through the growing point of the stalk was from Q87. Nodal sections were stained for polyphenolic compounds following the method of Hawker *et al.* (1972), where sections were rinsed briefly in water and then placed in 1% vanillin in concentrated HCl overnight. Vanillin-HCl produces a carbonium ion from compounds containing a 1, 3, 5 trihydroxybenzene nucleus, giving a red colour.

Heat inactivation of PPO

Stems of the commercial cultivars Q87, Q90, and Q96 and an *Erianthus* clone IK76-140, were sliced using a Berkel food processor and 15 g of material from each clone added to 50 mL of extraction buffer containing 0.1 M NaH₂PO₄, 1 mM MgCl₂ pH 5.0 and also to 50 mL of the same buffer heated to boiling. The tissue was homogenised using a Polytron blender. The extracts were filtered through Miracloth and PPO activity assayed as described in Chapter 2. After centrifugation at 40,000g for 10 minutes, the measurements of colour, total phenolics, and peroxidase activity of the extracts were carried out as described in Chapter 2.

Clonal studies

Some *Saccharum spp.* hybrid clones grown for the Northern zone of Queensland (referred to as the Northern clones) were used in the comparison with the *Erianthus arundinaceus* clones. 30-40 cm from the bottom half of the stalk were sliced, from which 20 g of tissue was subsampled and homogenised in 100 mL of 0.1 M NaH₂PO₄, 1 mM MgCl₂ pH 5.0 extraction buffer. The whole homogenate was then assayed for PPO activity as described in Chapter 2. Following filtering and centrifugation at 40,000g, the pH of the extract was adjusted to 4, 7, or 9 and the absorbance at 420 and 720 nm was measured at each pH. These results were used to calculate the IV value, the ratio of the absorbance at 420nm at pH 9.0 to that at pH 4.0. The IV value reflects the pH sensitivity of the colourants, a high IV value indicating a high proportion of pH-sensitive flavonoids and phenolics whilst a low value is indicative of pH-insensitive high molecular weight colourants. Juice soluble solids were measured using a Zeiss handheld refractometer and expressed in °Brix. Total phenolics were extracted in methanol as described for the comparison in Chapter 2. For each clone, measurements were made on two separate stalks.

Results

Localisation of tissue browning and phenolics

The most obvious browning of cut sugarcane was through the shoot apex. A longitudinal section through this region is shown in Figure 3.2. Browning was concentrated in the region of the apical meristem and in the young nodes. Browning occurred very rapidly, and was even more rapid and spectacular in the high browning cultivars such as Q96 (data not shown).



Figure 3.2. Longitudinal section through the shoot apex of sugarcane cultivar Q87, showing browning.

Browning observed in transverse and longitudinal stem sections left to brown was concentrated towards the periphery of the stem where the concentration of vascular bundles was greatest (Figures 3.3, 3.4). The brown colourants were localised primarily around the vascular bundles and the cells which constitute the rind. The browning was greatest in the young internodes decreasing with increasing age of the tissue (Figure 3.3). A longitudinal section through a node shows a concentration of browning through the node and along the vascular strands, again increasing at the periphery of the pith (Figure 3.4).

Staining for polyphenolics using vanillin showed a concentration of phenolics in the pith tissue beneath the rind (Figure 3.5) decreasing towards the centre of the section. There was also staining in the lateral bud. The longitudinal section (Figure 3.6) showed strong staining through the node but was much less obvious in the internode. It should be noted, however, that the vanillin stain is selective and does not detect hydroxycinnamoyl esters such as chlorogenic acid and therefore does not show the location of all potential substrates in sugarcane tissues.



Figure 3.3. Browning of stem sections. Transverse internode sections of sugarcane cultivar Q90 were left to brown, older internode (left) to younger internode (right).



Figure 3.4. Longitudinal section through a node of sugarcane cultivar Q90, showing location of browning.

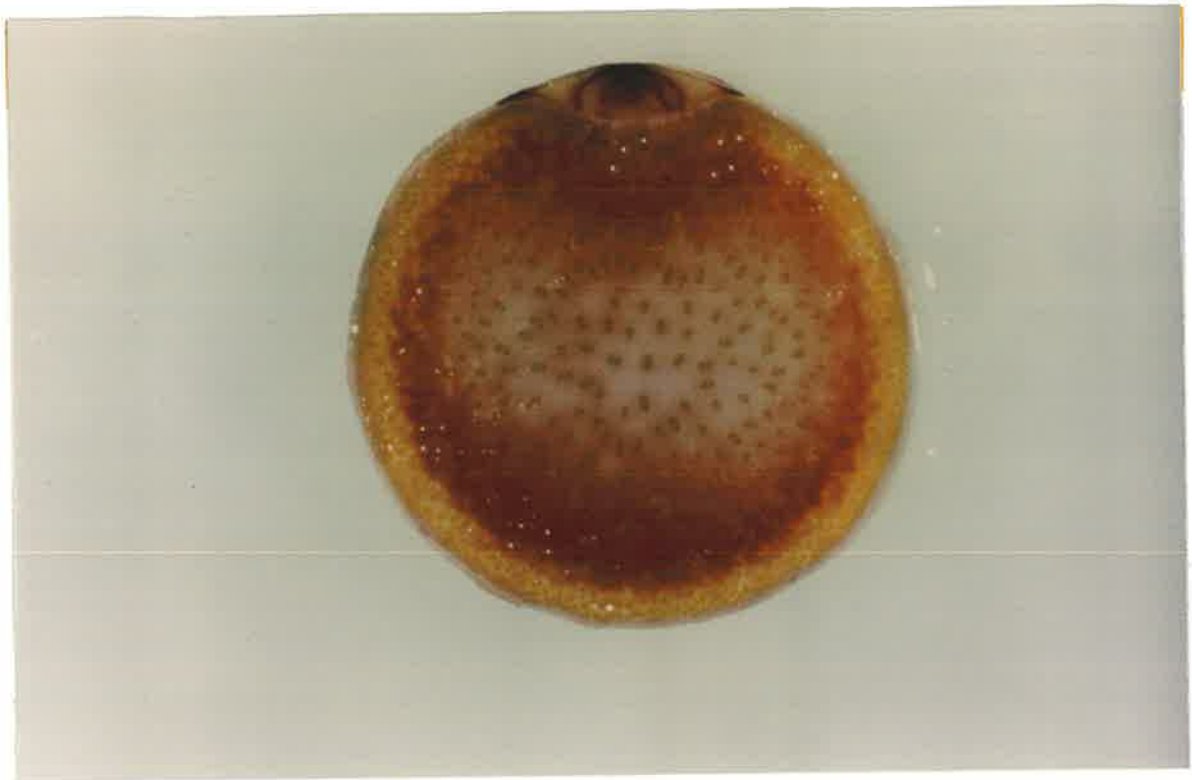


Figure 3.5. Transverse nodal section of sugarcane cultivar Q90, stained for polyphenolic compounds.



Figure 3.6. Longitudinal section through a node of sugarcane cultivar Q90, stained for polyphenolic compounds

PPO activity throughout the cane plant

The distribution of PPO activity in sugarcane stems was measured in Q87 and Q96, with both cultivars showing similar trends over three separate experiments. A typical set of results from an actively growing Q87 stalk is presented (Figure 3.7). PPO activity was much higher in the nodes than the internodes, and ^{substantially} significantly higher in the immature stem. In the mature cane, levels of PPO activity remained relatively constant down the stalk with activity remaining higher in nodes than internodes.

The tissue defined as the growing point includes the apical meristem and its derivative meristematic tissues that give rise to the entire plant body. PPO activity was highest on both a fresh weight and protein basis in this meristematic tissue (Table 3.1) and in the young stem immediately below the growing point. PPO activity was also high in the young leaf roll, and was highest in the 5 cm of soft tissue immediately above the growing point. Activity also was high in young leaf, and was extracted from both young leaf lamina and leaf sheath. Activity in the green leaf lamina was almost three times higher on a fresh weight basis than that in the leaf sheath. However, the specific activity of PPO extracted from the leaf sheath was twice that from the leaf lamina. A more extensive investigation of PPO activity in leaf material showed a similar situation to that in the stem with PPO activity decreasing on a fresh weight basis (Figure 3.8) with leaf age suggesting PPO synthesis takes place early in leaf development.

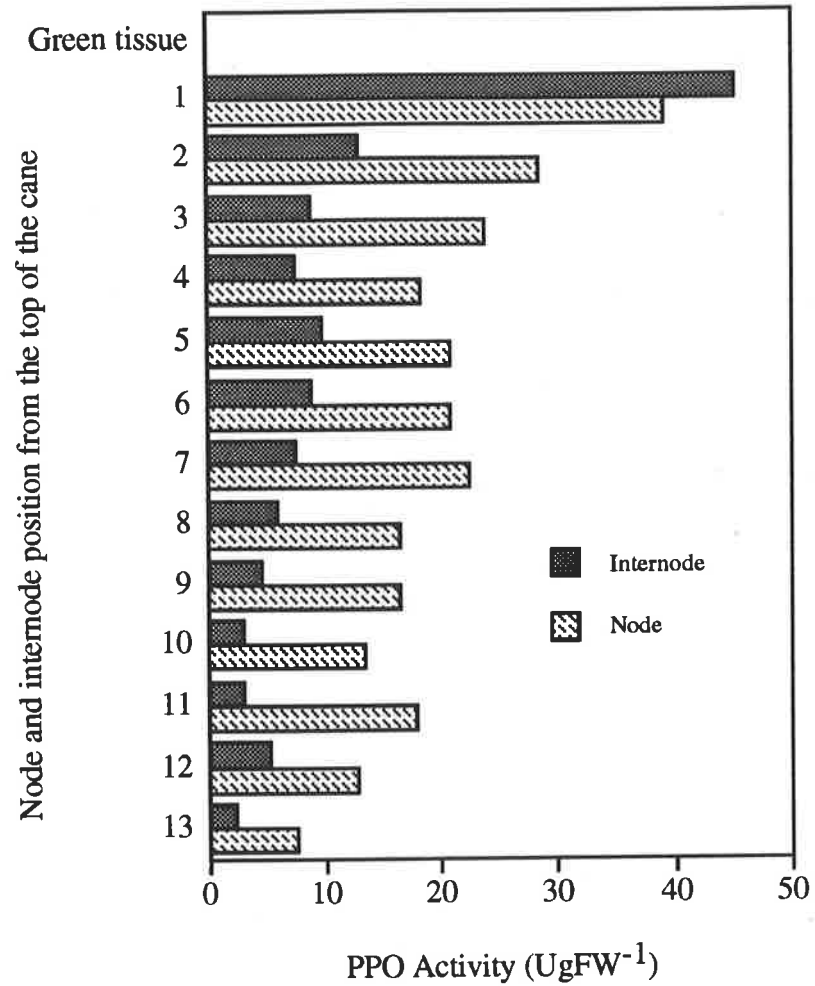


Figure 3.7. PPO activity measured in the nodes and internodes of a single stalk of cultivar Q87. Node and internode position were counted from the top of the plant with (1) representing the youngest node and internode. This profile is from a single stalk, however it was repeated three times with both Q87 and Q96.

Table 3.1. Distribution of PPO activity within young stalk tissues of cultivar Q87.

The growing point was dissected out and tissue taken 1 cm (C1) and 2 cm (C2) below the apex. The soft leaf roll was dissected out and sections 5, 10, 20, 30 and 40 cm above the growing point were measured for PPO activity and total protein. Young leaf refers to green leaves above leaf D1, and D1 refers to the first exposed dewlap (see Figure 3.1).

Tissue	PPO activity (U gFW ⁻¹)	Protein (mg gFW ⁻¹)	Specific activity (U mg ⁻¹)
Growing point	406.0	19.70	20.61
C1	126.4	12.74	9.92
C2	86.0	12.67	6.79
Leaf roll+5 cm	135.2	21.49	6.29
+10 cm	58.5	12.97	4.51
+20 cm	29.2	8.96	3.26
+30 cm	31.7	9.11	3.48
+40 cm	33.7	8.94	3.77
Young leaf	47.9	18.00	2.66
D1 Leaf blade	34.3	28.35	1.21
D1 Leaf sheath	12.0	4.96	2.42

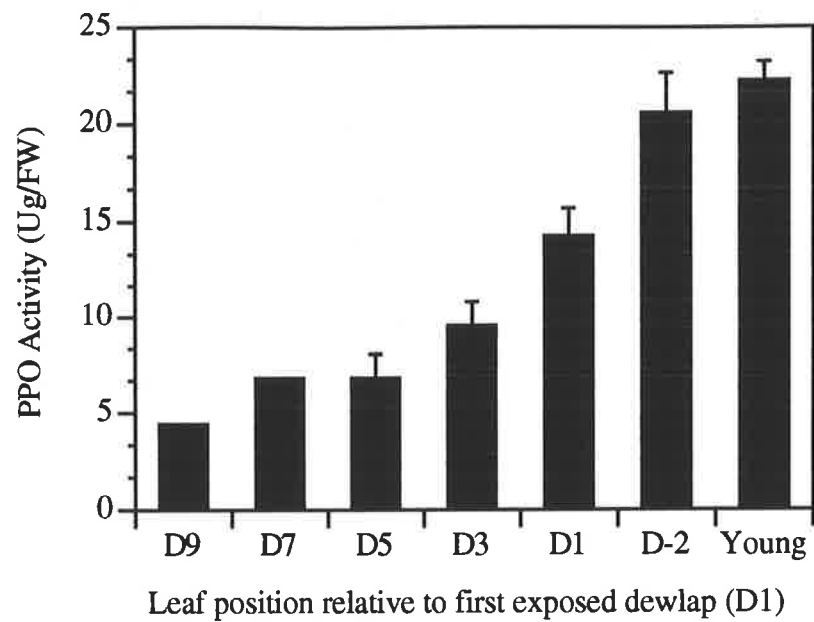


Figure 3.8. PPO activity in sugarcane leaves of different ages from Q87. Leaves were numbered relative to the first exposed dewlap, so that D9 was the oldest leaf. Each value is the mean and standard error of three replicates.

Extracts of sugarcane nodes had higher levels of PPO activity, total phenolics and colour than comparable extracts of sugarcane internodes (Table 3.2). PPO activity in the node was five times that in the internode as was colour measured at 420 nm. The total phenolics measured were twice that in the internode. A further dissection of the internode showed the bulk of the phenolics and colour to be contributed by the rind, however, higher levels of PPO activity were measured in the storage parenchyma.

Table 3.2. Comparison of PPO activity, total phenolics and colour in extracts of various stem tissues of cultivar Q87.

Internodes 8 and 10 and the nodes directly below each internode were used. Each value represents the mean and standard error of four replicates.

Tissue	PPO Activity (U gFW ⁻¹)	Phenolics (mg gFW ⁻¹)	Colour (A420 nm)
Node	25.24±0.61	723.5±42.0	0.685±0.041
Internode	4.62±0.57	342.5±28.2	0.137±0.017
Parenchyma	4.24±0.13	164.5±16.6	0.053±0.006
Rind	3.07±0.48	655.5±103.8	0.304±0.06

PPO activity in roots was very low on a fresh weight basis in both Q87 and Q96 (Table 3.3) compared to other tissues. In some plant tissues PPO is latent and can only be detected in extracts following activation by detergents in particular, sodium dodecyl sulfate (SDS) (Flurkey, 1986). To ensure that the PPO was fully activated in these tissues, extracts were also assayed for activity in the presence of the detergents SDS and Triton X-100 however there was no increase in activity (data not shown).

Table 3.3. PPO activity measured in the roots of a high and low browning cultivar.

Roots were taken from germinating setts and immediately frozen in liquid nitrogen. Each value represents the mean and standard error of two replicates.

Cultivar	PPO activity (U gFW ⁻¹)
Q87	0.97±0.21
Q96	0.57±0.01

Heat inactivation of PPO

In Chapter 2 a strong correlation was found between colour and total phenolics in nine different sugarcane clones. The correlation between PPO activity and colour however was poor. The factors influencing PPO-catalysed browning in different sugarcane clones was further investigated. Heat inactivation of PPO in extracts made from three commercial cultivars and the *Erianthus* clone IK76-140 illustrated dramatically not only the inhibition of colour formation but also the difference in the juice colour produced from these canes (Figure 3.9). In the extracts which had not been heated, the extracts of Q96 and IK76-140 were very similar in colour whilst the extracts of Q90, and particularly Q87, were much lighter in colour. PPO activity and total phenolics were highest in the *Erianthus* clone IK76-140 (Table 3.4). Colour measured in the extract of the commercial cultivar Q96 was more than four times that measured in Q87. However, the small difference in PPO activity between these two cultivars is unlikely to account for this. Where the extracts were heated and PPO inactivated, total phenolics measured in the heated Q96 extract were almost twice that measured in the heated extract of Q87. Further, the paired phenolics measurements for Q87 indicated very little of the phenolic substrate was utilised compared to Q96. PPO

activity measured in Q90 was lower than that measured in Q87 but there was greater colour formation with a corresponding decrease in total phenolics. The high colour measured in the heated extract of IK76-140 compared with the other heated extracts most likely indicates browning which occurred prior to heating. The high levels of both PPO activity and substrate in IK76-140 makes the control of browning during preparation of extracts more difficult.

Table 3.4. Effect of heat treatment on PPO activity, colour and total phenolics in extracts of an *Erianthus arundinaceus* clone and three *Saccharum* spp. hybrid clones.

Variety	PPO Activity (U gFW ⁻¹)	Phenolics (mg gFW ⁻¹)	Colour (A 420 nm)
IK76-140	11.4	0.8	0.644
Heated	0	1.27	0.363
Q87	4.7	0.53	0.179
Heated	0	0.61	0.104
Q90	2.3	0.47	0.329
Heated	0	0.70	0.106
Q96	5.6	0.72	0.761
Heated	0	1.12	0.230

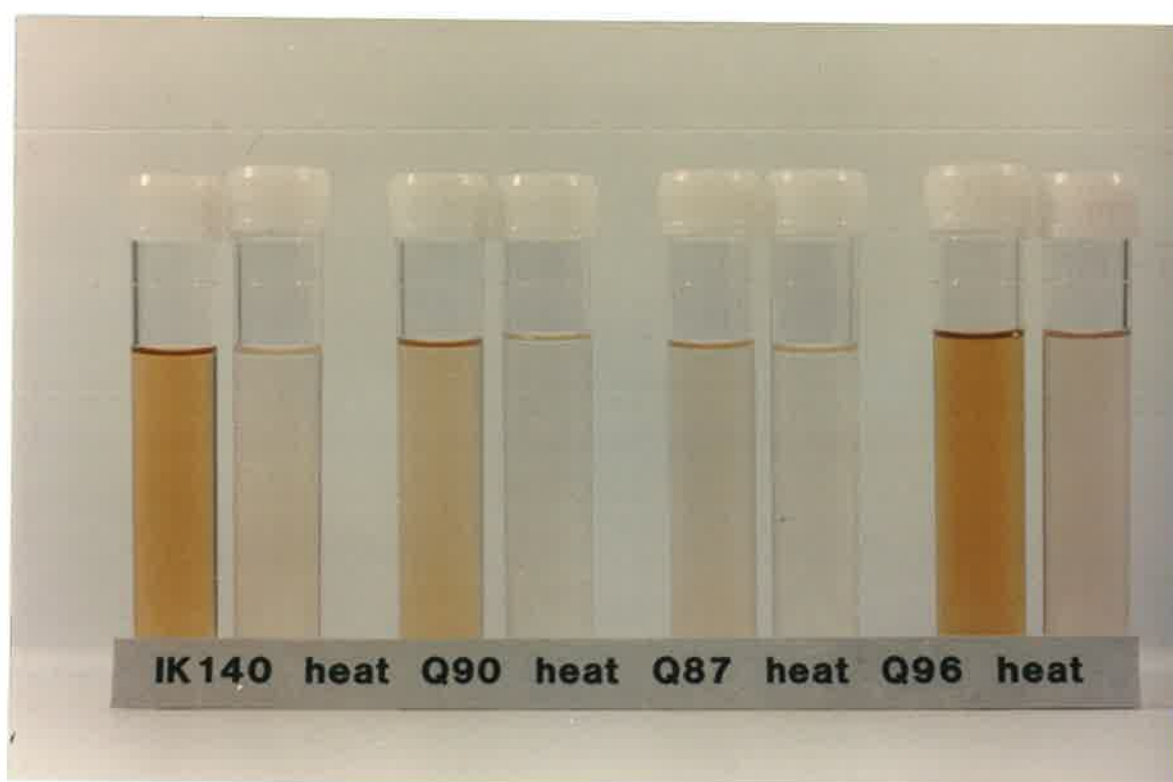


Figure 3.9. Colour differences between untreated (lefthand tube) and heated extracts shown in Table 3.4.

Erianthus arundinaceus - a high browning species

PPO activity in the *Erianthus arundinaceus* clones was more than eight times the activity measured in the Northern clones (Table 3.5). Given that PPO activity within the stalk decreases with tissue age (Figure 3.5), material was taken from the lower half of the stalk to minimise differences due to age. Phenolic levels were higher in the *Erianthus* clones, with the higher indicator (IV) ratio in the Northern clones reflecting the higher proportion of unoxidised, low molecular weight, pH sensitive colourants. Colour measured at pH 7.0 in the *Erianthus* clones was more than twice that measured in the Northern clones. Conversely °Brix measured in *Erianthus* was only half that of the hybrid clones.

Table 3.5. PPO activity, phenolics, colour, IV¹ and °Brix in a study of *Erianthus arundinaceus* clones compared with *Saccharum spp.* hybrids.

Five different *Erianthus arundinaceus* clones (two stalks of each) and nine North Queensland hybrid canes (two stalks of each) were investigated. Each value represents the mean and standard error of each group.

Cane	PPO Activity (U gFW ⁻¹)	Phenolics (mg gFW ⁻¹)	Colour at pH 7.0 (A420 nm)	IV	°Brix
<i>Erianthus</i>	16.84±2.01	1.41±0.11	0.63±0.07	2.21±0.0 7	13.8±0.5
Commercial hybrids	2.05±0.25	1.12±0.06	0.29±0.02	6.65±1.0 8	27.4±0.4

¹IV is the ratio of the absorbance at 420 nm at pH 9.0 to that at pH 4.0.

Discussion

Browning of cut sugarcane was most obvious in the region of the shoot apex (Figure 3.2). Transverse sections of sugarcane stem showed browning to be more intense in the immature internodes than mature internodes (Figure 3.3) and concentrated in the nodal tissues (Figure 3.4). From both the transverse and longitudinal sections of sugarcane stem, browning is most evident in the cells surrounding the vascular bundles. These vascular bundles are randomly scattered in the centre of the internode increasing to the periphery where they are smaller but more numerous. Within the internode the browning was most obvious in the epidermal layer and in the tissue immediately below it. According to Moore (1987) these outer layers of cells constitute the rind. The rind includes the epidermis and two or three layers of small, thick-walled, lignified cells which frequently contain anthocyanin. Below these are slightly larger, thinner walled cortical cells often containing chlorophyll, and then the sclerenchyma fibres of the fused vascular bundles. Obviously where browning has occurred in these node and internode sections, both PPO and its phenolic substrate must be present. PPO may be present in other cell types, but not detected because of the absence of an appropriate substrate.

The assay of PPO activity, phenolics and colour in extracts made from dissected internode tissue (Table 3.2) support the visual evidence. Phenolics and colour were much higher in the rind than the storage parenchyma, whilst PPO activity was slightly less than in the storage parenchyma. The staining of sections through the node with vanillin (Figure 3.5) does not support the phenolics measurements as the cells forming the rind did not stain red. The storage parenchyma tissue just inside the rind, and the lateral bud stained most strongly. The browning through the node, however, was less specific than through the internode which does support the red staining of the polyphenolics throughout the node seen in the longitudinal section (Figure 3.6). As the vanillin stain will not detect the hydroxycinnamate esters this may not be a good method for detecting all phenolic

substrates, particularly those in the rind. PPO activity in the node was more than five times that measured in the internode, as was colour, whilst phenolics measured were twice that in the internode. The browning through the node was distinct from the internode either side of it (Figure 3.4) again supporting the biochemical measurements.

PPO activity declined down the sugarcane stalk, with much higher activity in the young stem tissues. The immature stem also exhibited a greater propensity to brown (Figure 3.3). Independent of stalk age, PPO activity was higher in the nodes compared to the internode (Figure 3.7). This may reflect the potential for regrowth from the nodal region. The node consists of a growth ring (intercalary zone) which forms the boundary between the node and its internode above, the root band which contains the lateral bud, one to several rows of root primordia, and the leaf sheath scar. The growth ring can resume growth under certain circumstances, and thus is meristematic (see Moore, 1987).

PPO activity was highest in the region of shoot apex, the young stem tissues immediately below, and the leaf roll above it (Table 3.1). This supports the earlier work on sugarcane by Goodacre and Coombs (1978) which showed PPO activity to be highest in the growing point and higher in the nodes than in the internodes. They also suggested that the levels of phenolics and PPO activity were higher in the rind than internal pith tissue. Total phenolics measured in the rind of cultivar Q87 were also found to be much higher than in the storage parenchyma. Conversely, PPO activity was higher in the storage parenchyma than in the rind (Table 3.2) however, the difference in PPO activity measured in these tissues is not large. The discrepancy between this result and that reported earlier may be attributed to how carefully the rind was removed, or possibly to differences in growth conditions.

The high levels of PPO activity reported in young meristematic tissues is consistent with a number of other reports. In potatoes there was significant PPO activity in tubers, flowers, and roots but the highest activity was in young stolons and tuber buds (Thygesen *et al.*, 1994). Similarly, in tomato fruit the levels of PPO are highest in small immature fruit and

essentially negligible in mature fruit (Felton *et al.*, 1989). In Sultana grapes PPO activity on a fresh weight basis was high at fruit set and declined as the berry developed (Rathjen and Robinson, 1992b). In peaches, PPO activity was very high at an early development stage, declining to a constant level after the pit-hardened stage (Flurkey and Jen, 1978).

As in sugarcane stem, PPO activity in the leaf decreased on a fresh weight basis with increasing age. In potatoes, Thygesen *et al.* (1994) found PPO activity to be low in photosynthetic tissues but higher in young leaves than in fully expanded leaves. PPO activity decreased eight-fold from young to mature *Vicia faba* leaves (Lanker *et al.*, 1987). Delhaize *et al.* (1985) suggest that metalloenzymes including *o*-diphenol oxidase are only synthesised in early leaf development in subterranean clover. This was concluded after the failure of these enzymes in all but the leaf primordia to respond maximally to added copper following a period of copper deficiency. The activities measured per leaf remained constant or decreased with increasing maturity of the leaf after emergence, despite evidence that total protein increases about five-fold from leaf emergence to maturity (Williams and Rijven, 1970).

The oxidised phenolics and tanned proteins produced when plant tissue is wounded tends to reduce the digestibility and palatability of that tissue. Hence 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 and Harel, 1981). The high PPO activity measured in soft meristematic tissues of cane may serve a similar role in defence against predation. As cane matures and the deposition of secondary cell wall material occurs, forming a physical barrier, the stalk is less vulnerable to pathogen attack.

The heat inactivation of PPO in the four different sugarcane clones clearly illustrates the involvement of PPO in browning (Figure 3.9). The cultivar Q96 was chosen for the clonal comparison as it has been shown to produce juice of a dark colour, whereas Q87 reportedly

does not brown to as great an extent (Clarke *et al.*, 1990). The results here confirm these observations. Q96 produced juice of the highest colour, whilst Q87 produced the lowest juice colour (Figure 3.9; Table 3.4). As both showed similar PPO activities the difference in the browning of these two commercial cultivars cannot be attributed to PPO activity. In the heated extracts where oxidation had not been allowed to proceed the total phenolics measured in Q96 was twice that measured in Q87. This may explain the large difference in colour between the two extracts. Q90 was included in the comparison as was the *Erianthus* clone IK76-140. The juice colour of Q90 was intermediate and IK76-140 also produced juice of a high colour. PPO activity of Q90 was less than that of Q87 although juice colour was higher. The decrease in total phenolics measured for Q90 due to enzymic browning was almost three times that observed in Q87. This would suggest either the phenolics measured in Q87 are not readily oxidisable or that there is an endogenous inhibitor or a reducing agent such as ascorbic acid which is preventing oxidation. IK76-140 had the highest PPO activity and total phenolics. However, the final colour measured was less than that measured for Q96, which again suggests the difference in colour may be due to the type of phenolic present. The substrate specificity shown in Chapter 2 clearly indicates that sugarcane PPO has a high specificity for chlorogenic acid, although other phenolics and flavonoids can also be oxidised in the presence of chlorogenic acid. The presence of other reactive compounds in these extracts will also influence the final juice colour. Coombs and Baldry (1978) suggest that colourants are formed from the interaction between the enzyme-generated quinones and free amino acids.

Modern domestic sugarcane varieties are heterozygous and have highly complex polyploid, aneuploid genomes derived from several species of *Saccharum* and related genera. The genus *Erianthus* is believed to ^{be} one of a closely related interbreeding group involved in the origin of sugarcane (Mukherjee, 1957). Modern day sugarcanes have arisen from interspecific crosses. The progress in improvement of commercially-cultivated varieties has been achieved almost entirely with a germplasm base which is now over 60 years old. Attempts to widen the current genetic base have met with little success but would appear

necessary as a long term goal. Recently there have been attempts to introgress *Erianthus arundinaceus* with *Saccharum* spp. hybrids, with the aim of increasing hybrid vigour, however the *E. arundinaceus* clones have a tendency to produce very dark juice when crushed. Clones of *E. arundinaceus* were found to have much higher levels of PPO activity and phenolics than their commercial hybrid relatives resulting in higher colours in juice extracts (Table 3.5). If sugar content was taken into consideration, the colour on a per unit sugar basis would be doubled in *Erianthus* compared with the Northern clones.

The heat inactivation of PPO activity in the four clones studied reinforces the complexity of the enzymic browning in sugarcane as suggested in Chapter 2. Clearly PPO activity is required for browning to occur, but the extent of the reaction is determined not only by the concentration and type of substrate present but also by the presence of endogenous inhibitors and reductants. Further, the oxidation of the phenolics to the reactive quinones catalysed by PPO is only the first step in the browning process. These quinones are then able to polymerise and react with other cellular components such as amino acids or amino groups of proteins to give high molecular weight colorants. Obviously the phenolic substrate is necessary for browning to occur, but as phenolics are formed in a complex pathway, selection for low phenolic levels directly through breeding or genetic manipulation would be difficult.

There is considerable variation in the browning of sugarcane juice from different clones (compare Q87 and Q96). The planting of a particular clone is influenced mostly by factors such as yield and sugar content rather than colour. Whilst this may be the case, avoiding harvesting the leafy part of the cane plant during harvesting, where PPO activity is highest on a fresh weight basis in both young leaf and stem tissues, would seem desirable in reducing colour formation during milling. With the increase in green harvesting of sugarcane, care perhaps should be taken not to include too much of this top leafy material when harvesting.

Although PPO activity was found in both sugarcane stem and leaf tissues, it is not known at this stage if the same form of PPO is present in both tissues. PPO is localised in plastids and has been detected in a number of plastids including chloroplasts, etioplasts, root plastids, potato amyloplasts, leucoplasts and chromoplasts (Mayer and Harel, 1991). From the tissue sections (Figures 3.3 and 3.4) it appears the PPO is associated with the cells around the vascular bundles. Without specific localisation studies however, one can only speculate on where PPO may be located within these cells. In chloroplasts, PPO is said to be associated with the thylakoid membranes (see review by Mayer, 1987), in other plastid classes PPO has been detected in membrane-bound vesicles within the plastid e.g. in potato tuber amyloplasts (Czaninski and Catesson, 1974) and in leucoplasts of *Aegopodium podagraria* (Vaughn *et al.*, 1981). As sugarcane stem is largely composed of storage parenchyma and non-photosynthetic tissues, PPO may be similarly localised in a non-photosynthetic plastid, such as a leucoplast-type plastid. The localisation of PPO in sugarcane leaves may be similar to that in another C₄ plant, *Sorghum bicolor*, where PPO is present in the mesophyll plastids but not in the bundle sheath or guard cell plastids (Vaughn and Duke, 1981b).

CHAPTER 4. PROPERTIES OF THE PPO ENZYME IN SUGARCANE

Introduction

A knowledge of the properties of the PPO enzyme in sugarcane is imperative from a processing viewpoint, where manipulation of the processing conditions could reduce the activity of the enzyme and so inhibit colour formation in cane juice and, ultimately, lead to raw sugar of lower colour.

The original work on PPO in sugarcane was done by Alexander (1966b) who investigated the properties of a PPO in sugarcane which he called a cane tyrosinase. Later, Gross and Coombs (1976a) isolated two different PPO fractions from the leaves and stems of sugarcane and found the two forms to have different properties, which were again different to those reported by Alexander (1966b). The following experiments aimed to investigate the ambiguities in this earlier work and to conduct a more extensive investigation of the properties of cane PPO, both from a processing viewpoint and with respect to other polyphenol oxidases. Although the properties of the PPO enzyme have been studied in an extensive range of plants, in fruits and vegetables, the literature shows considerable variation and diversity. There are often conflicting reports of the enzyme from the same source (Mayer and Harel, 1979). Further to this, information on the properties of the enzyme (e.g. pH optimum, stability and solubility) will be necessary in planning a strategy for purification of the PPO protein to ensure measurement of PPO activity is optimised.

Materials and Methods

Plant Material

Glasshouse grown stalks of Q87 were harvested and crude extracts prepared from either leaf or stem tissues.

PPO solubility

Sugarcane leaf and stem were sectioned by hand and homogenised with a Polytron blender in a buffer of 0.1 M NaH₂PO₄, 0.4 M sucrose, 1 mM MgCl₂ pH 7.0 and 5 mM ascorbate . The extracts were centrifuged at 10,000g for ten minutes at 4°C, and the pellets resuspended in grinding media^{4m}. An aliquot of the supernatant was centrifuged at 40,000g for 40 minutes at 4°C and the pellets resuspended in grinding media. A further aliquot of the 40,000g supernatant was taken and centrifuged at 450,000g for one hour at 4°C and the pellet resuspended in grinding media. PPO activity in each of the fractions was assayed in the oxygen electrode as described in Chapter 2.

Chlorophyll determination

Chlorophyll was determined by mixing 1 mL of acetone with 100 µL of the leaf fractions and 200 µL of the stem fractions. The fractions were then centrifuged at 12,000g for five minutes, the supernatant removed and absorbance measured at 663, 645 and 652 nm. Total chlorophyll was calculated according to the method of Bruinsma (1963):

$$\text{Chlorophyll } a (C_a) = 12.7 A_{663} - 2.7 A_{645}$$

$$\text{Chlorophyll } b (C_b) = 22.9 A_{645} - 4.7 A_{663}$$

$$\text{Total chlorophyll } (C_a + b) = 20.2 A_{645} + 8.0 A_{663} = 27.8 A_{652}$$

Assay linearity

Sugarcane stem was sliced and homogenised with a Polytron blender in four volumes of ice-cold 50 mM NaH₂PO₄, 1mM MgCl₂ pH 5.0 and the homogenate filtered through Miracloth. The filtrate was assayed for PPO activity. Varying amounts of homogenate were added to the oxygen electrode with 2 mM chlorogenic acid to determine whether the assay was linear with respect to the amount of enzyme added.

pH Optima of sugarcane PPO

The optimal pH for assaying PPO activity was determined using the partially purified PPO (prepared as described in Chapter 2). PPO activity was assayed using the oxygen electrode. Three buffers were used at a concentration of 50 mM within their buffering range; MES, MOPS and Tricine. Buffer of a given pH was added to the electrode chamber and a constant amount of PPO was added. Chlorogenic acid (2 mM) was added finally to initiate the reaction.

Effect of substrate concentration

Increasing amounts of substrate (chlorogenic acid or 4-methyl catechol) were added to the oxygen electrode containing 50 mM NaH₂PO₄ at pH 5.0 and a constant amount of partially purified PPO. The resulting PPO activity was measured to determine the saturation point of the substrate. Michaelis constants and maximum velocities were calculated from Lineweaver-Burk plots. At pH 5.0, the effect of the addition of 5 mM NaCl on the reactions was also determined.

Heat inactivation

Heat treatments of PPO were carried out at 55, 60, 65 and 70°C for varying periods of time in a waterbath. Partially purified PPO in 50 mM NaH₂PO₄, pH 6.0 buffer was added to a glass tube equilibrated at the designated temperature. 50 µL was withdrawn at each time point and immediately placed on ice and remaining PPO activity determined.

Results

The following studies on the properties of the sugarcane PPO enzyme use both crude extracts of leaf and stem tissue and partially-purified PPO from stem tissue of cultivar Q87 (see Chapter 2). The type of extract used is clearly explained for each experiment.

Solubility of the PPO enzyme

Despite possible artefacts, the weight of evidence indicates the wide occurrence of membrane-bound polyphenol oxidases, particularly in chloroplasts (Mayer and Harel, 1979). In fruit, solubilisation tends to occur with fruit ripening as seen in grapes (Kidron *et al.*, 1978) and apples (Harel *et al.*, 1966). Sometimes more drastic conditions are required for solubilisation of membrane-bound PPO, such as the use of detergents or limited digestion with proteases (Vaughn and Duke, 1984a). In view of this, the solubility of the PPO enzyme in sugarcane was investigated with the aim of ensuring maximum extraction of PPO activity.

Leaf extracts were subjected to differential centrifugation to determine the subcellular localisation of PPO (Table 4.1). Most of the chlorophyll was recovered in the 10,000g or 40,000g pellets, suggesting it was associated with chloroplasts or thylakoid membranes. In contrast, 76% of the PPO activity recovered was found in the 450,000g supernatant suggesting that the enzyme is soluble or readily released from membrane fractions. The

same pattern of distribution of PPO activity was observed in stem extracts, with 81% of the PPO activity recovered in the supernatant of the 450,000g spin (Table 4.1). In the stem extracts the recovery of chlorophyll was greater than that measured in the original homogenate. This most likely reflects the inaccuracies in measuring low levels of chlorophyll in stem tissue. When protein was analysed in the various fractions much of the protein was found in the pellet fractions and as most of the PPO was present in the supernatant, there was an almost three-fold increase in specific activity following centrifugation (data not shown).

Table 4.1. PPO activity and total chlorophyll in fractions obtained by differential centrifugation of extracts of sugarcane leaf and stem.

Recovery is the total recovered PPO activity and chlorophyll from the various fractions. ND= Not determined

Fractions	PPO activity (Units)	Chlorophyll (mg)
Leaf homogenate	42.75	1.82
10,000g pellet	1.50	0.86
40,000g pellet	1.87	0.64
450,000g pellet	6.13	0.053
450,000g supernatant	30.63	ND
Recovery	40.13	1.53
Stem homogenate	49.50	0.234
10,000g pellet	3.25	0.192
40,000g pellet	1.73	0.156
450,000g pellet	3.45	0.009
450,000g supernatant	36.65	ND
Recovery	45.08	0.357

In many plants PPO exists in a latent state and can only be detected following activation by anionic detergents, fatty acids, treatment with proteases or acid treatment (Vaughn and Duke, 1984a; Steffens *et al.*, 1994). Detergents (SDS and CTAB) were added to the assay to try and activate any latent PPO activity. Unfiltered homogenate was added to the oxygen electrode together with the respective detergent and left stirring for two to three minutes before addition of the substrate. Neither of the detergents activated the enzyme, instead they both had an inhibitory effect (Figures 4.1 and 4.2). PPO activity was decreased by 70% with the addition of 0.1% SDS to both stem and leaf extracts of cultivar Q87, although the remaining PPO activity was not greatly inhibited by higher concentrations of SDS (Figure 4.1). CTAB decreased PPO activity but at much higher concentrations than that needed for SDS inhibition. The inhibition appeared to be linear, with approximately 50% loss of PPO activity with the addition of 0.4% CTAB (Figure 4.2). The inhibition of PPO activity with both SDS and CTAB did not appear to be time-dependent.

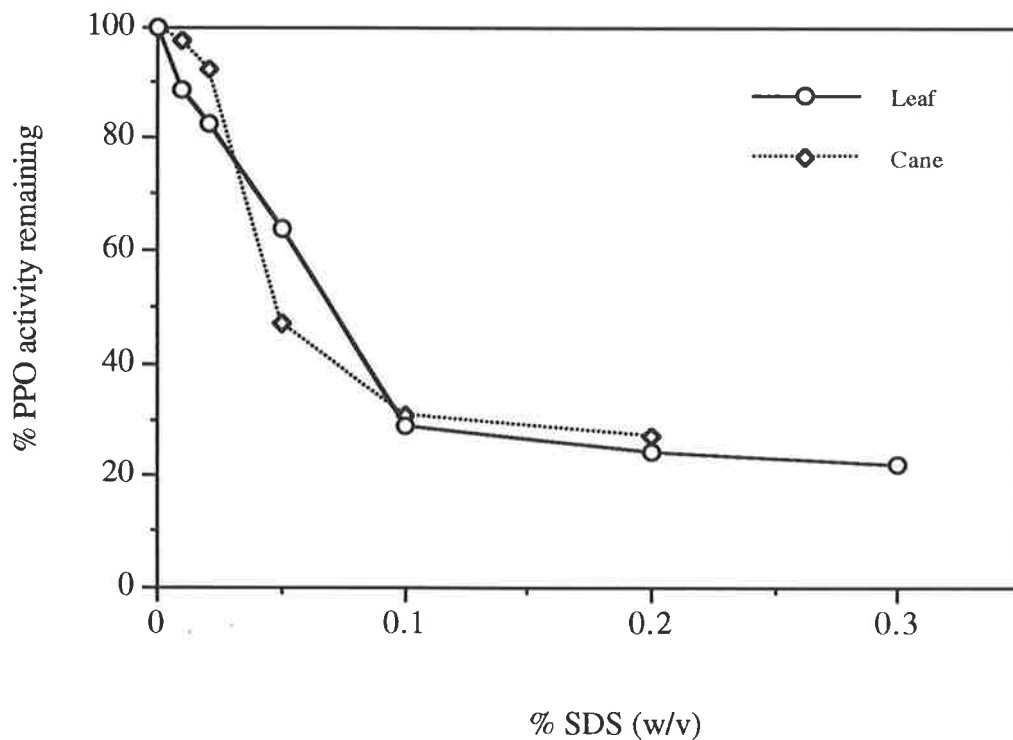


Figure 4.1 Effect of SDS on sugarcane PPO activity. Activity was measured in crude extracts of Q87.

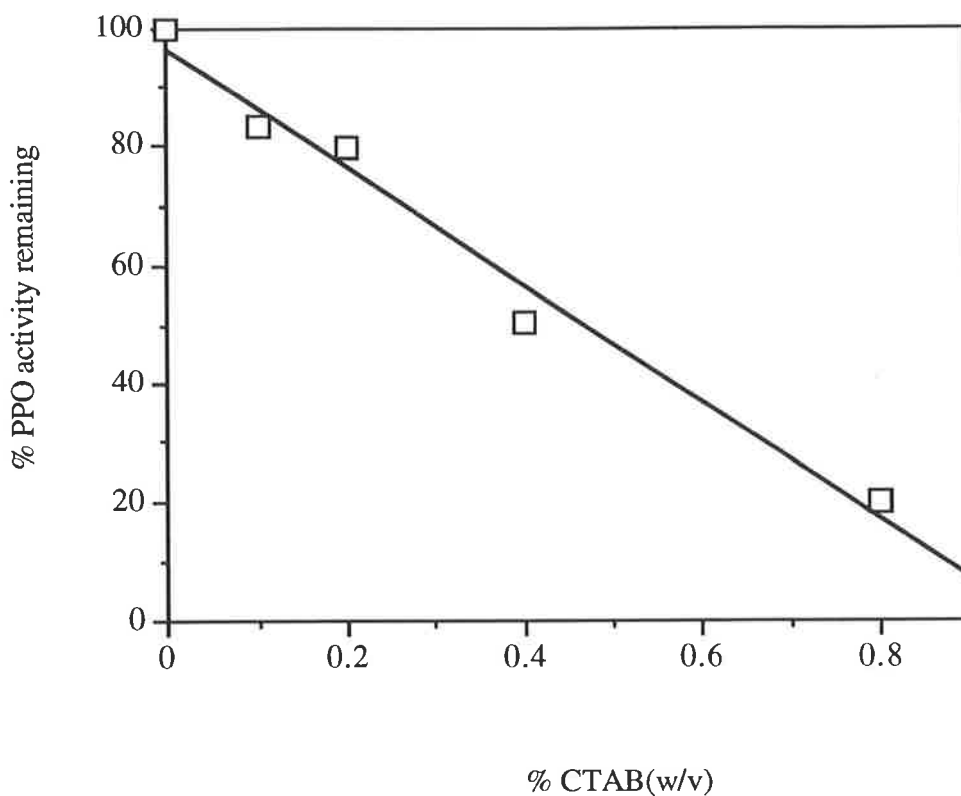


Figure 4.2. Effect of CTAB on sugarcane PPO activity. Activity was measured in crude extracts of sugarcane cultivar Q87.

Effect of enzyme concentration on reaction rate

PPO dependent oxygen uptake increased linearly with the addition of increasing amounts of homogenate (Figure 4.3). There was no inhibitory effect with the addition of 160 μL of homogenate indicating that the amount of added substrate exceeded that used by the enzyme, and that no significant endogenous inhibitors were present in the extract.

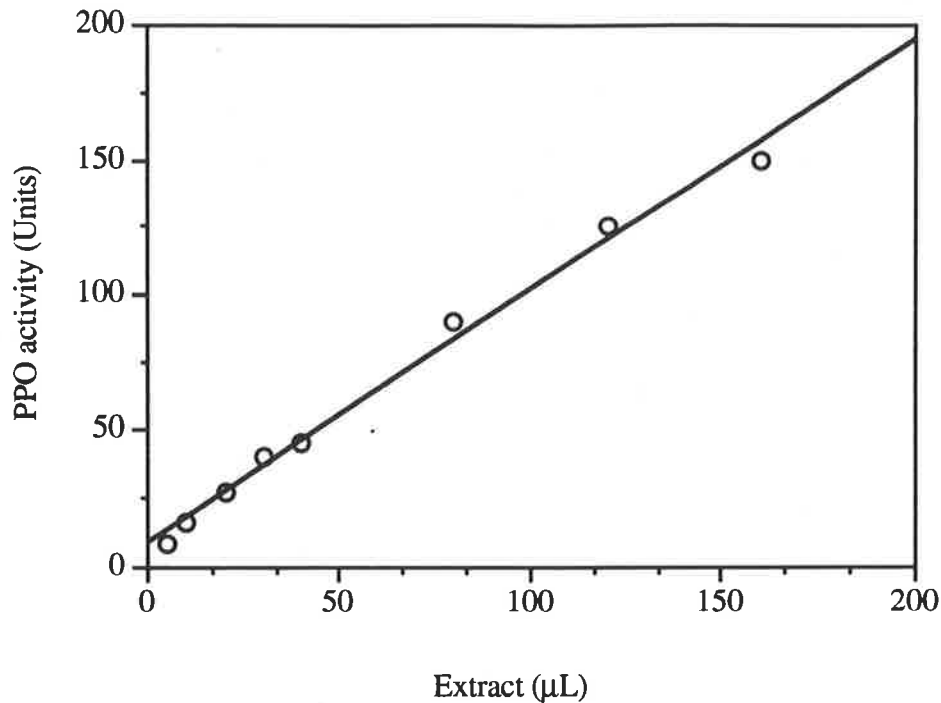


Figure 4.3. Linear increase in the rate of PPO dependent O₂ consumption with the addition of increasing amounts of homogenate.

pH optimum

The pH activity profile of partially purified sugarcane PPO was determined between pH 3.5 and 10 (Figure 4.4). Using MES, MOPS and Tricine buffers there was a relatively broad pH optimum, with maximum activity at pH 5.0. PPO activity declined more rapidly at higher pH values than at pH values below the optimum (Figure 4.4). Similar pH profiles were obtained with other buffer systems such as phosphate. In all instances, there was a broad pH optimum between pH 4.5 and 6.0 with an increased decline in PPO activity at alkaline pH values.

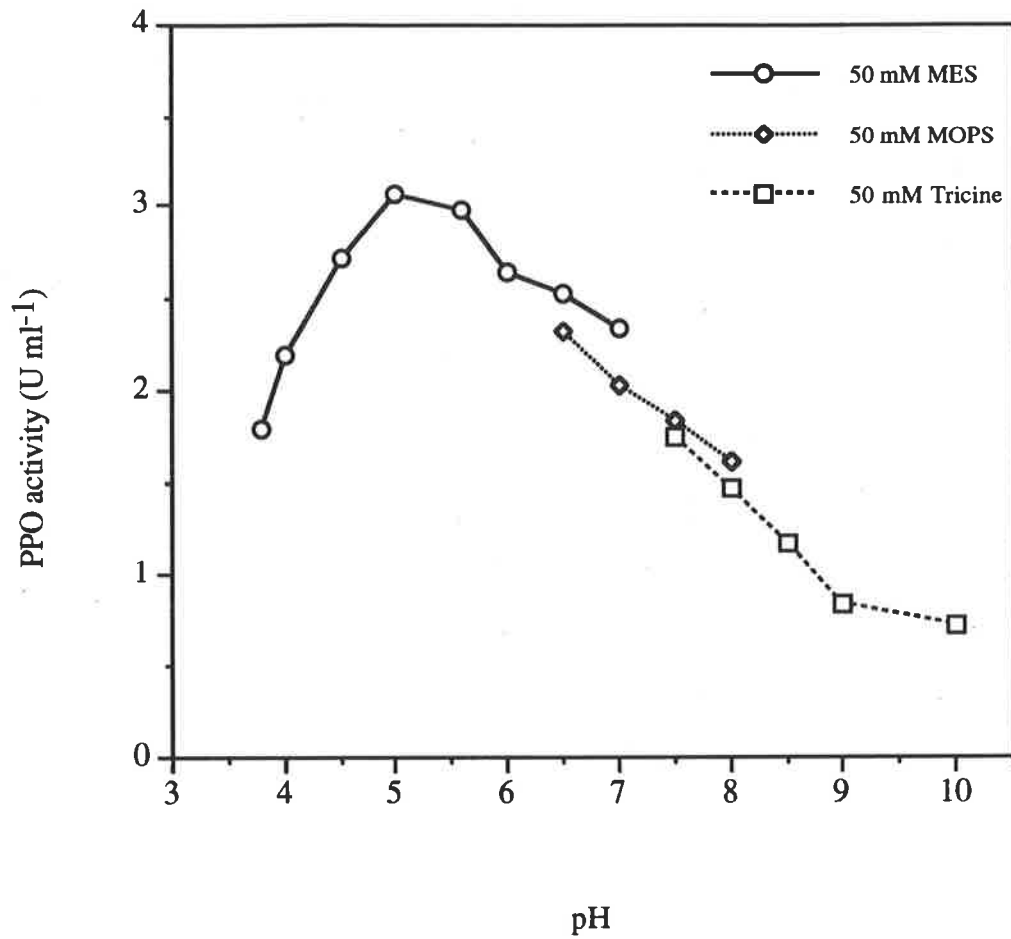


Figure 4.4. The effect of pH on the activity of partially-purified PPO. Activity was assayed using 2 mM chlorogenic acid.

Effect of substrate concentration on PPO reaction rate

As shown in Chapter 2, sugarcane PPO shows a distinct preference for the substrate chlorogenic acid over any of the other substrates tested. The effect of chlorogenic acid concentration on the rate of PPO-dependent O₂ uptake was further investigated. PPO activity increased with increasing substrate concentration up to 2 mM, after which there was a slight inhibitory effect with additional substrate (Figure 4.5).

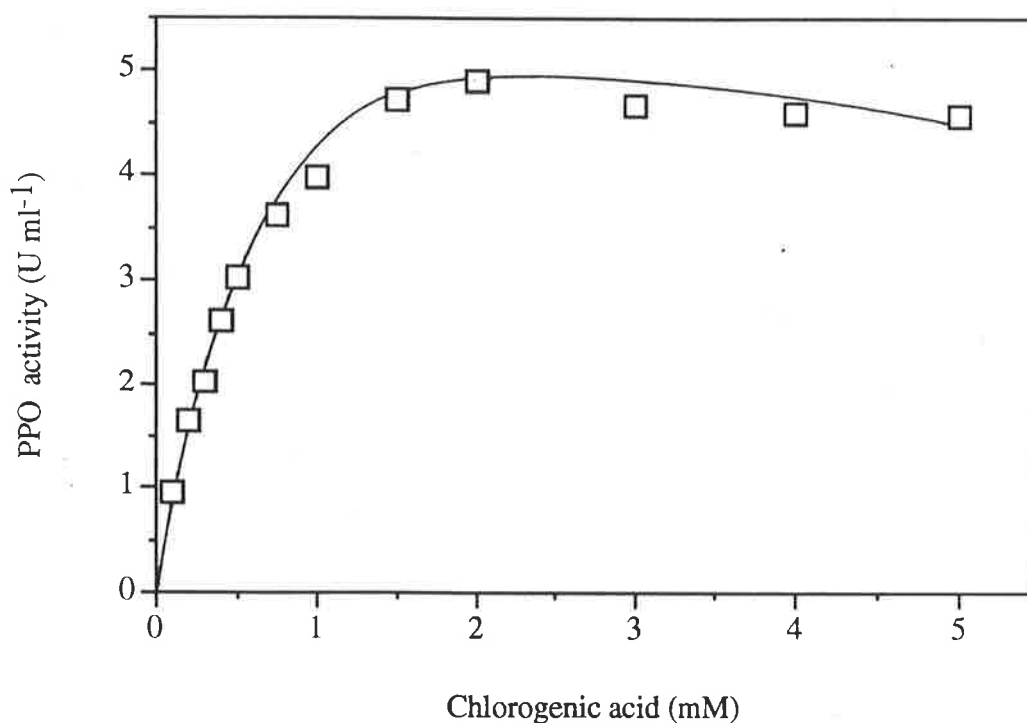


Figure 4.5. Effect of chlorogenic concentration on PPO activity. Activity was measured with partially purified PPO.

Lineweaver-Burk plots of the oxidation of chlorogenic acid and 4-methyl catechol are shown in Figures 4.6 and 4.7. For chlorogenic acid, the apparent K_M at pH 5.0 was 0.42 mM and the V_{max} was 6.54. Sugarcane PPO showed much lower affinity for 4-methyl catechol with a K_M of 15 mM and a V_{max} of 4.88 U mL⁻¹.

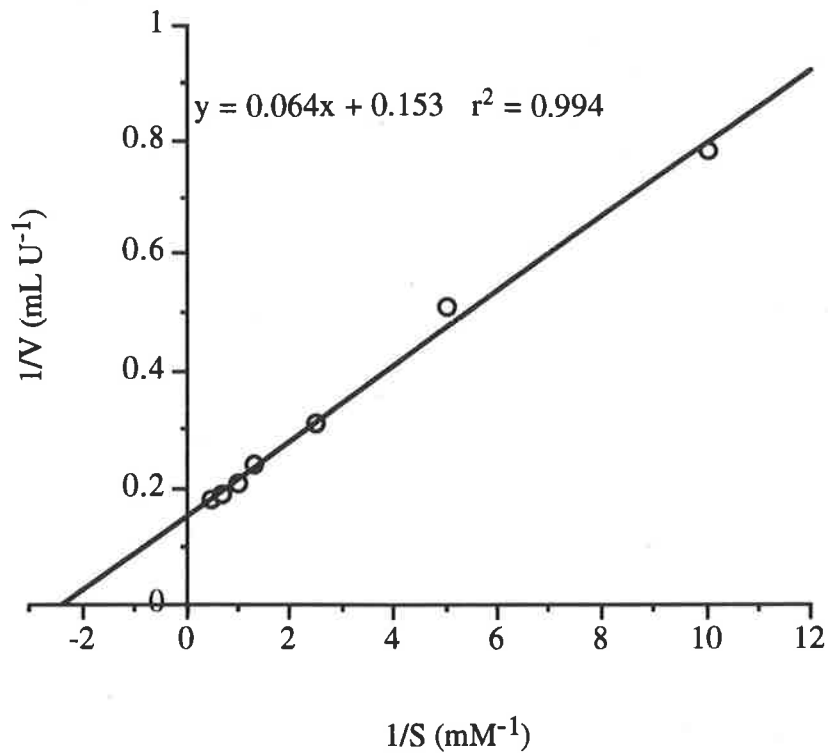


Figure 4.6. Lineweaver-Burk plot of sugarcane PPO activity versus chlorogenic acid concentration at pH 5.0.

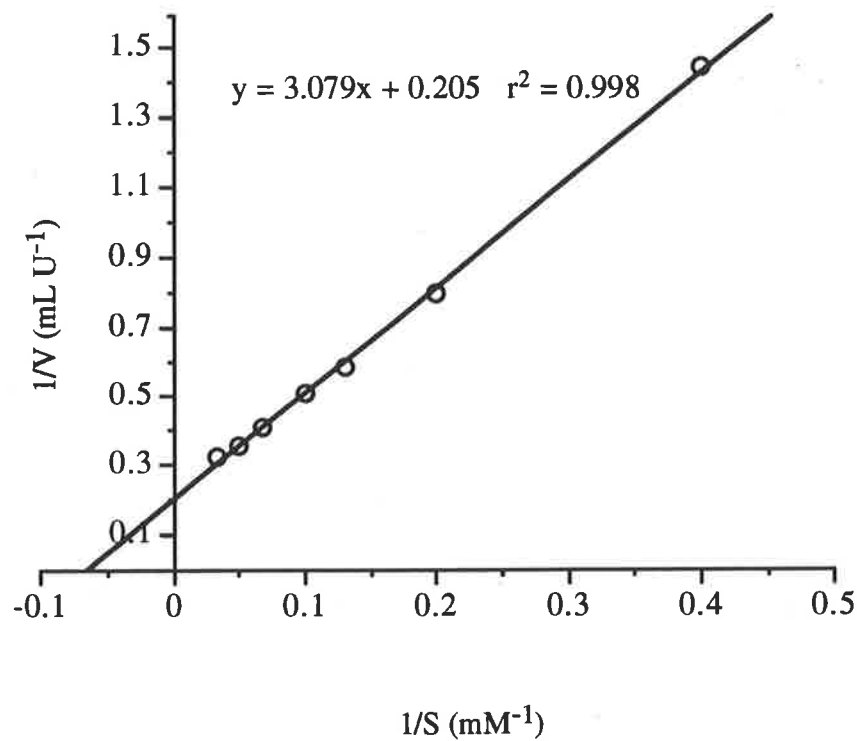


Figure 4.7. Lineweaver-Burk plot of sugarcane PPO activity versus 4-methyl catechol concentration.

Enzyme stability

Many enzymes lose activity when plant extracts are left standing. Loss of PPO activity in crude sugarcane extracts incubated on ice was found to be dependent on pH of the homogenate (Figure 4.8). At pH 7.5, loss of activity was most rapid, with 50% lost after two hours. Extracts at pH 5.0 were more stable with more than 70% of the initial PPO activity remaining after four hours on ice (Figure 4.8)

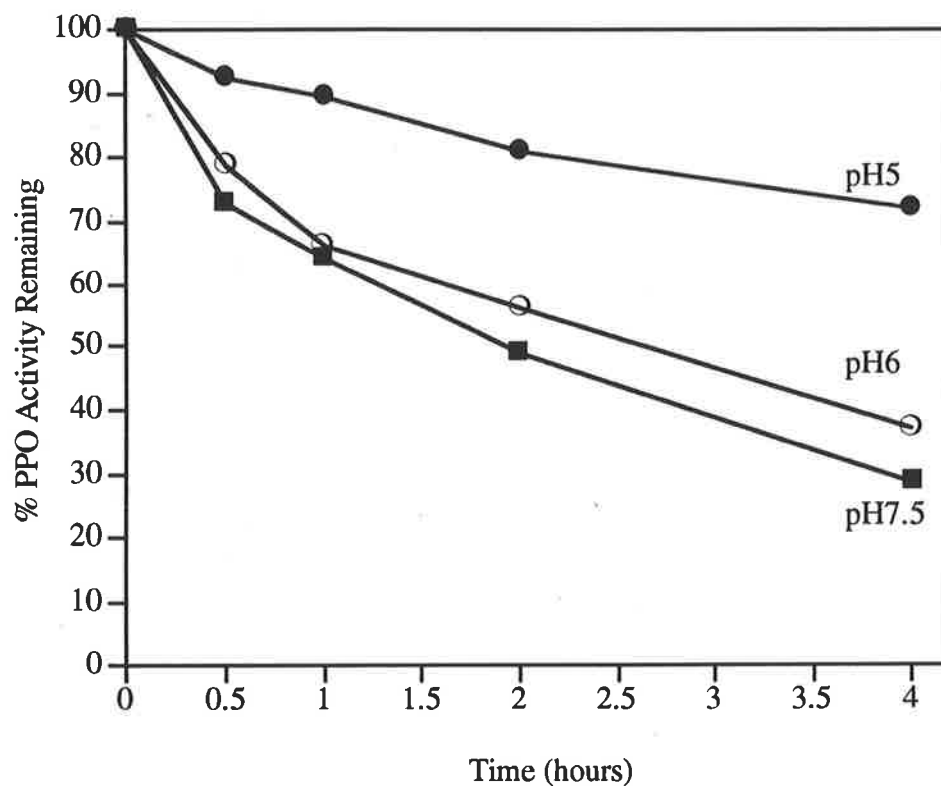


Figure 4.8. Effect of pH on the stability of PPO activity in sugarcane extracts. PPO activity was monitored in crude extracts of cultivar Q96 extracted in phosphate buffer at pH 5, 6 and 7 and left on ice over a period of four hours. An aliquot was withdrawn periodically and assayed for activity. The initial PPO activities were 4.4, 4.9 and 5.6 Units gFW^{-1} at pH 7.5, 6 and 5 respectively.

Heat inactivation

The thermal stability of the enzyme was measured by incubating extracts at various temperatures and measuring the PPO activity remaining over time. Partially purified sugarcane PPO was not a particularly thermostable enzyme, with almost all activity lost within five minutes at 70°C. At lower temperatures the rate of inactivation was much slower, with a sharp increase in the rate of inactivation from 55°C to 65°C (Figure 4.9). After ten minutes at 55°C just over 50% activity remained, the rate of inactivation then slowed as there was still 30% activity remaining after 25 minutes.

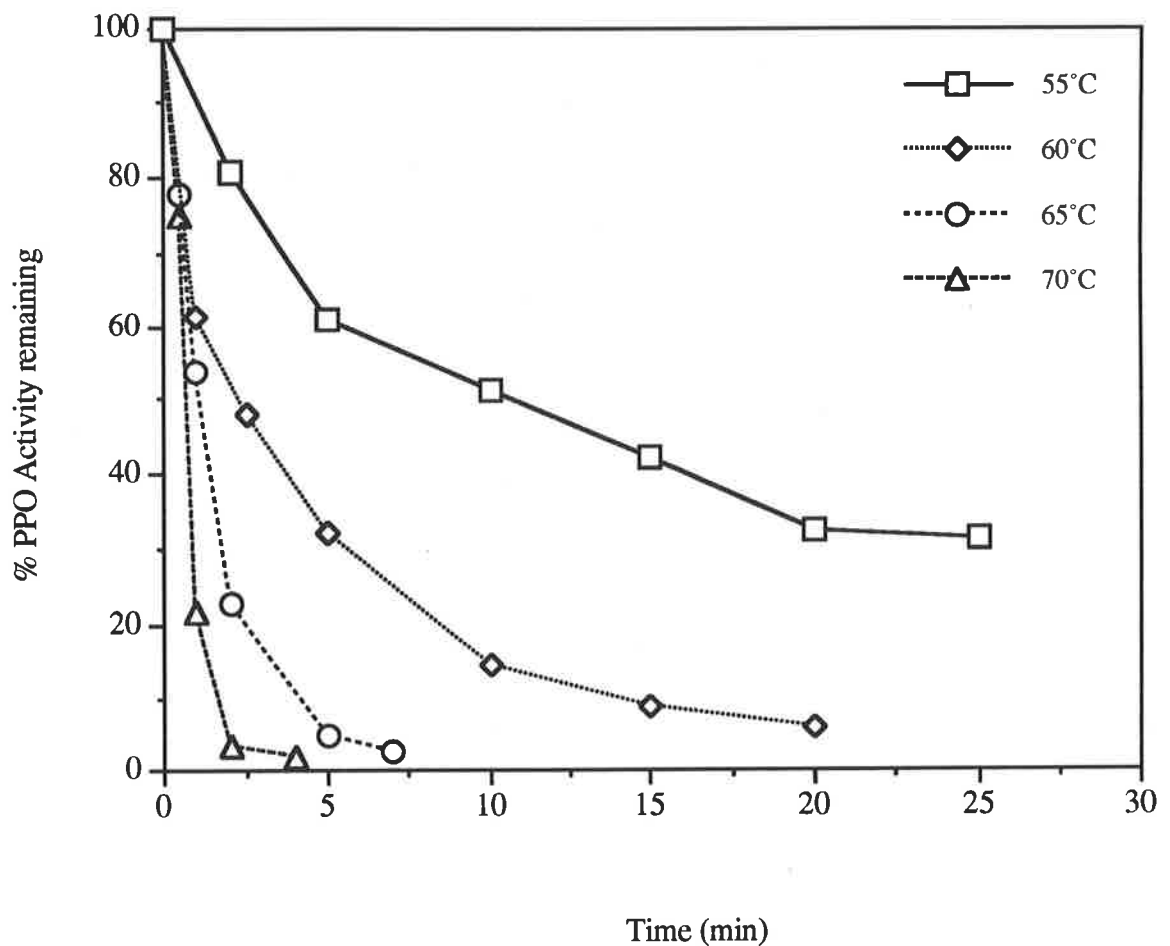


Figure 4.9 Thermal inactivation of partially purified sugarcane PPO at various temperatures.

At higher temperatures inactivation was much more rapid, however a log plot of % activity remaining against time showed at all temperatures the heat denaturation of the PPO enzyme did not follow first order kinetics (Figure 4.10). As the log plot was not linear, it might suggest two populations of enzyme, one being more temperature resistant, or a complex pattern of heat inactivation of the protein.

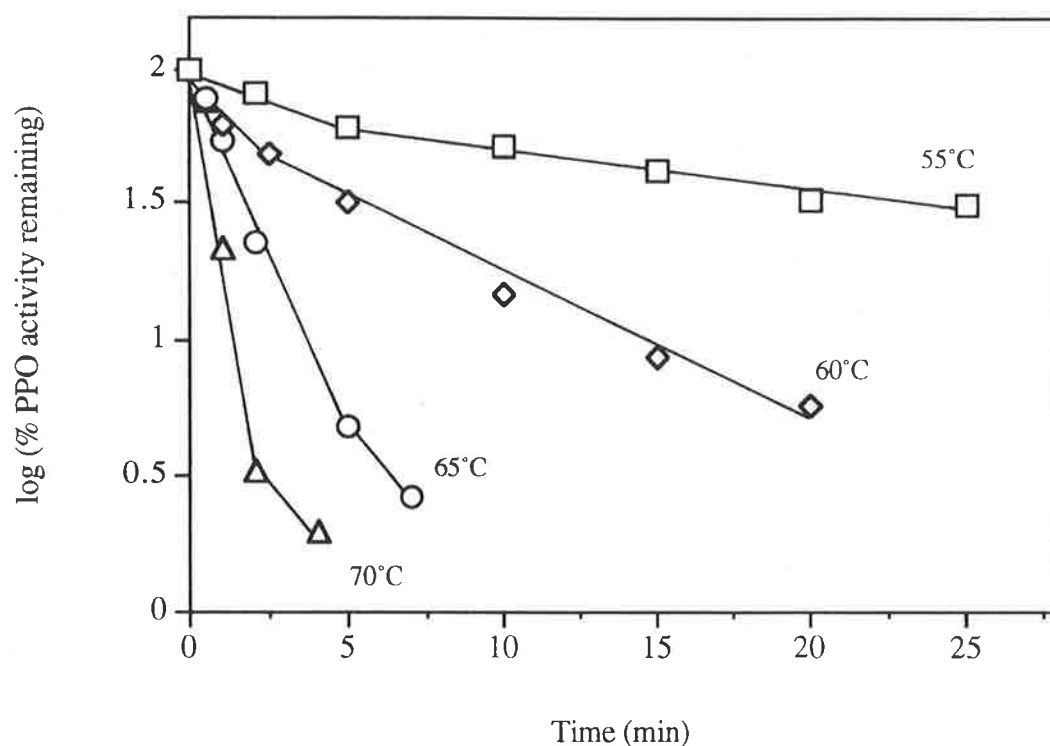


Figure 4.10. Log plot of thermal inactivation of PPO.

Halide inhibition

Inhibition by halides has been demonstrated for PPO from many sources (Mayer and Harel, 1979). PPO activity was assayed in the presence of a number of halides at both pH 5.0 and pH 7.0 (Table 4.2), as inhibition has been shown to be strongly pH dependent (Ben-Shalom *et al.*, 1977; Peñafiel *et al.*, 1984). All of the halides tested inhibited PPO activity strongly at pH 5.0, with little effect at pH 7.0. Sodium fluoride was the most

potent inhibitor followed by KCl, with NaBr the least effective. When NaH₂PO₄ was added to a concentration of 0.5 M at pH 5.0 (data not shown) there was no decrease in activity, suggesting it was the halide anion which is the inhibitory factor.

Table 4.2. Effect of different halides on sugarcane PPO activity at different pH. Values are expressed relative to activity measured without halide. Activity assayed with 2 mM chlorogenic acid and partially purified PPO enzyme.

Halide	PPO activity assayed at pH 5.0 (expressed as % of control)	PPO activity assayed at pH 7.0 (expressed as % of control)
KCl (10 mM)	10.7	98.3
NaBr (10 mM)	22.8	87.9
KI (10 mM)	16.9	75.6
LiCl (10 mM)	12.1	87.9
NaCl (10 mM)	10.0	88.3
NaF (6.7 mM)	0.0	71.9

The effect of pH on inhibition by halides was more closely studied using NaCl. PPO activity was assayed at pH 4.0, 5.0 and 7.0 with increasing NaCl concentration. There was virtually no effect of increasing NaCl concentration on activity at pH 7.0, while at pH 5.0 and 4.0 PPO activity measured was decreased by more than 80% with the addition of only 5 mM NaCl (Figure 4.11). Two series of measurements were then carried out with a range of chlorogenic concentrations with and without 5 mM NaCl at pH 5.0. A Lineweaver-Burk plot showed the NaCl at 5 mM to be a competitive inhibitor (Figure 4.12) changing the apparent K_M from 0.42 mM to 1.72 mM.

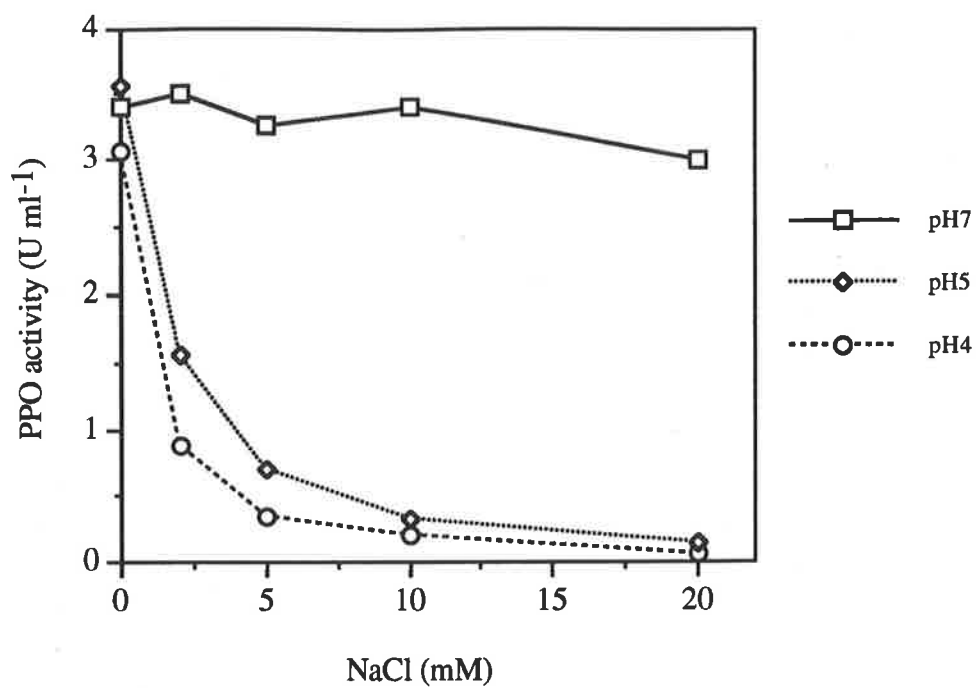


Figure 4.11. Effect of increasing NaCl on PPO activity Activity was measured with partially purified PPO.

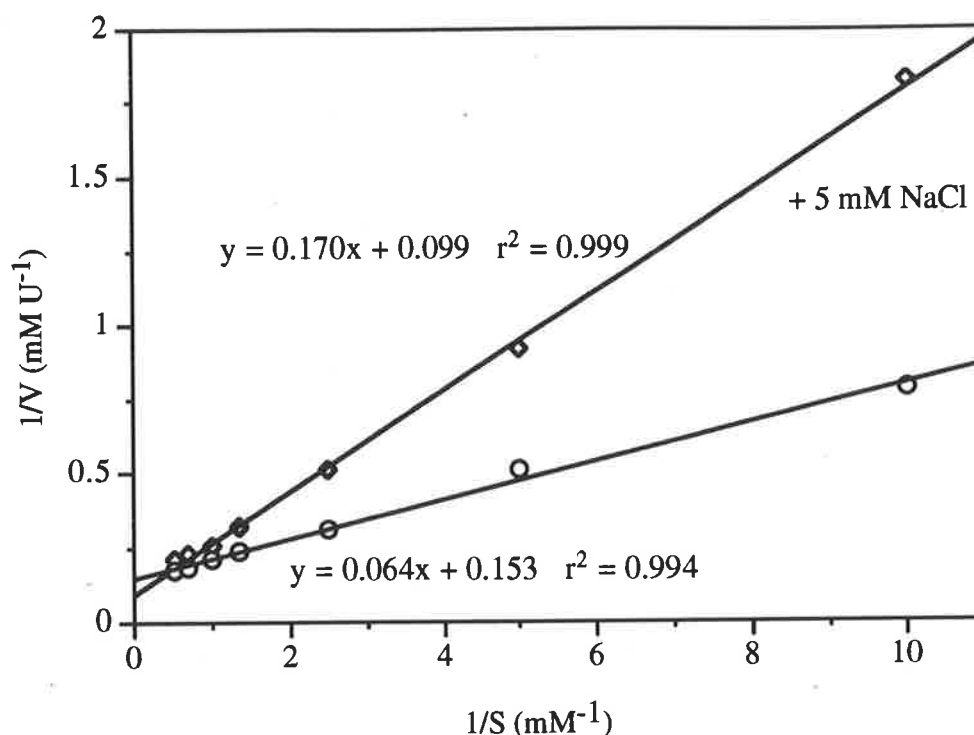


Figure 4.12. Lineweaver-Burk plots of sugarcane PPO activity versus chlorogenic acid concentration in the presence and absence of 5 mM NaCl. The assay was carried out using partially purified PPO at pH 5.0.

Other inhibitors

The effects of other known inhibitors of PPO on sugarcane PPO activity were investigated. SHAM which inhibits catechol oxidases but not laccases (Allan and Walker, 1988) was the most effective inhibitor of sugarcane PPO, with 50% inhibition observed with only 20 μ M. Laccases are inhibited by the cationic detergent CTAB, but this compound has no effect on catechol oxidases (Walker and McCallion, 1980). Sugarcane PPO, however, was inhibited by 80% with 0.8% CTAB (Figure 4.2). Cinnamic acid, which has been reported to inhibit catechol oxidases, but not laccases (Walker and McCallion, 1980), inhibited PPO activity by 50% at 2.5 mM (data not shown). DIECA, a known inhibitor of PPO (Mayer and Harel, 1979), inhibited sugarcane PPO by 50% at a concentration of 0.2 mM (Figure 4.13).

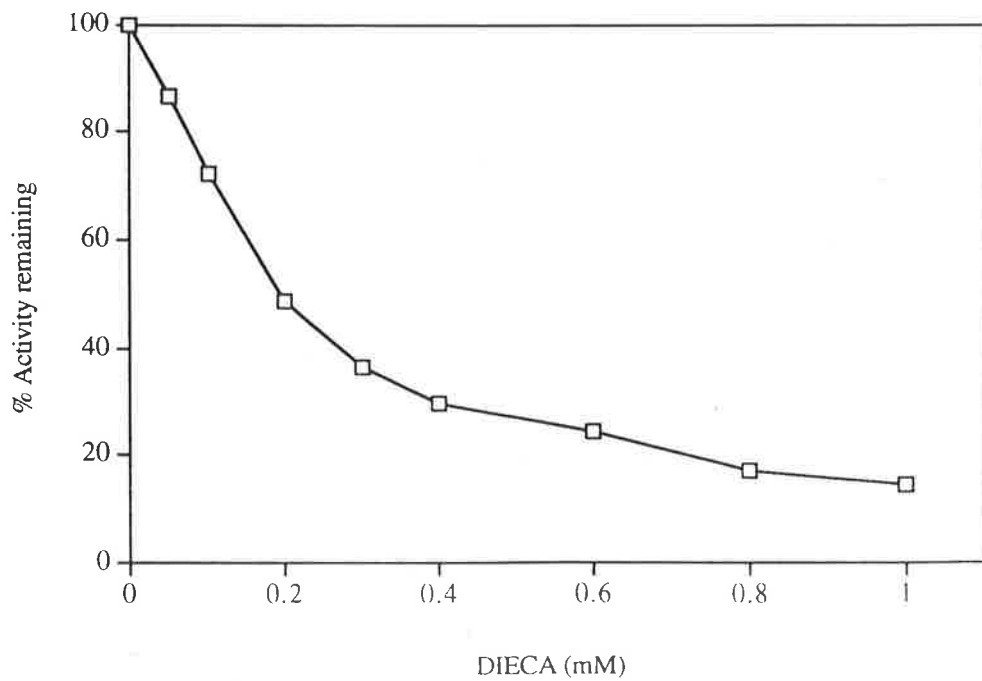
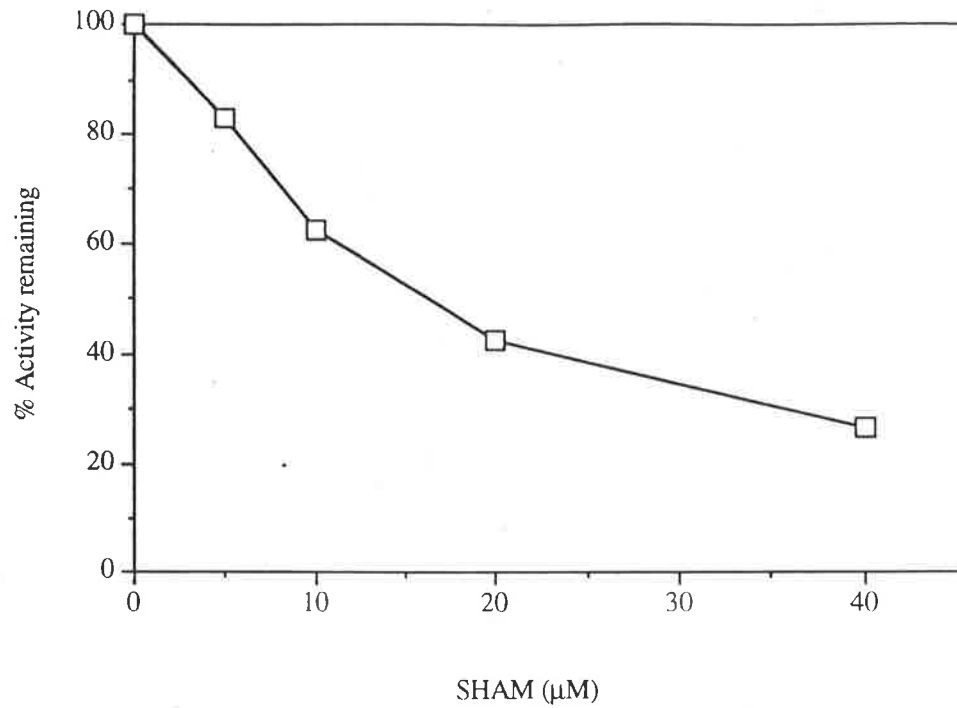


Figure 4.13. Effect of SHAM and DIECA on PPO activity. Activity was measured with partially purified PPO.

Discussion

It is generally accepted that the PPO enzyme is localised in plastids bound to the thylakoid membranes (Mayer, 1987) but it can be released from the thylakoids by sonication, detergent treatment or protease treatment (Golbeck and Cammarata, 1981; Flurkey, 1986; King and Flurkey, 1987). Sugarcane PPO appears to be unusual in that it was readily soluble in both leaf and stem extracts even when extracts were prepared in buffers containing sucrose and $MgCl_2$ to preserve the integrity of chloroplast membranes (Table 4.1). Sugarcane PPO was not closely associated with chlorophyll following centrifugation, remaining in the supernatant in both leaf and stem extracts, suggesting that it is not attached to plastid membranes. PPO from glandular trichomes of *Solanum berthaultii* is also reported to be very soluble, with 80% of the PPO activity remaining in the supernatant after high speed centrifugation (Kowalski *et al.*, 1992). More recently, based on the deduced amino acid sequences of PPO genes isolated from tomato, Newman *et al.* (1993) have suggested that tomato possesses both soluble and membrane-bound PPOs. Following the work of Sommer *et al.* (1994) on the import, targeting and processing of PPO, they suggest that some reports of PPO tightly bound to the thylakoid membrane could be due to cross-linking reactions occurring during isolation of the proteins.

In most, but not all plant extracts, PPO activity is latent and maximum activity can only be observed in extracts following activation with detergents, acid treatment or protease treatment (Vaughn and Duke, 1984a; Steffens *et al.*, 1994). In many plant extracts the anionic detergent SDS is commonly used to activate PPO enzymes (Flurkey, 1986; Moore and Flurkey, 1990). However, SDS failed to increase the activity of PPO in sugarcane extracts and actually inhibited activity at quite low concentrations (Figure 4.1). CTAB had a similar inhibitory effect at higher concentrations. This suggests that there may be an endogenous agent which activates PPO when the sugarcane tissues are disrupted, or that sugarcane PPO is simply not latent.

Generally, the affinity of plant catechol oxidases for their phenolic substrates is relatively low, with a K_M of around 1 mM (Mayer and Harel, 1979). The affinity for the preferred substrate, chlorogenic acid, was higher than this, with a K_M of 0.42 mM whilst the affinity for the next preferred substrate, 4-methyl catechol was significantly lower, the K_M being 15 mM. Alexander (1966b) previously found the K_M of sugarcane PPO for catechol to be around 4.0 mM. Gross and Coombs (1976a) reported the K_M for two isolated fractions of sugarcane PPO for chlorogenic acid to be 2.4 and 1.1 mM respectively, however they do not state the pH at which the assay was made. The higher K_M they report, may indicate the assay was not performed at the optimal pH.

The pH optimum of most of the polyphenol oxidases studied to date is between 5.0 and 7.0 (Mayer and 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 (Vámos-Vigyázó, 1981). A common feature is the presence of two peaks within the pH curve; a peak and a prominent shoulder, (Harel *et al.*, 1965; Robb *et al.*, 1966; Harel and Mayer, 1971) or a wide optimum (Ben-Shalom *et al.*, 1977). Gross and Coombs (1976a) found the pH optimum of both sugarcane PPO fractions to be between 4.5 and 7.5, while Alexander (1966b) showed a pH optimum of around 7.5. The pH optimum of sugarcane PPO found in this study was between pH 4.5 and 6.0. The same pH profile was observed with different buffer systems (data not shown). As the pH optimum was quite broad between 4.5 and 6.0, PPO was subsequently assayed throughout at pH 6.0. The enzyme was also more stable at acid pH, with stability decreasing at higher pH (Figure 4.8).

PPO is not thought to be a particularly heat-stable enzyme. Short exposures, in tissue or in solution, to temperatures of 70-90°C, are in most cases sufficient for partial or irreversible destruction of its catalytic function (Vámos-Vigyázó, 1981). There are however exceptions, for example, the PPO enzyme from mango required more than 15 minutes at

80°C for 50% loss of activity (Robinson *et al.*, 1993). In grape the PPO enzyme is also quite heat stable requiring 15 minutes at 75°C for complete inactivation (Valero *et al.*, 1988). Sugarcane PPO however, does not appear to be a particularly heat-stable enzyme which is important from a processing point of view. A heat treatment at 70°C for five minutes was found to completely inactivate the enzyme (Figure 4.9) and at higher temperatures the process would presumably be even more rapid. Gross and Coombs (1976a) found the sugarcane fractions they isolated to be even more sensitive to temperature, being fairly rapidly denatured at 55°C, with a $t_{1/2}$ of 1.6 and three minutes respectively. Factors such as pH, ionic strength, protein concentration can affect the inactivation rate (Dixon and Webb, 1958) and so give rise to the differences reported on the inactivation of PPO from the same species (Vámos-Vigyázó, 1981). Lourenço *et al.* (1992) found sweet potato PPO was more thermostable at 80°C in the presence of added sucrose.

The kinetics of sugarcane PPO heat inactivation are not straightforward as it does not appear to follow classic first-order kinetics (Figure 4.10), suggesting possibly two populations of PPO. Many reports on the heat stability of PPO enzymes show the inactivation to follow first order kinetics, as seen in grape (Wissemann and Lee, 1981; Lee *et al.*, 1983), cherries (Benjamin and Montgomery, 1973) and mango (Robinson *et al.*, 1993). However, heat inactivation of sweet potato PPO was also shown to be biphasic between 60-80°C (Lourenço *et al.*, 1992).

Given the substrate specificity (see Chapter 2) of sugarcane polyphenol oxidase, it would appear to be a catechol oxidase-type enzyme and not a laccase. The inhibition of PPO activity with low concentrations of SHAM is further evidence for this, but it is atypical in that it was inhibited by both SDS and CTAB. DIECA (a copper chelator) has been shown to be a potent inhibitor of other polyphenol oxidases. For example, the activity of olive PPO was inhibited by 50% in the presence of only 5 μ M DIECA (Ben-Shalom *et al.*,

1977). Similarly DIECA inhibited sugarcane PPO activity at quite low concentrations with 50 % inhibition at 0.2 mM.

The inhibition of sugarcane PPO by halides was found to be strongly pH dependent and from the Lineweaver-Burk plots is of the competitive type. Ben-Shalom *et al.* (1977) showed the pH dependence of inhibition of PPO by chloride from three distinct sources. It was found that avocado and olive PPO which have low pH optima (approximately 5.0) were more effectively inhibited by chloride ions than mushroom tyrosinase which has a higher pH optimum (pH 6.5). Ben-Shalom *et al.* (1977) and Peñafiel *et al.* (1984) postulated a mechanism in which chloride binds to the enzyme in competition with the substrate only when the enzyme is in its protonated form. Janovitz-Klapp *et al.* (1990) suggested that as chloride was found to be a noncompetitive inhibitor for apple PPO it can also interact with the enzyme-substrate complex in the protonated form. Both Peñafiel *et al.* (1984) and Janovitz-Klapp *et al.* (1990) also found NaF to be the most potent halide inhibitor, followed by NaCl, NaBr and NaI.

The inhibition of PPO by chloride is interesting from a commercial point of view as most other inhibitors of PPO are toxic to humans and therefore not suitable in food processing industries or are expensive. At the pH of cane juice (5.0-5.5) even relatively low concentrations of NaCl would significantly inhibit PPO activity.

CHAPTER 5. PURIFICATION OF A 45 KD PROTEIN SHOWING PPO ACTIVITY

Introduction

Purification of PPO from a number of different sources has led to varying and often conflicting estimates of its molecular weight and properties (Mayer and Harel, 1979; Vámos-Vigyázó, 1981). Multiple forms of PPO have been reported in a range of plant species including banana (Thomas and Janave, 1986) and kiwifruit (Park and Luh, 1985) where 14 and eight isoforms were reported respectively.

Extraction and isolation of PPO enzymes is complicated by the fact that high levels of enzyme are often found in tissues rich in phenolic substrates. It is therefore likely that at least some of these multiple forms arise from artefacts generated during isolation and purification either by quinone alkylation, partial denaturation or proteolysis (Lanker *et al.*, 1987; Ganesa *et al.*, 1992; Robinson and Dry, 1992). Failure to inhibit quinone formation has been an important source of heterogeneity in PPO molecular weight estimates. Inadequate inhibition of this quinone mediated cross-linking during the isolation of a potato trichome PPO led to an estimate of its molecular weight to be in excess of 10^6 , but it was later shown to be 59 kD (Bouthyette *et al.*, 1987; Kowalski *et al.*, 1992).

A 40-45 kD isoform has been purified from a number of species including spinach beet (Vaughan *et al.*, 1975), spinach (Golbeck and Cammarata, 1981), grapes (Nakamura *et al.*, 1983), sunflower seeds (Raymond *et al.*, 1993) and green olives (Ben-Shalom *et al.*, 1977). Immunoblots probed with a polyclonal antibody raised to broad bean PPO indicated a 43-45 kD band in broad bean, bush bean, lettuce, mung bean, soybean, spinach, and tobacco (Lanker *et al.*, 1988). *In vitro* translation of mRNA isolated from each of these species also produced a PPO protein of approximately 45 kD suggesting that PPO is synthesised as a mature 45 kD protein (Flurkey, 1986).

Active 59-65 and 70-72 kD isoforms and higher, however, have also been reported (Mayer and Harel, 1979; Ganesa *et al.*, 1992; Kowalski *et al.*, 1992; Robinson and Dry, 1992). Recently Robinson and Dry (1992) purified an active 60 kD PPO from broad bean leaf which, in the presence of proteases, could be cleaved *in vitro* to a 42 kD protein. They suggested that the 45 kD protein described by Flurkey (1985;1989) may be the result of proteolytic cleavage of the 60 kD protein.

Previous work by Coombs *et al.* (1974) and Gross and Coombs (1976a) led to the isolation of a non-particulate PPO from the leaves and stems of sugarcane. Gel filtration produced two fractions: PPO I (MW 130 kD) and PPO II (MW 32 kD). PPO I could be converted quantitatively to PPO II by eluting columns with buffer of high ionic strength, using aged preparations or eluting with sodium dodecyl sulphate or urea.

The aim of the following work was to purify PPO from sugarcane with a view to understanding better its properties with respect to manipulating the enzyme to ultimately reduce colour in raw sugar.

Materials and Methods

Protein purification

Method 1-Purification of active PPO (see Figure 5.1)

Stems (750 g) of sugarcane cultivar Q87 were sliced transversely into 2-3 mm thick discs and homogenised with a Polytron blender in 1.5 L of ice-cold buffer containing 0.1 M NaH₂PO₄, 10 mM ascorbic acid and 1 mM MgCl₂ (pH 5.0) and the protease inhibitors phenylmethylsulfonylfluoride (PMSF) and leupeptin at concentrations of 1 mM and 2 μM respectively. The homogenate was then filtered through Miracloth and centrifuged at 15,000g for 30 minutes at 4°C and the supernatant retained.

Ammonium sulphate was added to the supernatant to saturation over a period of 90 minutes at 4°C and the extract then centrifuged at 15,000g for 40 minutes at 4°C. The pellets were resuspended in 0.1 M NaH₂PO₄, 1 mM MgCl₂ and 2 mM ascorbic acid (pH 5.0) and centrifuged at 40,000g for 15 minutes. The supernatant was desalted on a 3.8 x 40 cm Sephadex G25 column equilibrated with 20 mM piperazine, pH 5.0 at a flow rate of 10 mL min⁻¹ and the active fractions were collected and pooled. The pooled extract was applied to a 1.6 x 5 cm Q-Sepharose column equilibrated with 20 mM piperazine, pH 5.0 and the active fractions which did not bind to the column were pooled and desalted on the 3.8 x 40 cm Sephadex G25 column equilibrated with 20 mM MES, pH 5.5. The desalted fraction was then loaded onto an 1 x 10 cm S-Sepharose column equilibrated with 20 mM MES, pH 5.5. The PPO was eluted with a 0-500 mM NaCl gradient and the active fractions pooled and concentrated on an Amicon YM30 ultrafiltration membrane. The concentrate was then applied to a 2.6 x 90 cm Sephacryl-300 column equilibrated with 25 mM MES, 150 mM NaCl (pH 6.0) at 2 mL min⁻¹. Fractions were collected, assayed for PPO activity and the active fractions pooled. Active fractions were concentrated on an Amicon YM30 ultrafiltration membrane.

The Biorad Model 491 Prep Cell is a preparative electrophoresis apparatus which purifies specific proteins from complex mixtures by continuous-elution electrophoresis. Proteins are electrophoresed vertically through a cylindrical acrylamide sieving gel. As individual bands migrate off the bottom of the gel, they pass directly into an elution chamber consisting of a thin frit. A dialysis membrane underneath the elution frit traps proteins within the chamber. Elution buffer enters the chamber and is drawn into the elution frit and inward to an elution tube using a peristaltic pump. The eluted protein is collected as individual liquid fractions.

For preparative nondenaturing gel electrophoresis, the discontinuous buffer system of Laemmli was used. A 7.5 cm high 8% acrylamide (30 acrylamide: 0.8 bis-acrylamide) resolving gel, and a 0.5 cm 4% stacking gel, were cast in the 28 mm ID gel tube of the Model 491 Prep Cell. One mL of the Sephacryl concentrate was loaded in 50 mM Tris (pH 6.8), 10% glycerol, 0.1% SDS, 0.002% bromophenol blue. The gel was electrophoresed at 40 mA with the same electrophoresis buffer in the upper and lower tanks (25 mM Tris-HCl, 192 mM Glycine, 0.1 % SDS pH 8.3). The lower electrophoresis buffer was cooled by circulation through an ice waterbath. The elution buffer contained 25 mM Tris-HCl and 192 mM Glycine (pH 8.3). Following elution of the bromophenol blue (approximately 100 min), 5 mL fractions were collected and assayed for PPO activity. The active fractions were pooled and concentrated in an Amicon Centricon-30.

Another 1 mL Sephacryl concentrate aliquot was desalted on a 0.8 x 16 cm Sephadex G25 column equilibrated with 20 mM Bis-Tris propane-HCl, pH 7.2 at 1 mL min⁻¹. The active fractions were pooled and loaded on a 1 x 10 cm Q-Sepharose column equilibrated with the same buffer at 1 mL min⁻¹. PPO activity was eluted with a 0-300 mM NaCl gradient and the active fractions pooled. The fractions were then concentrated in an Amicon Centricon-30.

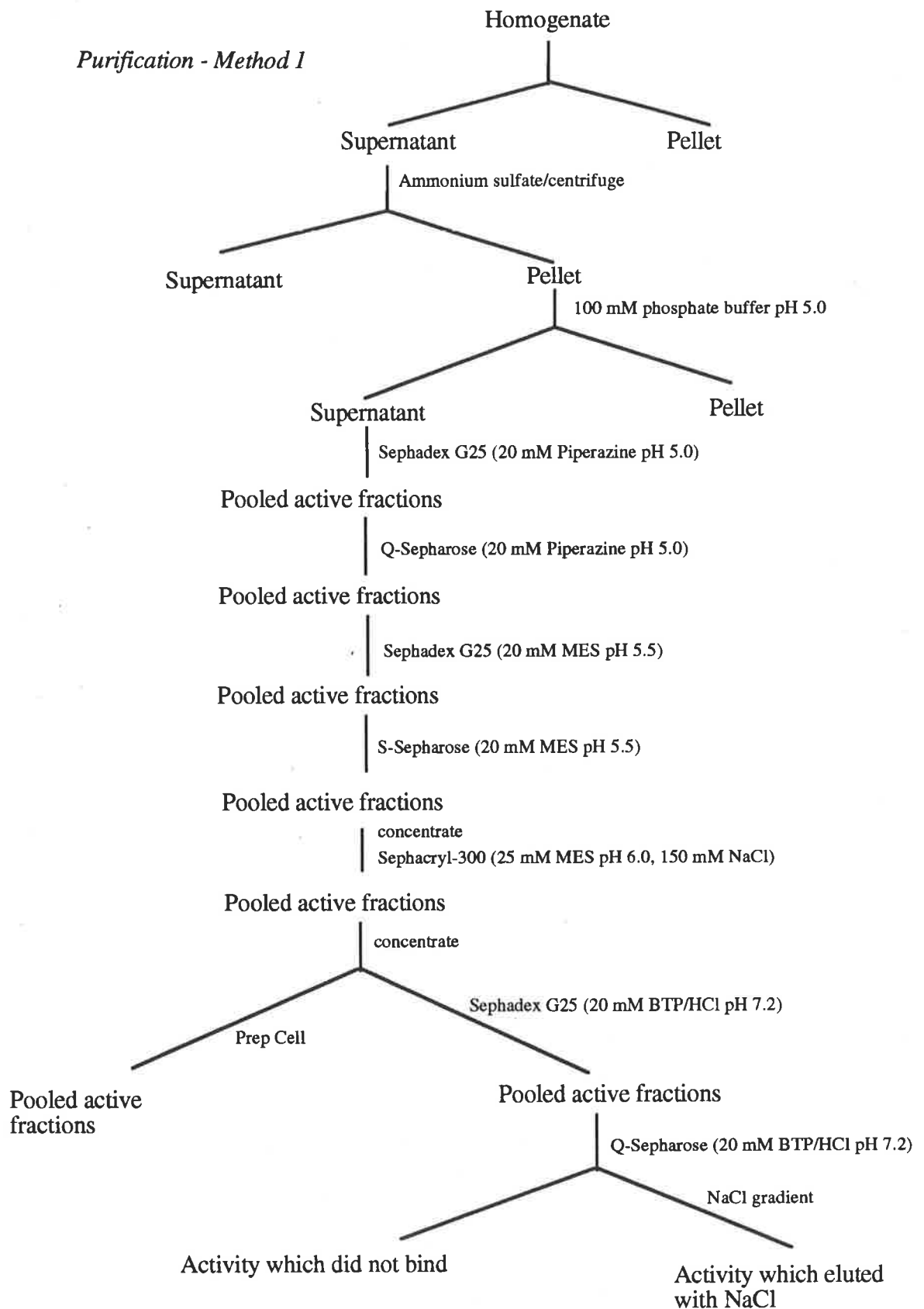


Figure 5.1 Diagrammatic representation of the steps in the first purification method

Method 2- Purification for antibody production

Young stem tissue of Q87 was sliced and 1.5 kg of tissue homogenised in two stages in three litres of 20 mM MES, 15 mM *L*-ascorbic acid, 1 mM MgCl₂ (pH 5.0), 1 mM PMSF and 2 µM leupeptin using a Polytron blender at 4°C to minimise browning. The homogenate was then filtered through Miracloth and centrifuged at 15,000g for 40 minutes at 4°C. The first preparation was loaded onto a 2.5 x 12 cm column of S-Sepharose equilibrated with 20 mM MES, 2 mM *L*-ascorbic acid (pH 5.0) at 10 mL min⁻¹. The column was washed with approximately 100 mL of buffer and loaded with the second preparation. The column was washed with 170 mL of 20 mM MES, 2 mM *L*-ascorbic acid (pH 5.0) and 0.5 mM PMSF. PPO activity was eluted with a 0-300 mM NaCl gradient at 5 mL min⁻¹ and the active fractions pooled. The extract was desalted on a 3.8 x 40 cm Sephadex G25 column equilibrated with 20 mM Tris-HCl, 2 mM *L*-ascorbic acid (pH 7.5) and eluted in the presence of 0.5 mM PMSF at 10 mL min⁻¹. The pooled fractions were loaded onto a 2.2 x 10 cm Q-Sepharose column equilibrated with 20 mM Tris-HCl and 2 mM *L*-ascorbic acid (pH 7.5) and washed with 120 mL buffer and 0.5 mM PMSF. PPO activity was eluted with a 0-300 mM NaCl gradient.

The active fractions were pooled and concentrated on an Amicon PM10 ultrafiltration membrane. There were two separate groups of PPO activity: that which did not bind to the Q-Sepharose (RT) and that which eluted in the NaCl gradient (BP). As the RT fraction contained the majority of the activity, it was concentrated and loaded onto a 2.6 x 90 cm Sephacryl-300 column equilibrated with 25 mM MES, 150 mM NaCl pH 6.0 at 2 mL min⁻¹ and the active fractions pooled.

For preparative denaturing gel electrophoresis, the Tricine Page (Schägger and Von Jagow, 1987) system was used. A 6.1 cm high 10% acrylamide resolving gel, and a 0.3 cm high 4.0% acrylamide stacking gel were cast in the 37 mm ID gel tube of the Model 491 Prep Cell

and cooled during polymerisation. The upper tank buffer contained 0.1 M Tris-HCl, 0.1 M Tricine and 0.1% SDS (pH 8.25) and the lower tank buffer contained 0.2 M Tris-HCl, pH 8.9. The sample was loaded in denaturing load buffer (225 mM Tris (pH 8.45), 10% glycerol, 2% SDS, 100 mM DTT, 0.0025% bromophenol blue) after heating at 100°C for 3 minutes. The gel was electrophoresed initially at 30 V for 60 minutes, with the lower tank buffer cooled to 8°C. The voltage was then raised to 100 V and run overnight. The elution chamber outlet was pumped at 0.5 mL min⁻¹ to a fraction collector and 3 mL fractions were collected. In order to detect the fractions containing the PPO, 15 µL of every fifth fraction after the fraction containing bromophenol blue marker dye was analysed by the Tricine Page system. After the elution position was determined, every fraction near the peak was analysed. The purest fractions were pooled and concentrated on an Amicon YM30 ultrafiltration membrane.

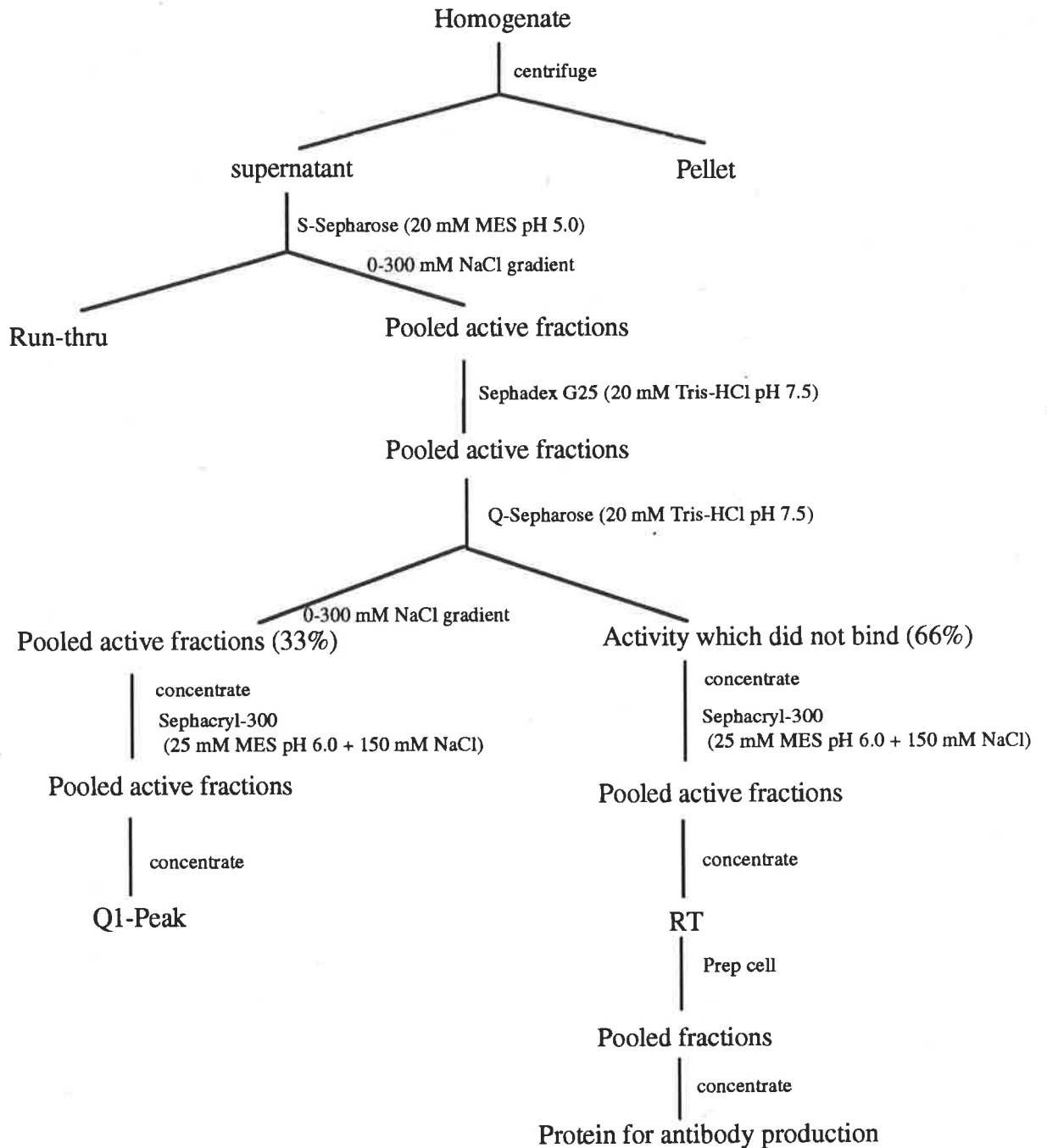


Figure 5.2. Diagrammatic representation of the steps in the Method 2 purification.

Measurement of PPO activity and protein estimation

PPO activity was assayed as described in Chapter 2 as oxygen uptake in a Hansatech oxygen electrode at 25°C. The reaction chamber contained 50 mM NaH₂PO₄, pH6.0 and extract in total volume of 1 mL and the reaction was initiated by addition of 2 mM chlorogenic acid. Protein concentration was estimated using the Biorad protein assay based on the method of Bradford (1976) using a combination bovine gamma globulin as the standard.

Electrophoresis

Electrophoresis was conducted using a mini-gel system (Hoeffer Mighty Small II) and Tricine polyacrylamide slab gels (Schägger and Von Jagow, 1987), comprising a 10% (w/v) resolving gel and a 4% (w/v) stacking gel in a discontinuous buffer system. For denaturing gels the protein sample was diluted into 225 mM Tris (pH 8.45), 10% glycerol, 2% SDS, 200 mM DTT, 0.0025% bromophenol blue and heated at 100°C for three minutes prior to loading. Gels were electrophoresed for 30 minutes at 30 V followed by 60-90 minutes at 150 V. Apparent molecular weights on Coomassie and silver stained gels were calculated by comparison with known standards. For gels stained for PPO activity apparent molecular weight was determined by comparison with prestained protein standards obtained from Biorad which, in turn, were calibrated by electrophoresis in the same gel system with unstained protein standards.

Gels were stained for total protein with Coomassie stain (0.15% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid) then destained in 50% (v/v) methanol, 10% (v/v) acetic acid for 1-2 hours. Remaining background colour in the gels was removed by washing in 5% (v/v) methanol, 7% (v/v) acetic acid before drying on a slab dryer. For more sensitive protein detection a silver stain based on the method of Merrill *et al.* (1981) was used.

For activity stained (partially denaturing) gels, samples were diluted into loading buffer (50 mM Tris-HCl (pH 6.8), 0.1% SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue) and the gels electrophoresed with 12°C cooling. The gels were washed twice for five minutes in 20 mM NaH₂PO₄, pH 6.0 and then stained in 20 mM NaH₂PO₄ (pH 6.0), 1 mM chlorogenic acid, 2 mM L-3,4-dihydroxyphenylalanine, 0.5 mM *p*-phenylenediamine and 180 Units mL⁻¹ catalase. After staining the gels were rinsed briefly in 20 mM NaH₂PO₄, pH 6.0 and dried on a slab dryer.

Protein sequencing

Determination of the N-terminal sequence of the sugarcane PPO proteins was carried out according to the method of Ploug *et al.* (1989). A total of 300 µg of partially purified extract was loaded into six tracks of a 1.5 mm, 10% Tricine gel. The gel was pre-electrophoresed in a cathode buffer containing 1 M Tris-HCl (pH 8.45), 0.1% SDS, 10 mM reduced glutathione (GSH) for 100 minutes. Reduced ^{glutathione} was also added to the cathode running buffer (0.1 M Tris-HCl (pH 8.25), 0.1 M Tricine, 0.1% SDS, 10 mM GSH) in an attempt to prevent amino terminal blockage of the protein (Simpson *et al.*, 1989). Separated proteins were transferred onto Transblot Protein Sequencing PVDF membrane (Biorad) in sequencing transfer buffer (10 mM CAPS-NaOH pH 11.0, 20% methanol, 0.5 mM DTT) for 1 hour at 300 mA. After washing in water 3 x 3 minutes, the membranes were stained in 0.025% Coomassie blue, 40% methanol for three minutes. The membranes were subsequently destained in 50% methanol, the stained protein bands excised, washed in sterile water and left to air dry.

Results

Purification-Method 1

A number of attempts were made to purify PPO from sugarcane. The first method was based on ammonium sulphate precipitation (see Figure 5.1). As the sugarcane PPO enzyme is soluble (Chapter 4), extraction of the enzyme led to large volumes of extract. The enzyme was concentrated by precipitation with ammonium sulphate. The specific activity of the sugarcane PPO in the homogenate was relatively high (Table 5.1). Following centrifugation, there was a significant loss in PPO activity (40%) in the ammonium sulphate precipitation step and only a two-fold increase in purification over the homogenate. Subsequent desalting on a Sephadex G25 column led to an increase in total activity measured, suggesting the removal of an inhibitory agent. The extract was then fractionated by anion exchange chromatography on Q-Sepharose and the peak of activity further fractionated by cation exchange chromatography on S-Sepharose. The most significant increase in purification occurred with this fractionation, with a more than five-fold increase in purification over that achieved with the Q-Sepharose column step (Table 5.1).

Table 5.1. Purification table using Method 1.

	Activity (Units)	Protein (mg)	Specific activity (Units mg ⁻¹)	Purification (-fold)	Yield (%)
Homogenate	4428	587.2	7.5	1.0	100
15,000g supt	4105	344.2	11.9	1.6	93
Ammonium sulfate pellet	2450	148.7	16.5	2.2	55
Sephadex G25	2717	105.0	25.9	3.5	61
Q-Sepharose	2059	46.2	44.6	6.0	47
S-Sepharose	1320	7.2	234.4	31.2	38
Sephacryl-300*	1012	2.6	389.2	51.9	25

* The Sephacryl concentrate was subsequently used in a number of different procedures.

After concentration, the enzyme was fractionated on a size exclusion column, Sephacryl-300, and the active fractions pooled. The specific activity was $389 \text{ Units mg}^{-1}$ and the purification was almost 52 fold over the homogenate. Electrophoresis under denaturing conditions revealed three strong protein bands (Figure 5.3) the strongest being at 60 kD. As PPO activity can be easily measured using the oxygen electrode, tracing the presence of the enzyme through the various chromatography steps was relatively straightforward. Identifying which of the protein bands shown in Figure 5.3 was the PPO enzyme also appeared to be quite simple given that PPO can be activity-stained when electrophoresed under partially denaturing conditions. Based on partially-denaturing activity stained gels, the sugarcane PPO enzyme appeared to be an approximately 60 kD protein. An activity stained gel of the peak fractions eluted from the Sephacryl-300 column (Figure 5.4), shows in fractions one and two a sharp band at 60 kD with a much fainter band in fraction three corresponding to lower amounts of the 60 kD protein seen in the protein stained gel (not shown). These 60 kD bands, when stained for activity, excised and re-run under fully denaturing conditions still migrated as 60 kD bands (data not shown).

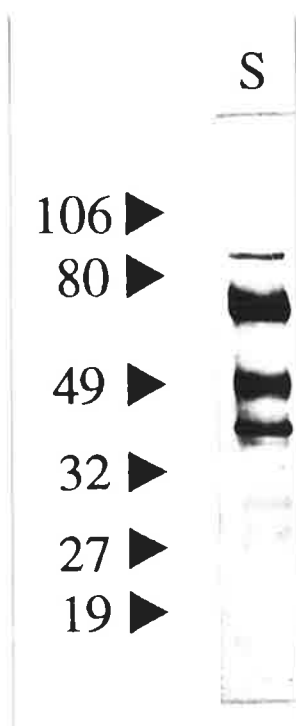


Figure 5.3. Silver stained denaturing Tricine gel of the Sephacryl-300 concentrate. 6 μg of total protein was loaded.

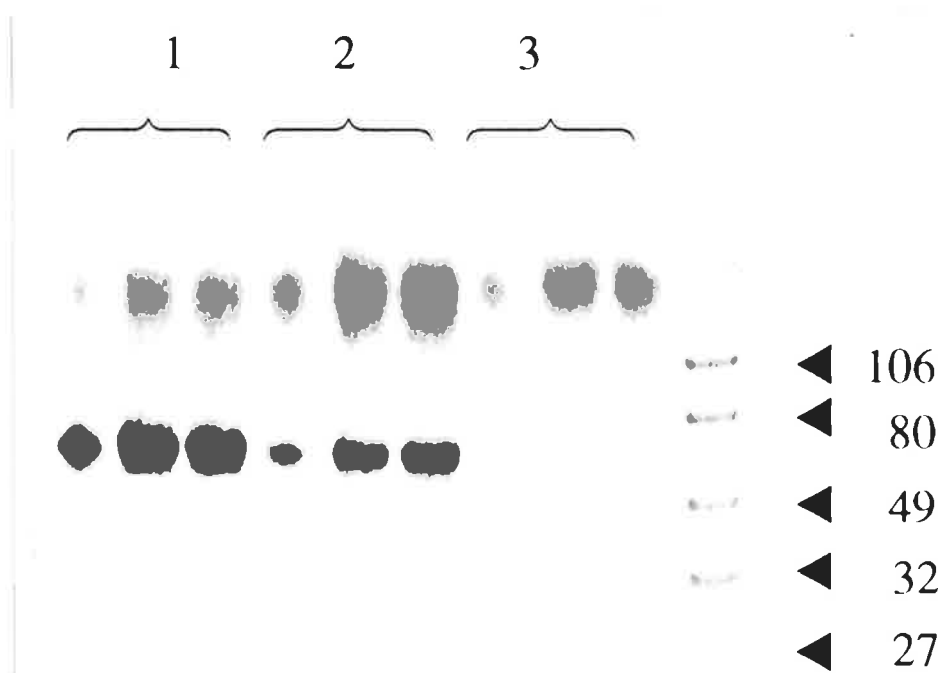


Figure 5.4. Activity staining of Sephacryl-300 fractions. Fractions which had eluted from the Sephacryl-300 column were electrophoresed on a partially denaturing Tricine gel and stained for PPO activity. The fractions are labelled as 1, 2 and 3. The three lanes of each fraction were loaded with 2, 5 and 5 μL .

To separate the 60 kD protein from the other proteins shown in Figure 5.3 the Sephacryl-300 extract was further fractionated using Preparative electrophoresis and by anion exchange chromatography on a Q-Sepharose column. 274 units of PPO activity (0.6 mg protein) were loaded onto the Prep Cell and the gel was electrophoresed until the bromophenol blue had eluted, at which point 3 mL fractions were collected and assayed for activity. PPO activity eluted in fractions 27-34 with the peak in fractions 30 and 31 (Figure 5.5). There was, however, a low recovery rate with only 22% of the PPO activity recovered (data not shown). Fractions 27-34 were pooled into three larger fractions and concentrated. Analysis of these three concentrated fractions on a denaturing Tricine gel revealed a predominant band of protein with an apparent molecular mass of approximately 60 kD and a fainter band at around 45 kD (Figure 5.6).

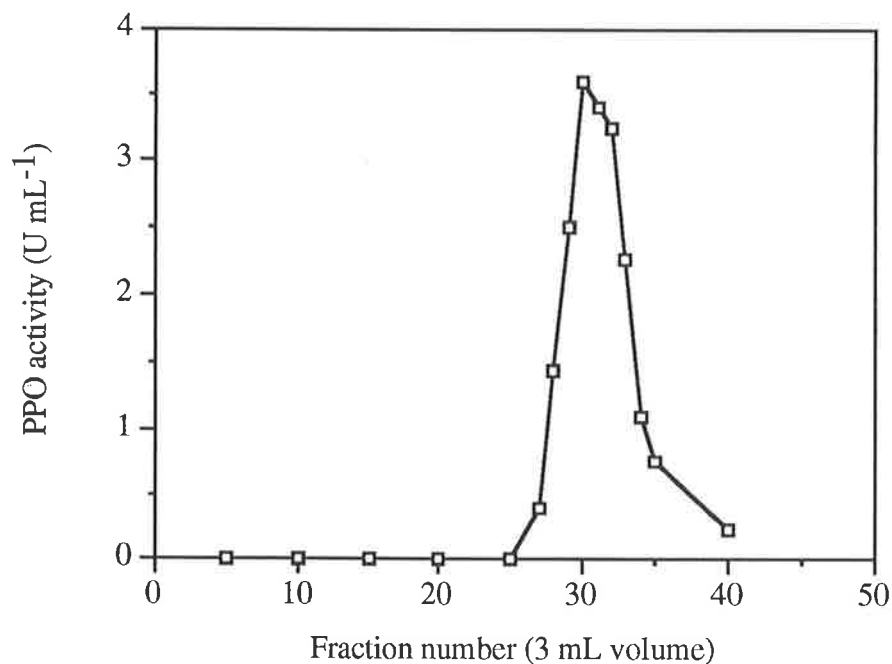


Figure 5.5. Elution profile of PPO activity from the Prep Cell.

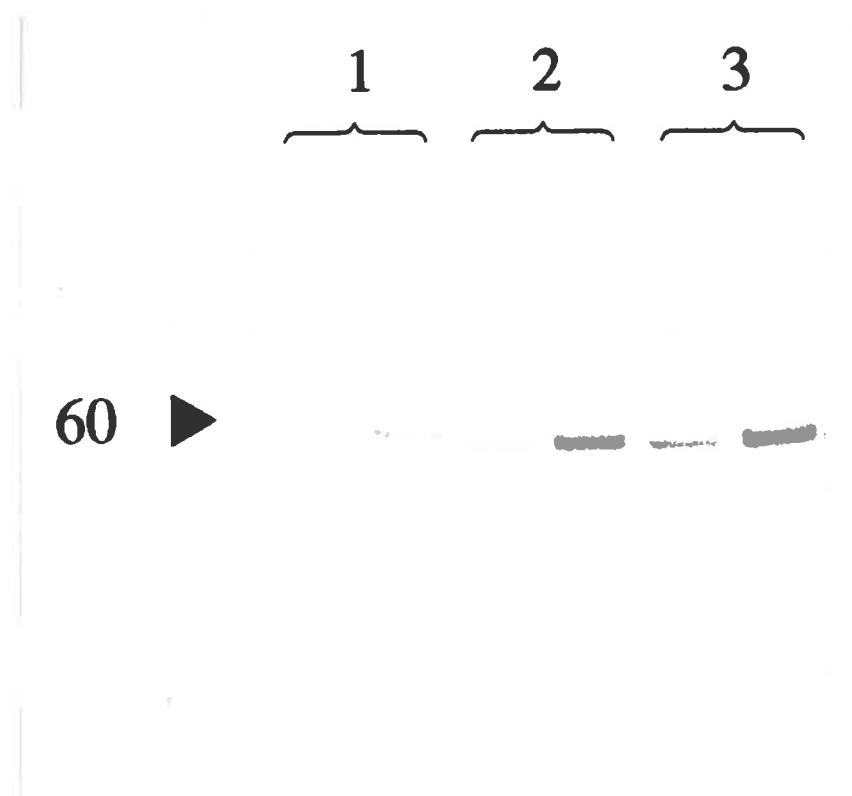


Figure 5.6. Purification of 60 kD PPO protein using preparative electrophoresis. Coomassie blue stained denaturing 10% Tricine gel of fractions collected from the Prep Cell. 5 and 10 μ L of each of the pooled and concentrated fractions were loaded. Where (1) fractions 27, 28 and 29, (2) fractions 30, 31 and 32 and (3) fractions 33 and 34.

A second portion of the Sephacryl fraction was fractionated on a Q-Sepharose column equilibrated with 20 mM BTP/HCl, pH 7.2. At this pH some of the PPO activity bound to the column whilst the remainder ran straight through. The peak which eluted from the column under a NaCl gradient was very broad (Figure 5.7). The two peaks of activity were concentrated separately and analysed by denaturing electrophoresis (Figure 5.8). In the peak which did not bind to the column (RT) there were two strong bands of protein at around 60 and 45 kD. In the peak which eluted from the column the predominant band was at 60 kD. The column was not overloaded as the capacity of the column was far in excess of that which was loaded onto it.

The 60 kD ^{protein} isolated under partially denaturing conditions using the Prep cell (Figure 5.6) still showed PPO activity, confirming that it was indeed a PPO. It appeared, however, that although the protein was being recovered not all the activity was, with only a 22% recovery of activity from the Prep Cell. Fractionation by anion exchange chromatography (Figure 5.7) suggested two PPO populations; one which was predominantly the 60 kD protein (Q) and another which contained both a 60 kD protein band and a 45 kD protein band. Despite purifying the 60 kD protein almost to homogeneity using the Prep Cell, the low yield of activity limited further purification. Scaling up this first purification procedure was not a viable option as the limiting step was the ammonium sulphate precipitation. Instead a second strategy was employed which eliminated this precipitation step.

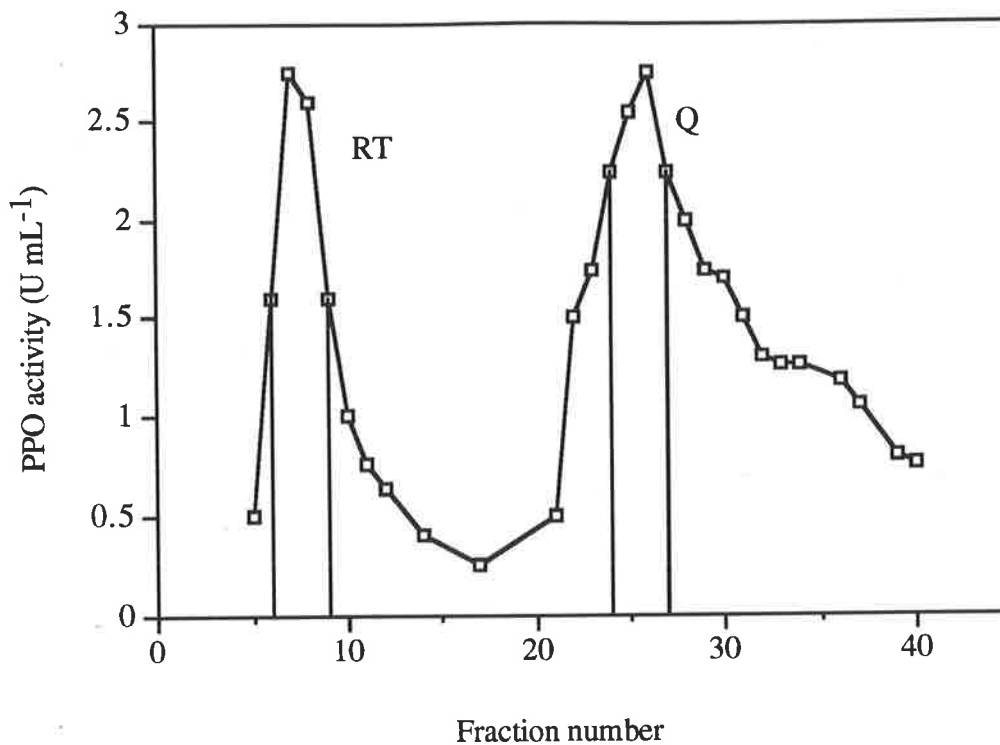


Figure 5.7. Elution profile of PPO activity from Q-Sepharose equilibrated with 20 mM BTP/HCl pH 7.2. RT is the activity which did not bind to the column and Q was eluted with a 0-300 mM NaCl gradient. The vertical lines indicate the pooled fractions.

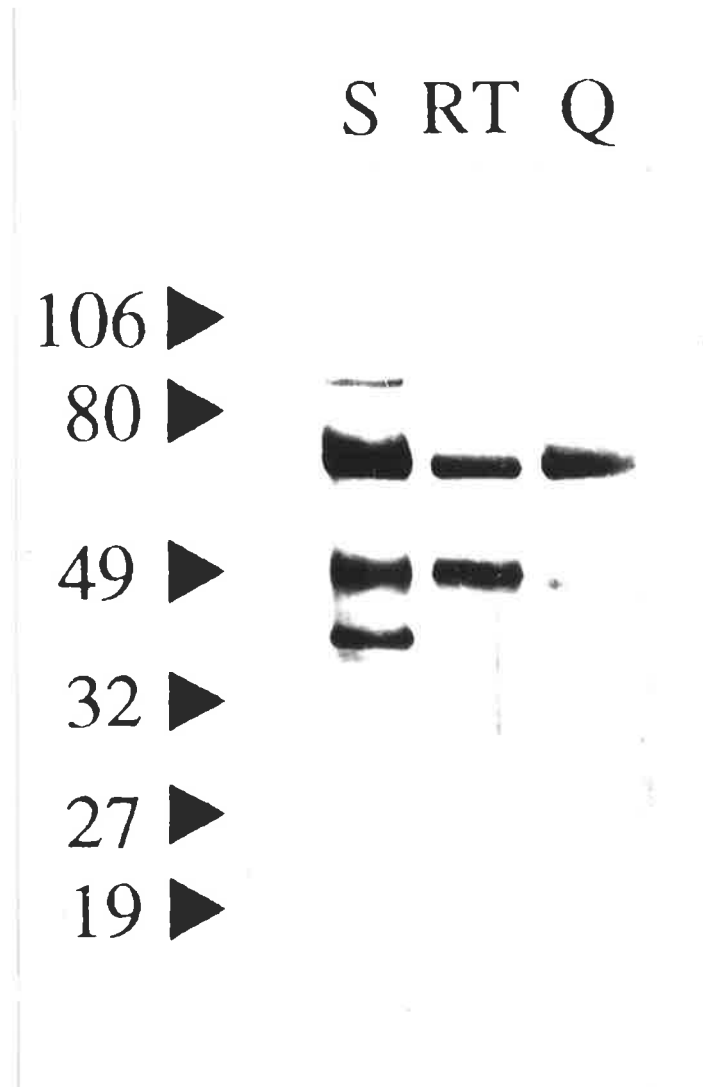


Figure 5.8. Comparison of two peaks of activity collected from the additional Q-Sepharose chromatography step. Silver stained denaturing 10% Tricine gel, lane S contains 6 μg of the Sphacryl-300 concentrate, lanes RT and Q were loaded with approximately 2 μg of protein (see Figure 5.6).

Purification-Method 2

The purification was scaled up such that the initial total activity was more than six times that of the first purification procedure (Table 5.2) and the procedure eliminated the need for the ammonium sulphate precipitation step (see Figure 5.2). The supernatant from the 15,000g spin was loaded directly onto a cation exchange column and the PPO activity eluted with a NaCl gradient, the peak of activity eluting at a concentration of 125 mM (Figure 5.9). There was an 11-fold increase in specific activity with a 42% recovery of activity (Table 5.2).

Table 5.2. Purification of PPO from 1.5 kg of cane by Method 2.

	Activity (Units)	Protein (mg)	Specific activity (Units mg ⁻¹)	Purification (-fold)	Yield (%)
Homogenate	29260	2057.0	14	1	100
15,000g supernatant	21755	1001.0	22	1.5	74
S -Sephrose	12420	83.7	149	11	42
Q-Sephrose RT	4710*	9.1	518	37	16
Amicon YM30 conc.	4602	5.7	802	57	16
Sephacryl-300	2850	2.6	1096	77	10
Amicon YM conc.#	2312	1.8	1284	90	8

* The fractions were left on ice for five days. As a consequence some PPO activity was lost.

This preparation was subsequently fractionated using Preparative electrophoresis.

The active fractions were pooled and desalted into 20 mM Tris-HCl, pH 7.5 for fractionation on a Q-Sephrose column. A large component of the activity (66%) was recovered in the fraction which did not bind to the column (Run-thru). The rest of the activity was recovered in two peaks E1 and E2, representing 16 and 17% of the total activity respectively, which eluted from the column with NaCl (Figure 5.9). Due to unforeseen circumstances, the

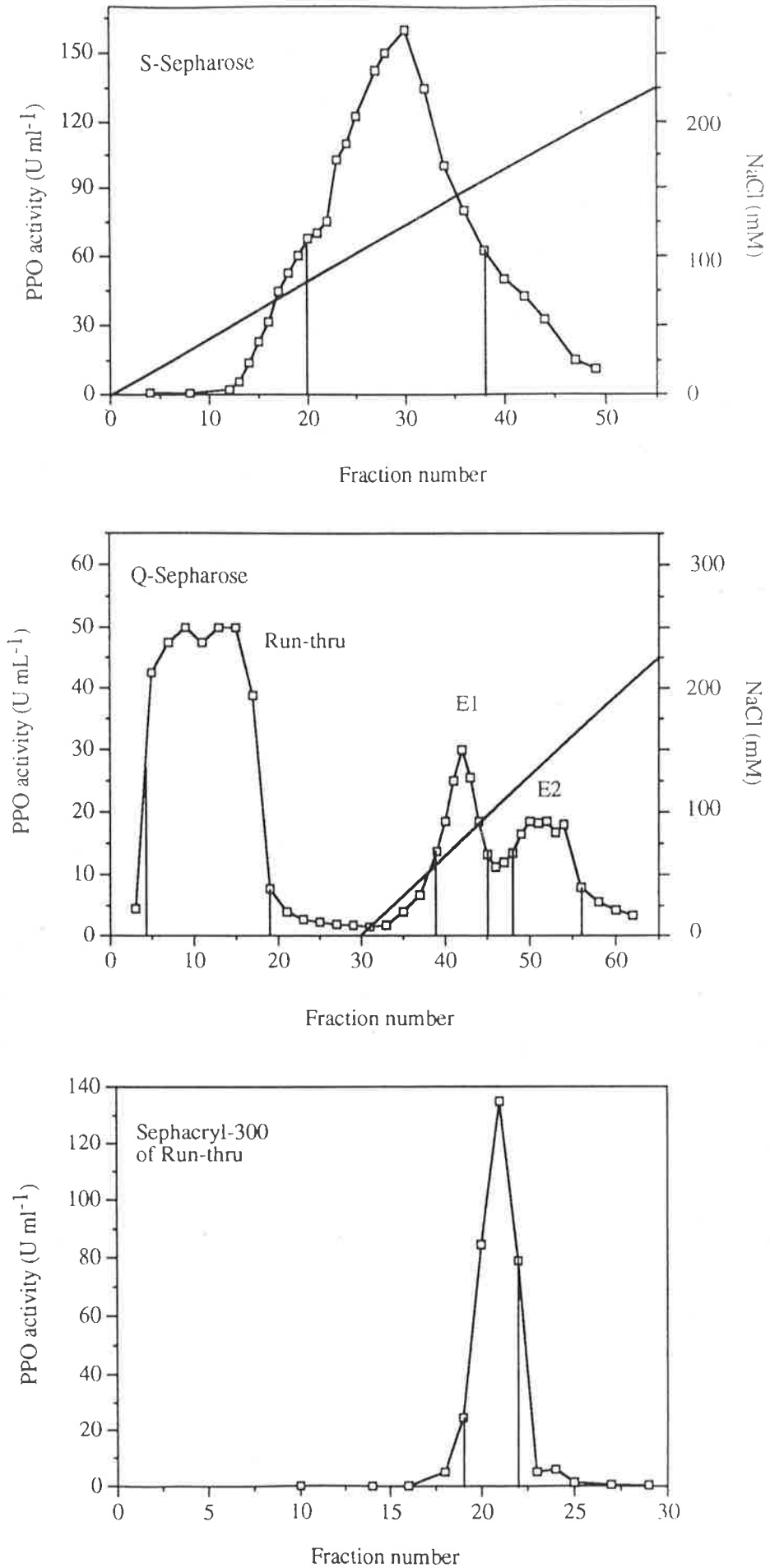


Figure 5.9. Elution profiles of PPO activity during purification. Vertical lines indicate the fractions which were pooled from each step.

column fractions were left on ice for five days and as a result some activity was lost. The Run-thru fractions however, when pooled, gave a specific activity of 518 units mg^{-1} and a 37-fold increase in purification. There was a further increase in specific activity following concentration on an Amicon YM30 ultrafiltration membrane (Table 5.2).

Size fractionation on the Sephacryl-300 column gave a sharp peak of activity (Figure 5.9) with a 77-fold purification over the homogenate and a specific activity of 1096 Units mg^{-1} . Again, concentration using an Amicon YM30 ultrafiltration membrane resulted in an increase in the specific activity. A gel of fractions from different stages of the purification shows the predominant protein band in the final Sephacryl-300 concentrate to be at approximately 45 kD (Figure 5.9).

It can be seen with this purification procedure that the 60 kD protein was in much lower abundance even in the initial chromatography steps involving cation and anion exchange chromatography (Figure 5.10). The same three bands seen in the Sephacryl concentrate from the first procedure were still evident in this concentrate (the 60, 45 and 30 kD proteins), but the 45-kD protein band was now the most predominant.

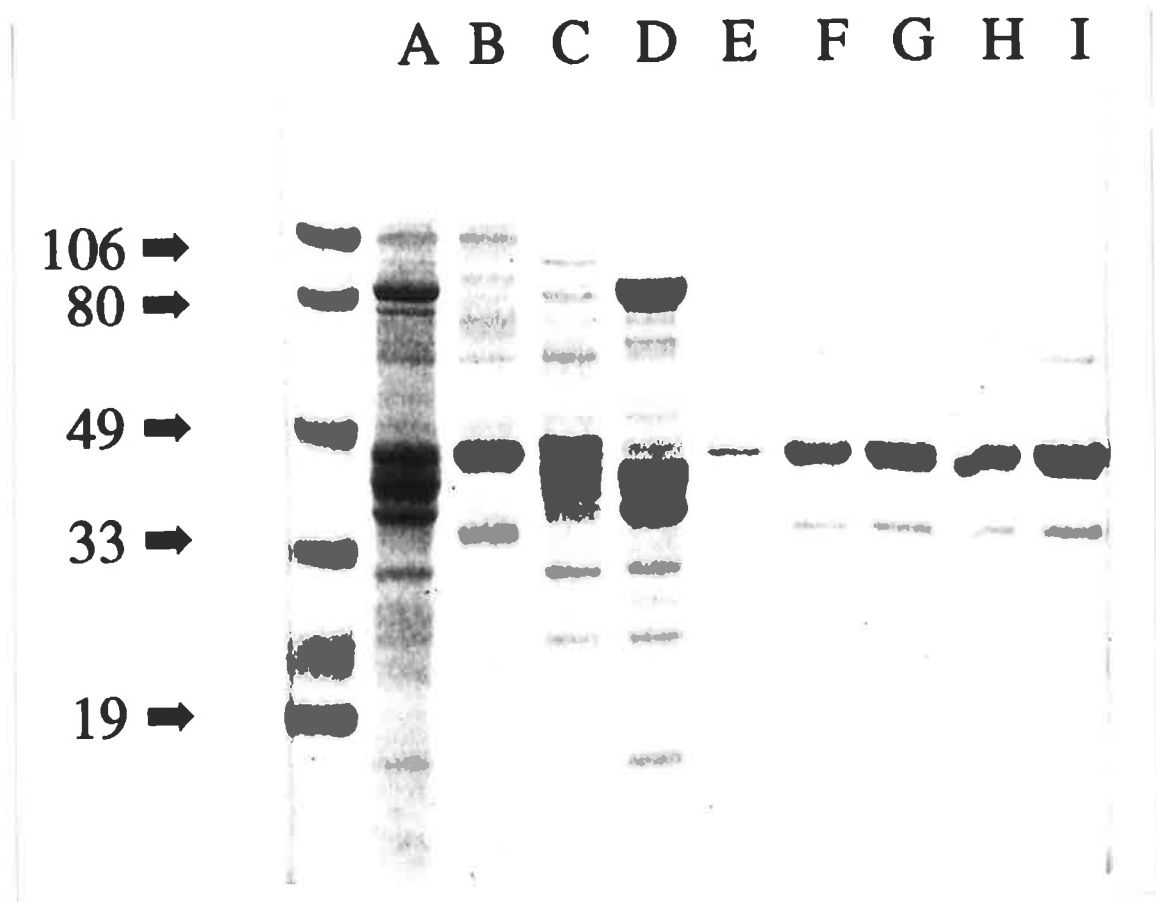


Figure 5.10. Comparison of different stages of the Method 2 purification. Coomassie blue stained 10% denaturing Tricine gel. Lane (A) S-Sepharose concentrate (9.3 μg), (B) Q-Sepharose RT concentrate (5.8 μg), (C) Q-Sepharose E1 concentrate (7.2 μg), (D) Q-Sepharose (12.7 μg), (E, F, G and H) peak fractions collected off the Sephacryl-300 column following fractionation of the Q-Sepharose RT (0.6, 2.5, 3.6 and 2.5 μg respectively), (I) Sephacryl-300 pooled concentrate (3.5 μg).

As these proteins were co-purifying on the basis of activity, it was possible they were all PPOs, and would not be separated by following activity and using conventional chromatography. Thus to purify the predominant 45 kD protein away from the other proteins, the Sephacryl concentrate was denatured and fractionated using denaturing Preparative electrophoresis (Prep Cell). The fractions eluting off the Prep Cell were collected and 15 μ L of every fifth fraction was run on denaturing Tricine gels to locate the 45 kD PPO protein. Once the approximate location of the 45 kD protein was established, 15 μ L of every second fraction was run (Figure 5.11). Fractions 38-50 were pooled and concentrated. Figure 5.12 compares the Sephacryl concentrate loaded onto the Prep Cell and the purified 45 kD PPO which was subsequently isolated. Due to the presence of SDS on the denatured protein it was not possible to measure protein by the Biorad protein assay, instead aliquots of the purified protein were run with known amounts of bovine serum albumin on a Tricine gel and then stained with Coomassie Blue. The amount of PPO protein was determined by comparison with the protein bands of the bovine serum albumin and estimated to be approximately 300 μ g in total.

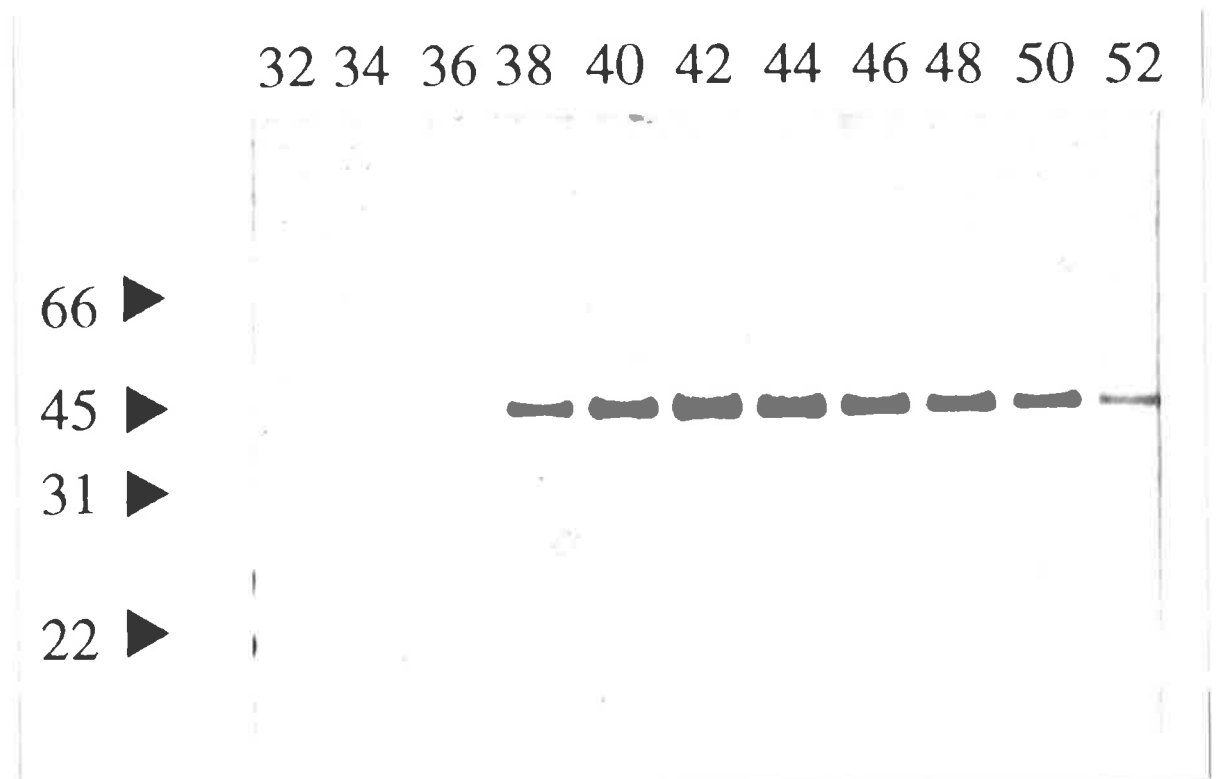


Figure 5.11. Fractions collected during the purification of PPO by Preparative electrophoresis. 15 μ L of every second fraction was run on a 10% denaturing Tricine gel and then silver stained. Numbers across the top indicate the fraction number.

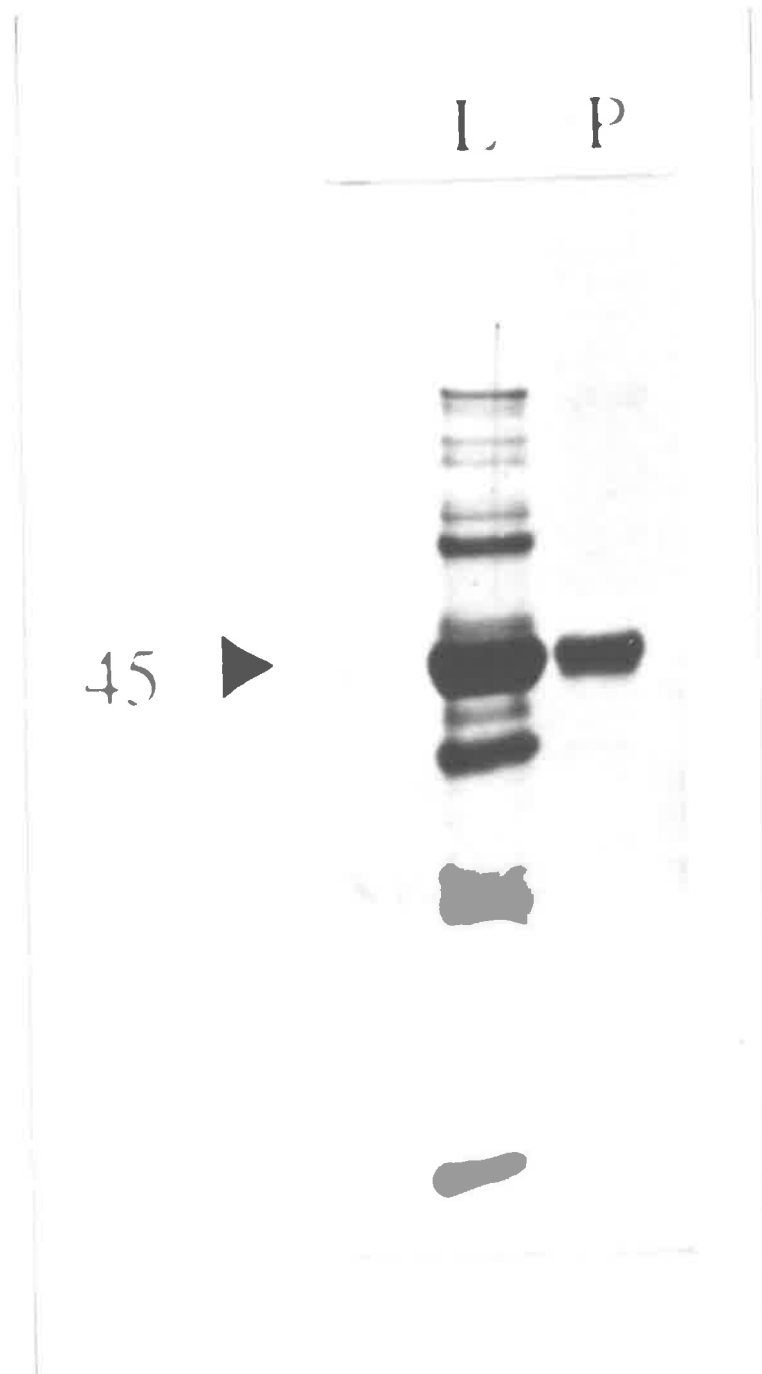


Figure 5.12. Purification of the 45 kD PPO using Preparative electrophoresis. Silver stained 10% denaturing Tricine gel showing the Sephacryl-300 concentrate (L) loaded onto the Prep Cell and the purified 45 kD PPO (P).

Fractions containing predominantly the 60 kD protein band and the 45 kD were run on partially denaturing Tricine gels and stained for PPO activity. The (a) fractions containing the 60 kD protein showed an activity smear down to 60 kD with a predominant band at around 60 kD (Figure 5.13) whilst the (b) fractions containing predominantly the 45 kD protein band showed a smear of activity starting and concentrated well above the 106 kD prestained marker down to the 60 kD mark. For the same amount of PPO activity loaded, staining of the 45 kD fraction was weaker than for the 60 kD protein.

Protein sequencing

Very little N-terminal amino acid sequence was obtained from either the 45 or 60 kD proteins as they were N-terminally blocked. However, both proteins had the same N-terminal amino acid sequence: ADSS.

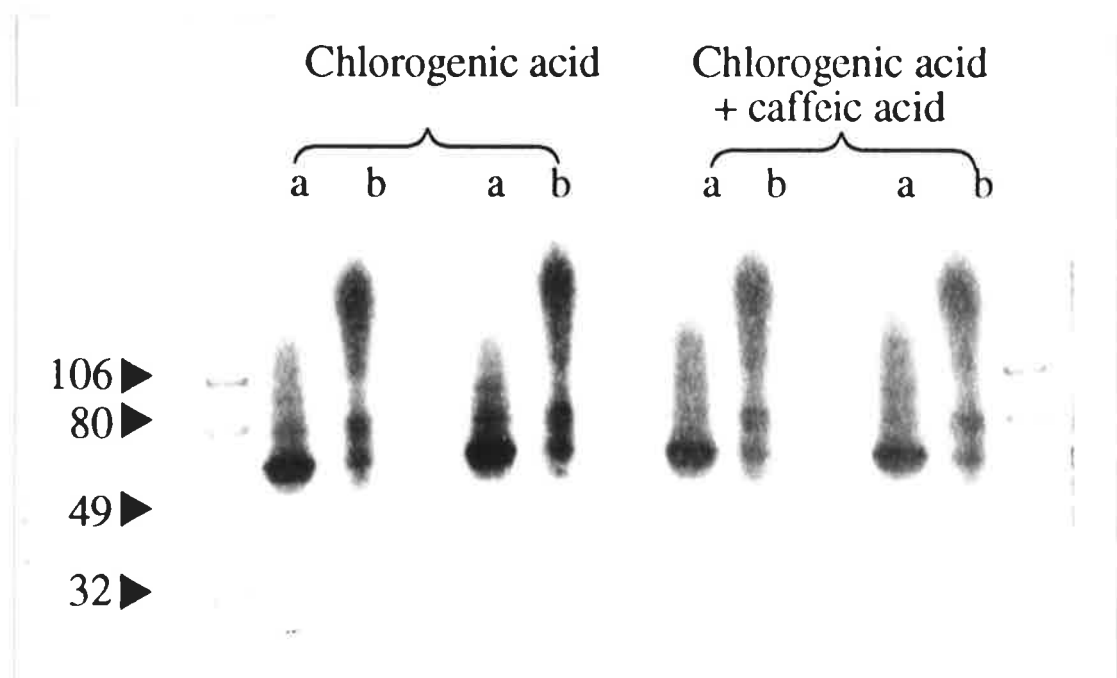


Figure 5.13. Activity stain of partially purified fractions of PPO. Fractions containing predominantly the 60 kD protein (a) and the 45 kD protein (b) were electrophoresed on a partially denaturing Tricine gel and stained for PPO activity. The first four lanes had chlorogenic acid as the major phenolic substrate and the second four had a combination of chlorogenic acid and caffeic acid. Each of the lanes contained 0.5 Units of PPO activity.

Discussion

The initial purification of the sugarcane PPO enzyme isolated low amounts of a 60 kD protein as estimated under fully denaturing conditions by gel electrophoresis. When stained for activity under partially denaturing conditions an activity band at approximately 60 kD was observed. Although this procedure yielded relatively pure 60 kD PPO protein, the yields were extremely low and scaling up of this method was not feasible. The observation that all of the PPO activity could be bound to S-Sepharose at pH 5.5 was utilised to devise an improved procedure. Modification of the purification protocol eliminated the ammonium sulphate precipitation step which had proved to be cumbersome. This modification (Figure 5.2) however, increased the proportion of the 45 kD protein detected at each of the purification steps (Figure 5.10).

Unlike the 60 kD PPO, the 45 kD protein did not stain as a single band of activity on a partially denaturing gel which led to speculation as to whether it really was a PPO (Figure 5.13). It is possible that the 45 kD protein is more affected by the electrophoretic conditions than the 60 kD protein and becomes inactivated. The high molecular weight smear of activity in extracts containing predominantly the 45 kD protein suggest that under partially denaturing conditions this protein runs as an aggregate. Angleton and Flurkey (1984) electrophoresed extracts of grapes, mung beans, yams, broad beans, lettuce and mushrooms in the presence and absence of SDS. They found that in the absence of SDS, the pattern of staining in all but the extracts of lettuce and mushrooms was a broad zone of activity but that this changed into sharp bands in the presence of SDS. If a protein is not fully denatured and saturated with SDS, the relationship between its apparent molecular weight and mobility on a gel is tenuous (See and Jackowski, 1989).

The 45 kD protein could not be totally separated from the 60 kD protein using conventional chromatography as this was based on fractionation using activity as the marker, and the

possibility that there may be other smaller forms of the PPO protein could not be ignored. It is possible that the 30 kD protein co-purifying with the 45 and 60 kD proteins is also a PPO (see Figures 5.3 and 5.10). The 45 kD protein was ultimately isolated from the other proteins in a denatured form, so that it could not be confirmed as a PPO. However, given the same limited N-terminal sequence in both forms, it is suggested that the 45-kD protein may be the *in vitro* cleavage product of the 60 kD PPO, a similar situation to that observed by Robinson and Dry (1992) with broad bean leaf PPO.

Despite the number of reports of the 45 kD protein as the predominant PPO form, the most compelling work is that on PPO in *Vicia faba*. Flurkey (1985) partially purified an active 45 kD PPO from broad bean leaf. Poly A⁺mRNA was isolated, translated *in vitro* and immunoprecipitated with an antibody to broad bean PPO yielding a 45 kD protein. This led him to suggest that, unlike other nuclear coded proteins targeted to the chloroplast, PPO was synthesised without a transit peptide to facilitate transport into the chloroplast. He subsequently translated *in vitro* poly A⁺mRNA isolated from a number of different plants and showed that the products immunoprecipitated with the broad bean antibody had an apparent molecular weight of 45 kD. When Lanker *et al.* (1988) tested the cross-reactivity of the broad bean antibody against other species they identified, in addition to the 43-45 kD protein, a band at 60-63 kD. Flurkey (1989) purified both the 60 and 45 kD forms and showed them to have almost identical N-terminal amino acid sequence. More recently, Ganesa *et al.* (1992) purified broad bean PPO to apparent homogeneity in the presence of protease inhibitors and reducing agents, to prevent covalent modification and phenolic oxidation, to give a 65 and a 68 form of PPO with no evidence of a 45 kD enzyme. They suggested that the broad bean PPO may be larger than originally thought and susceptible to proteolysis during purification. Robinson and Dry (1992) purified a 60 kD PPO from broad bean leaves in the presence of protease inhibitors and showed that it is susceptible to *in vitro* proteolytic cleavage at the C-terminal end, yielding an active 42 kilodalton protein. They concluded that PPO is synthesised as the 60 kD form but that it can be cleaved to the 45 kD form without loss of activity.

The increase in the prevalence of the 45 kD protein with the Method 2 purification suggests that this procedure resulted in proteolytic cleavage of the 60 kD PPO protein to yield the 45 kD form. This may have resulted from inadequate inhibition of proteolysis or acid mediated cleavage of the 60 kD protein promoted by the acidic nature of the cation exchange chromatography step early in the purification procedure. Harel *et al.* (1973) reported that the predominant form of grape catechol oxidase (55-59 kD) underwent dissociation with exposure to acid pH or urea forming 31-33 kD and 20-21 kD subunits. Similarly, when purification of peach polyphenol oxidase was carried out in the presence of protease inhibitors the number of isozymes observed decreased (Flurkey and Jen, 1980). It is also possible that the ammonium sulphate step utilised in the first strategy may have removed proteases involved in the cleavage.

The apparent discrepancy between the estimates of molecular weight reported by Gross and Coombs (1976a) for sugarcane PPO and those reported here may reflect the method of size determination. The molecular weight estimates they report were based on gel filtration which separates the proteins in a native state and does not take into consideration any aggregation which may occur. They also reported the presence of brown bands when the preparations were run on electrophoresis gels, suggesting the proteins had not been isolated under sufficiently reduced conditions. They found the larger form, PPO I (130 kD), could be quantitatively converted to PPO II (32 kD) by eluting with buffer of high ionic strength, using aged preparations or eluting with SDS or urea. In many cases crude or partially purified preparations show a multiplicity of forms which may occur through association or dissociation. Harel and Mayer (1968) observed three forms of catechol oxidase from apple fruit, with molecular weights of 30-40, 60-70 and 120-130 kD which were shown to undergo interconversions. Thus the 60-70 kD fraction could be partially converted to the 120-130 kD fraction in the presence of 1 N NaCl.

Whilst there continues to be confusion over the estimates of the structural size of PPO, all PPO genes isolated to date from tomato (Shahar *et al.*, 1992; Newman *et al.*, 1993), potato (Hunt *et al.*, 1993), broad bean (Cary *et al.*, 1992; Robinson and Dry, 1992), apple (Boss *et al.*, 1995) and grape (Dry and Robinson, 1994) encode 56-62 kD mature peptides with 8-12 kD putative transit peptides. This supports the hypothesis that the mature PPO protein has a molecular weight of 57-62 kD and that reported smaller PPOs may be the product of *in vitro* cleavage as a result of the extraction procedure. However Dry and Robinson (1994) and Rathjen and Robinson (1992a) suggest that in the Sultana grape berry cleavage may be an *in vivo* process necessary for activation of the enzyme. In a variegated mutant of Sultana with low levels of the active 40 kD PPO in the white regions of the leaf, it appears that the specific protease necessary for cleavage of the inactive 60 kD form to the active 40 kD PPO is absent or dysfunctional, leading to the accumulation of the inactive 60 kD form. Similarly, in *Neurospora crassa*, a protyrosinase with a molecular weight of 75 kD is cleaved to produce the mature 46 kD tyrosinase (Kupper *et al.*, 1989). When the gene encoding the protyrosinase was cloned it revealed cleavage involved the removal of 213 amino acids from the C-terminus. The authors suggested that this C-terminal extension was involved in shielding the enzyme's active site, its cleavage resulting in activation of the enzyme.

Clearly there is still considerable debate as to both the size of PPO and the number of forms present in plants. In sugarcane, a 45 kD PPO has been purified and a 60 kD form shown to stain for PPO activity. The two proteins were shown to have identical N-terminal amino acid sequence, however, the relationship between these two proteins in sugarcane is still uncertain. A polyclonal antibody was raised to the purified 45 kD protein with a view to determining the relationship between these two proteins and the results are presented in the following chapter.

CHAPTER 6. SUGARCANE POLYPHENOL OXIDASE IS A 60 KILODALTON PROTEIN SUSEPTIBLE TO *IN VITRO* CLEAVAGE

Introduction

In the previous chapter, a 60 kD protein was partially purified which could be stained for PPO activity in partially denaturing gel systems, however significant quantities of this protein could not be obtained. A 45 kD protein also believed to be a PPO was purified to homogeneity but as it was purified in a denatured state the activity of this 45 kD protein could not be demonstrated. The relationship between these two proteins remains unclear although previous results suggest that the 45 kD protein may be a cleavage product of the 60 kD protein (Rathjen and Robinson, 1992a; Robinson and Dry, 1992; Dry and Robinson, 1994). To clarify this relationship a polyclonal antibody was raised to the 45 kD protein and used to further characterise this enzyme in sugarcane.

Materials and Methods

Antibody preparation

The purified 45 kD protein was used to raise polyclonal antibodies in a New Zealand white rabbit. For the initial injection, 1.2 mL of the Prep Cell purified PPO desalted into PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4), was added to 1 mL Freund's Complete Adjuvant. The protein was added to the Adjuvant 200 µL at a time, mixing with a syringe after each addition until a thick emulsion was formed. This was injected intramuscularly into the hind legs of the rabbit. After four weeks a further 750 µL of the protein in PBS was added to 0.5 mL of Freund's Incomplete Adjuvant and injected intradermally in approximately ten different positions over the rabbit. A final injection occurred three weeks later when 0.5 mL of the protein in PBS was injected intravenously.

Ten days later the rabbit was test bled and an Ouchterlony test showed an antibody to the injected protein.

Blood was removed from the rabbit by cardiac puncture and the serum separated. IgG was purified on a Protein A column and concentrated to a final concentration of 7 mg mL⁻¹. Aliquots were stored at -70°C in 25% (v/v) glycerol to give a final concentration of 5.2 mg mL⁻¹.

For immunoblotting, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH~8.3) using a Biorad Mini Trans-Blot apparatus. Prior to transfer, both the gel and membrane were washed in transfer buffer for 20 minutes. Transfer was for 60 minutes at 100 V, the membrane was then removed and washed in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) for 15 minutes. The membrane was placed in block solution (3% gelatin in TBS) for 45 minutes, and subsequently washed three times for five minutes in TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% (v/v) Tween-20, pH 7.5). It was then incubated for one to two hours in primary antibody (diluted 1:3000 in TTBS, 1% gelatin, 0.02% azide) and subsequently washed a further three times in TTBS. The secondary antibody was a goat anti-rabbit antibody conjugated with alkaline phosphatase (Biorad) and was made to a 1:2000 dilution in TTBS. This was incubated with the membrane for a further 1-2 hours, followed by three rinses, each of five minutes, in TBS. The alkaline phosphatase substrates, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), were prepared according to the manufacturers instructions (Promega) and incubated with the membrane for up to ten minutes. The reaction was stopped by rinsing in water and the membrane was allowed to air-dry. All washing and incubations were carried out on a rotary shaker.

Preparation of different sugarcane tissues for blotting

Various tissues were homogenised in 50 mM NaH₂PO₄, 10 mM ascorbate (pH 6.0); the tissue:buffer ratio dependent on the expected level of activity. The homogenates were filtered through Miracloth and assayed for PPO activity and total protein. For electrophoresis, an aliquot was removed, centrifuged at 12,000g for ten minutes, the supernatant removed and denatured in loading buffer at 100°C for three minutes. Following electrophoresis, PPO protein was detected by immunoblotting. Equivalent loadings were made in each lane based on PPO activity determined in each extract.

Acid-mediated cleavage of sugarcane PPO

Extracts of sugarcane cultivar Q87 were prepared by homogenising 7.5 g of internode tissue in 20 mL of cold grinding medium (20 mM NaH₂PO₄, 10 mM ascorbate, pH 7.5). When the tissue was extracted in the presence of protease inhibitors, a cocktail containing 1 µM pepstatin, 1 µM leupeptin and 1 mM PMSF was included in the grinding medium. Where extracts were immediately heat-denatured, tissue was added directly to boiling buffer in the presence of 2% SDS and reheated to boiling. The various homogenates were filtered through Miracloth and centrifuged at 40,000g for ten minutes at 4°C. From each of the extracts an aliquot was taken and the pH adjusted to 4. The extracts at both pH 4 and 7.5 were then left overnight at 4°C. Total protein measurements and, where possible, assays for PPO activity were made and the samples were then denatured as described previously and loaded onto a 0.75 mm 10% Tricine gel. Immunoblots were performed as described above.

Protease digestion

Stem portions of cultivar Q87 were homogenised in 25 mM Tris-HCl, 2mM ascorbate (pH 7.0), filtered through Miracloth and centrifuged at 40,000g for ten minutes at 4°C. The supernatant was concentrated and desalted in an Amicon Centricon-30 into 25 mM Tris-HCl,

pH 7.8. For digestion with Lys-C, 1 mM EDTA was added to the buffer. The proteases trypsin, thermolysin and Lys-C were added to a concentration of $30 \mu\text{g mL}^{-1}$ and incubated at room temperature. Aliquots were removed after zero, one and three hours and added directly to denaturing buffer to stop further digestion. Additional aliquots were taken to measure PPO activity and aliquots were also taken from control tubes, incubated under the same conditions without added proteases. The denatured extracts were then loaded on a 0.75 mm 10% Tricine gel and PPO protein was detected by immunoblotting.

Cross reactivity of plant PPOs

Known amounts of PPO purified from sugarcane, bean and grape were loaded onto 0.75 mm 10% Tricine gels and the proteins were subsequently transferred onto PVDF membrane and probed with antibodies to grape PPO, bean PPO and the 45 kD sugarcane protein. A control lane was run on each gel of bovine serum albumin to check for non-specific binding of the antibodies.

Immunoblotting of activity stained gel slices

A 0.75 mm 10% Tricine gel was run with extracts at varying stages of purification under partially denaturing conditions and the gel was stained for PPO activity as described earlier. Gels slices were cut from the activity stained smears as shown in Figure 6.8 and added to 5 μL of denaturing buffer and heated at 100°C for three minutes. The gel slices were then trimmed and loaded onto a second 1.5 mm 10% Tricine gel. The presence of bromophenol blue allowed the slices to be seen. It was found to be essential for the gel slices to be placed at the bottom of each well. Following electrophoresis, the positions of PPO bands were detected by immunoblotting.

Results

The antibody raised against the purified 45 kD protein was used to detect PPO proteins in immunoblots of extracts of various sugarcane tissues. The specificity of this antibody was determined by probing a western blot of the purified 45 kD protein with pre-immune and post-immune serum. Pre-immune serum did not react with the purified 45 kD extract, whereas the post-immune serum gave the expected single band at 45 kD (data not shown).

When PPO partially purified from stem and leaf extracts of Q87 using the cationic exchange step described in Chapter 2, was separated on an SDS gel and probed with the sugarcane PPO antibody, not only was a band at 45 kD detected but also an antigenic protein of 60 kD. This suggested they were either closely related proteins or that the 45 kD protein was a cleavage product of the 60 kD protein (Figure 6.1). There were different proportions of the two forms, with more of the 45 kD band in the stem preparation, which may reflect the fact that the stem preparation was left at 4°C overnight. In addition to the two major bands, a further band at around 35 kD was detected in the leaf extract.

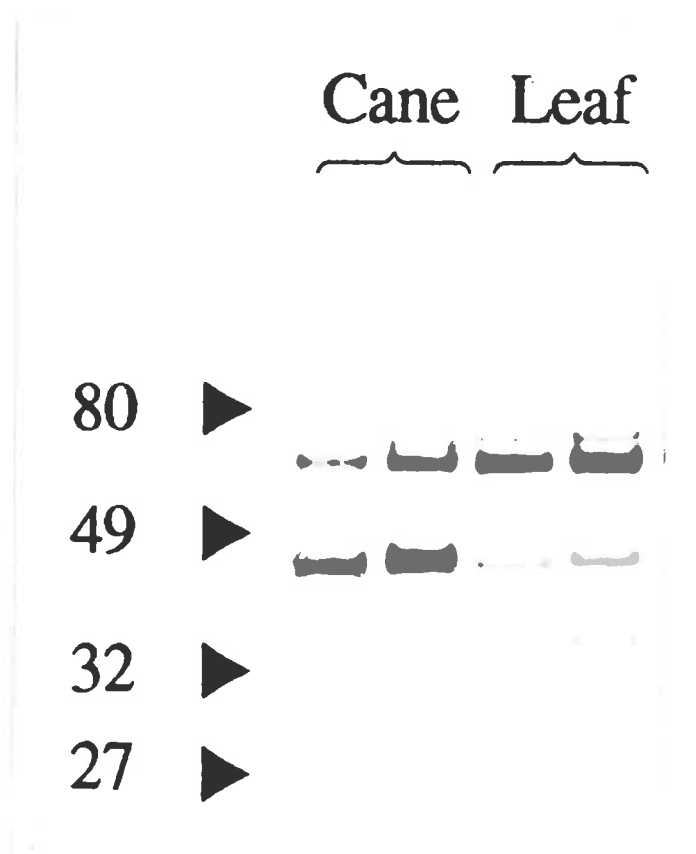


Figure 6.1. Western blot of partially PPO in stem and leaf extracts probed with the sugarcane PPO antibody. In the two lanes of stem extract, 60 and 120 mU of PPO activity were loaded respectively and in the two lanes of leaf extract, 30 and 60 mU.

Detection of PPO in different tissue types

Western blot analysis was used to detect the presence of PPO in different sugarcane tissues. Extracts were made from immature stem, the nodes and internodes of mature stem and from leaves. When these samples were run on a gel under partially denaturing conditions and stained for PPO activity, the active 60 kD band showed up strongly with very little smearing (Figure 6.2). When the extracts were electrophoresed under fully denaturing conditions and probed with the antibody to the 45 kD protein the active 60 kD band was detected in all the tissue types, with very little of the 45 kD protein apparent except in the extract of nodal tissues (Figure 6.3). Trace amounts of an antigenic 35 kD protein were also observed. Similarly, in other tissue types where the extracts were made at pH 6.0, the 60 kD band was the predominant band detected by immunoblotting (Figure 6.4) in all tissues. Despite the same activity loaded in each track, the intensity of the 60 kD band detected by the antibody was not consistent. In extracts with high levels of PPO activity, which brown more readily, the PPO protein was less antigenic, as seen in the tracks GP, C1, LR1 and LR2 (Figure 6.4).

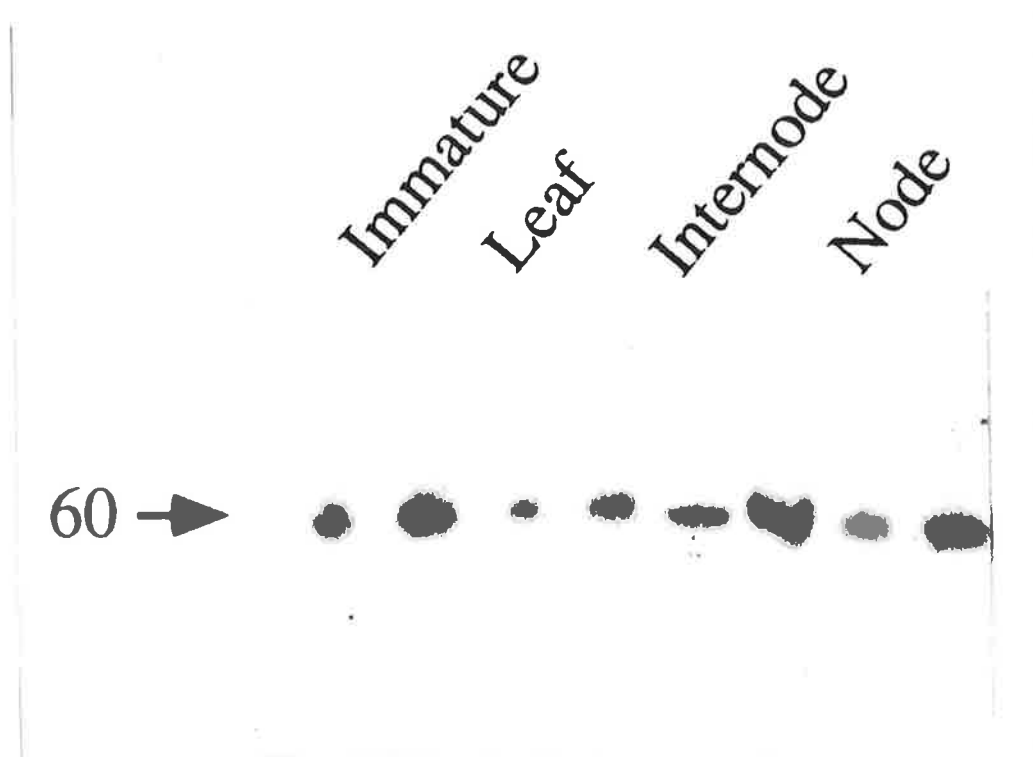


Figure 6.2. Crude extracts of immature stem, the internodes and nodes of mature stem and sugarcane leaf electrophoresed on a partially denaturing Tricine gel and stained for PPO activity. For each extract there are two lanes representing 25 and 50 mU of PPO activity except for the leaf extract where only 15 and 30 mU of activity was loaded.

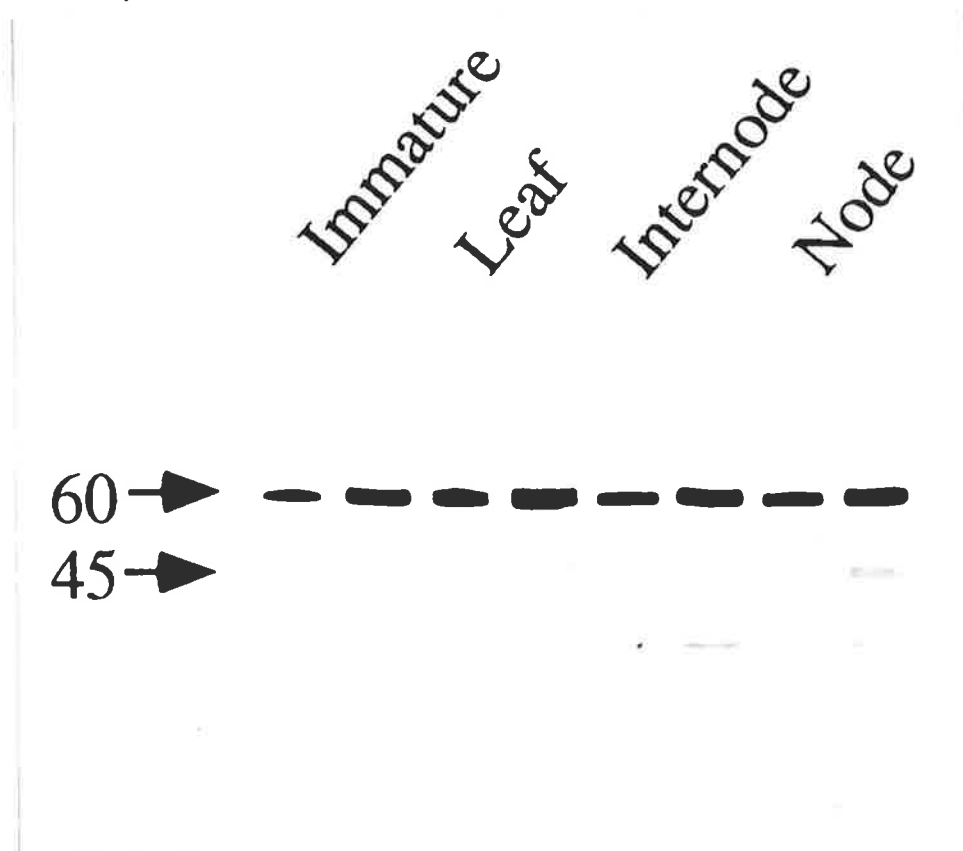


Figure 6.3. Western blot of extracts of immature sugarcane stem, the internodes and nodes of mature stem and sugarcane leaf. For each extract there are two lanes with the same units of activity loaded as for the activity stained gel (Figure 6.2).

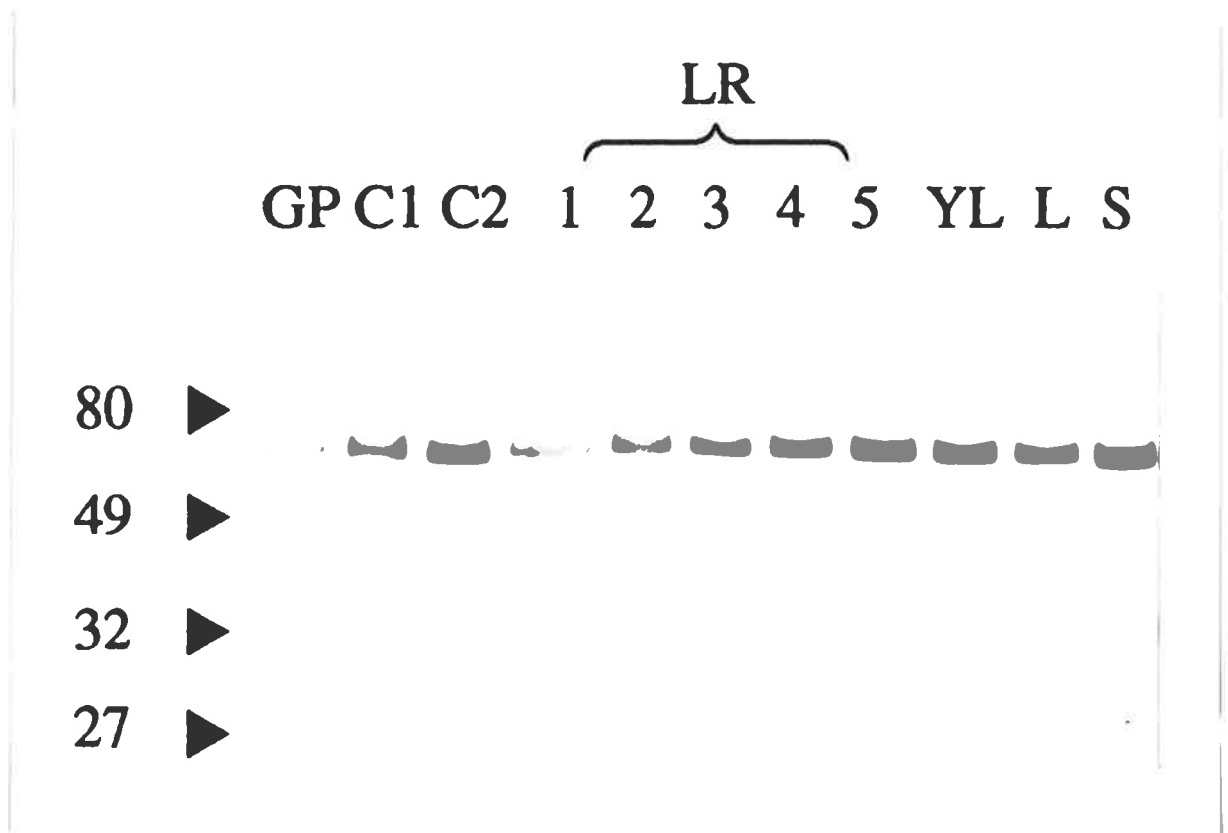


Figure 6.4. Immunoblot of extracts made from young sugarcane tissues containing high levels of PPO activity. GP-growing point; C1-stem tissue taken 1 cm below GP; C2-stem taken 2 cm below GP; leaf roll 1-5, (1)-the 5 cm immediately above the GP, (5)-40 cm above the GP; YL-young furled green leaf; L-D1 leaf lamina; S-D1 leaf sheath. Equivalent loadings were made on the basis of PPO activity.

Protease digestion

Crude protein extracts containing predominantly the 60 kD protein were subjected to proteolytic digestion by trypsin, thermolysin and Lys-C over a period of three hours and the products analysed by immunoblotting (Figure 6.6). There was almost complete conversion of the 60 kD protein to the 45 kD form in extracts digested with trypsin after one hour and with thermolysin and trypsin after three hours. There was also the appearance of a smaller antigenic band at around 20 kD in the extract digested with thermolysin. Aliquots were taken through the time course and PPO activity measured. PPO activity in the control extracts decreased over time (Figure 6.5) which was not unexpected as the enzyme was in an alkaline environment.

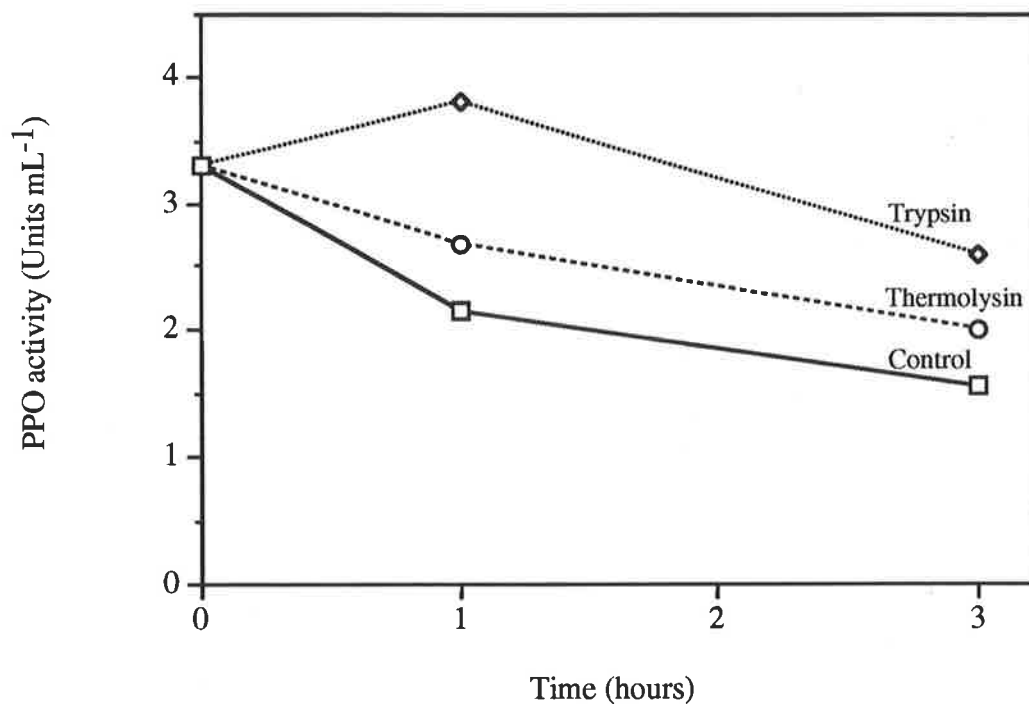


Figure 6.5. Effect of trypsin and thermolysin treatment on PPO activity in crude sugarcane extracts. Extracts were incubated with proteases as described in Figure 6.6 and samples were taken at times corresponding to those taken for western analysis.

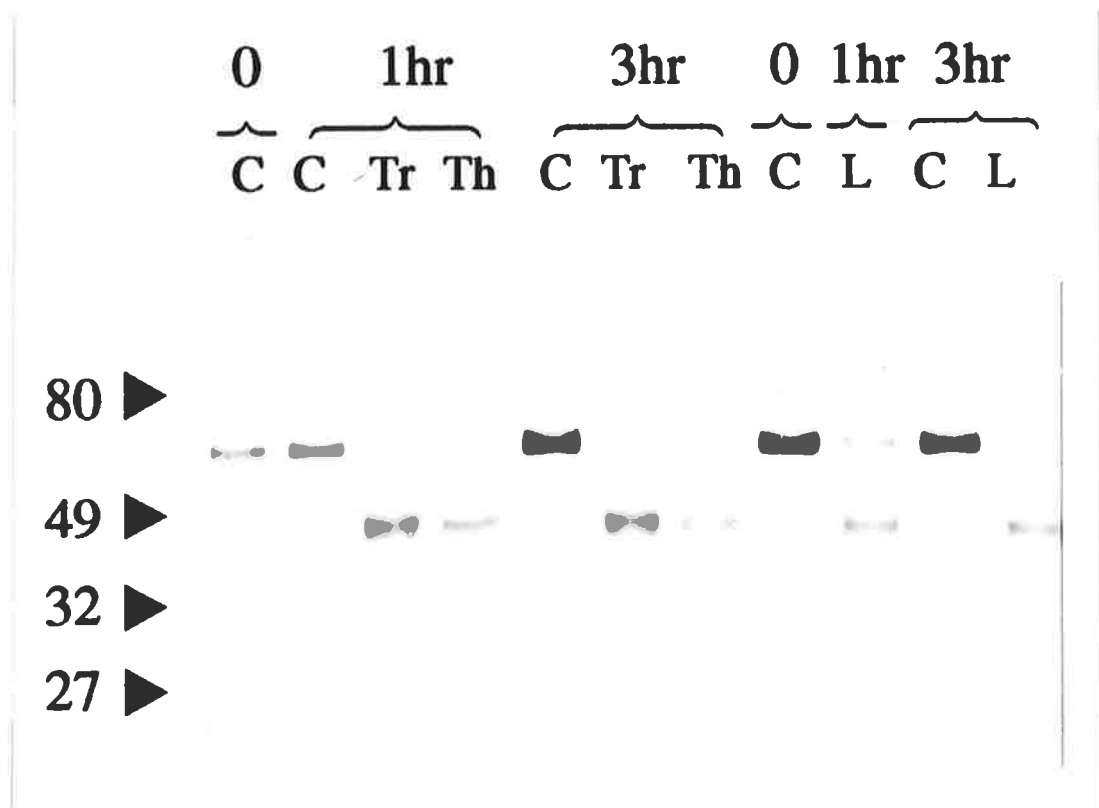


Figure 6.6 Western blot of proteolysis of PPO in sugarcane extracts. Crude extracts of Q87 were digested with $30 \mu\text{g mL}^{-1}$ of the proteases over a period of three hours. Samples were taken after one and three hours, denatured, and run on a 10% Tricine gel. Lane C is the control sample and the other lanes are samples from extracts treated with trypsin (Tr), thermolysin (Th) and Lys-C (L), taken at the times indicated.

After an one hour incubation with trypsin, PPO activity had increased relative to the control at time zero and remained greater than the control after three hours incubation. Similarly, activity in the thermolysin treated sample was higher than that measured in the controls after one and three hours. These results indicate that cleavage of the 60 kD protein to the 45 kD form did not result in loss of PPO activity, rather there was activation relative to the control.

Acid-mediated cleavage

Given the greater proportion of the 45 kD protein observed in the Method 2 purification (Chapter 5) where there was an acidic step early in the procedure, it was possible the 45 kD form was a breakdown product of the 60 kD form resulting from the acidic conditions. In order to determine whether this was the case, extracts were made at pH 7.5 in the presence and absence of protease inhibitors and a portion subsequently adjusted to pH 4. After incubation overnight, PPO activities were assayed and the antigenic proteins identified in a western blot. Activity was higher in all the extracts adjusted to pH 4 (Table 6.1). In the extracts at pH 7.5 the major band was at 60 kD, whilst at pH 4 most of the 60 kD form had been cleaved to the 45 kD form (Figure 6.7). In the extracts denatured immediately in the presence of SDS and then adjusted to pH 4 and incubated overnight, there was no cleavage to the 45 kD form. Where protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin, 100 μ M EDTA and 1 mM PMSF) were included in the extraction media, the same patterns of proteins were observed, thus the 60 kD form was still cleaved to the 45 kD form at pH 4 (data not shown).

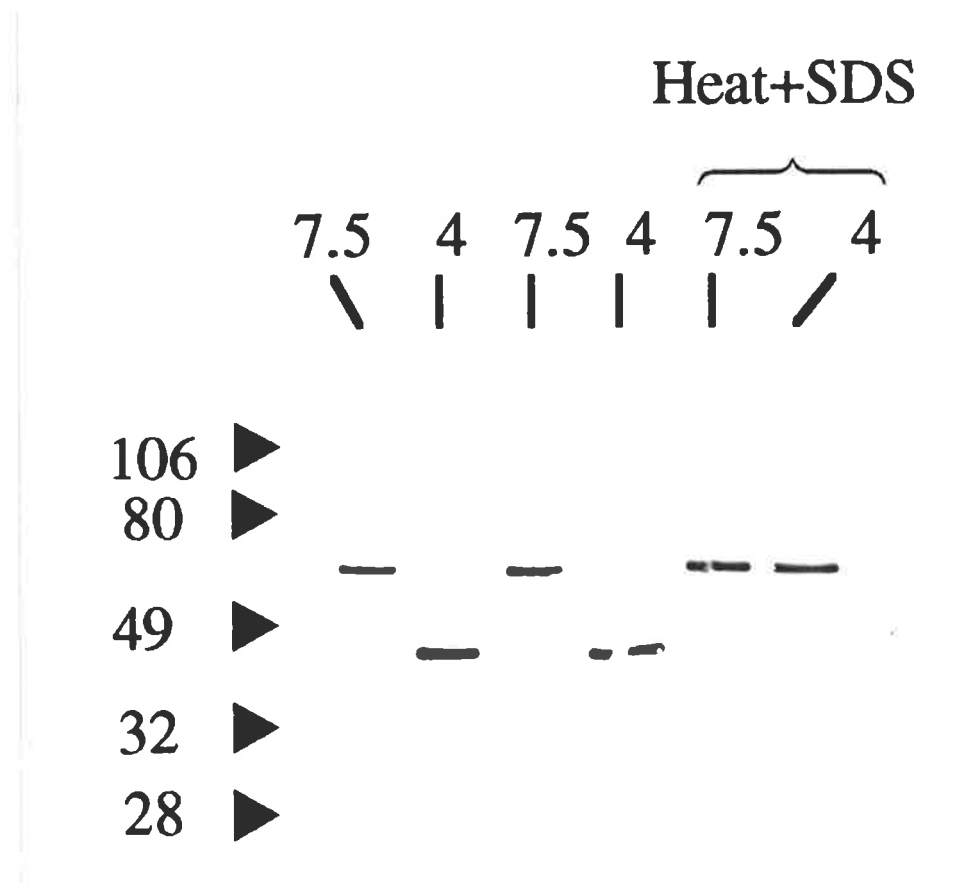


Figure 6.7. Immunoblot showing the effect of pH on the cleavage of the PPO protein. Two separate extracts were prepared at pH 7.5 and half of each subsequently adjusted to pH 4. Cane was also extracted at pH 7.5 at 100°C in the presence of 2% SDS and the pH then adjusted to 4. The extracts were incubated overnight on ice and subsequently electrophoresed. 5 μ L of each of the extracts was loaded.

Table 6.1. Activation of PPO activity in sugarcane extracts by incubation at low pH. Tissue was extracted at pH 7.5 and then adjusted to pH 4 and incubated overnight on ice. PPO activity was then assayed. Each value represents the mean and standard error of two measurements. The samples were subsequently electrophoresed, blotted and probed with the sugarcane antibody (Figure 6.7).

	PPO Activity (Units mL ⁻¹)
Extract 1 - pH 7.5	2.27 ± 0.02
- pH 4	3.88 ± 0.00
Extract 2 - pH 7.5	2.78 ± 0.03
- pH 4	4.47 ± 0.03

Active PPO can be detected by separating extracts on partially denaturing gels and staining with a phenolic substrate (Chapter 5). When preparations containing predominantly the 45 kD protein were run on gels under partially denaturing conditions and stained for PPO activity a discrete band was not detected. This inability to identify the 45 kD protein by staining for PPO activity was of concern, especially as the active 60 kD PPO could be readily identified as a single band on the same gels. Various preparations of partially purified PPO from different purification procedures were run on partially denaturing gels and stained for PPO activity and gel slices were excised from various positions within the smear of activity (Figure 6.8). The five tracks on the righthand gel show the activity staining pattern prior to excision. Lane A shows a large smear of activity of high molecular weight down to around 60 kD, but in the other tracks the 60 kD band is the major staining zone. In the lefthand gel the areas of gel excision in an identical gel can be seen. When these gel slices were re-run under fully denaturing conditions and analysed by western blotting, the 60 kD bands which were activity stained (5 and 6) showed the same mobility under fully denaturing conditions (Figure 6.9). Where gel slices were cut from the smear of lane A (Figure 6.8: 1, 3, 4 and 7) the 45 kD protein was the main band detected in the immunoblot although there were

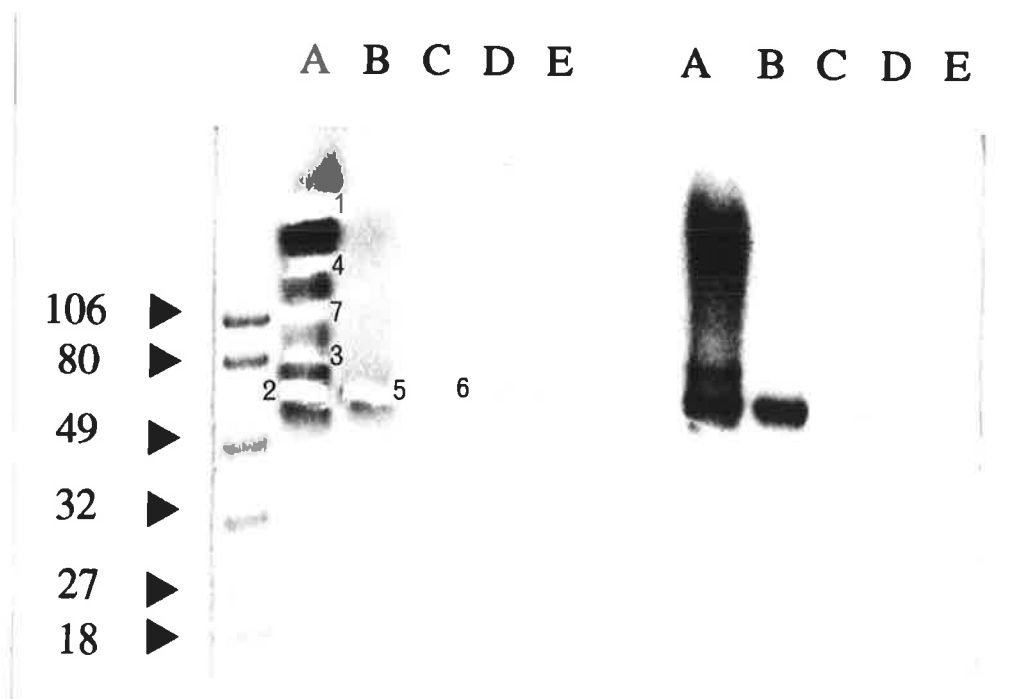


Figure 6.8. Partially denaturing Tricine gel of various partially purified extracts of sugarcane PPO stained for PPO activity. The two halves of the gel have the same protein loadings, the five lanes (A, B, C, D, E) on the right side show the activity stain before excision of the gel slices, as seen on the left side.

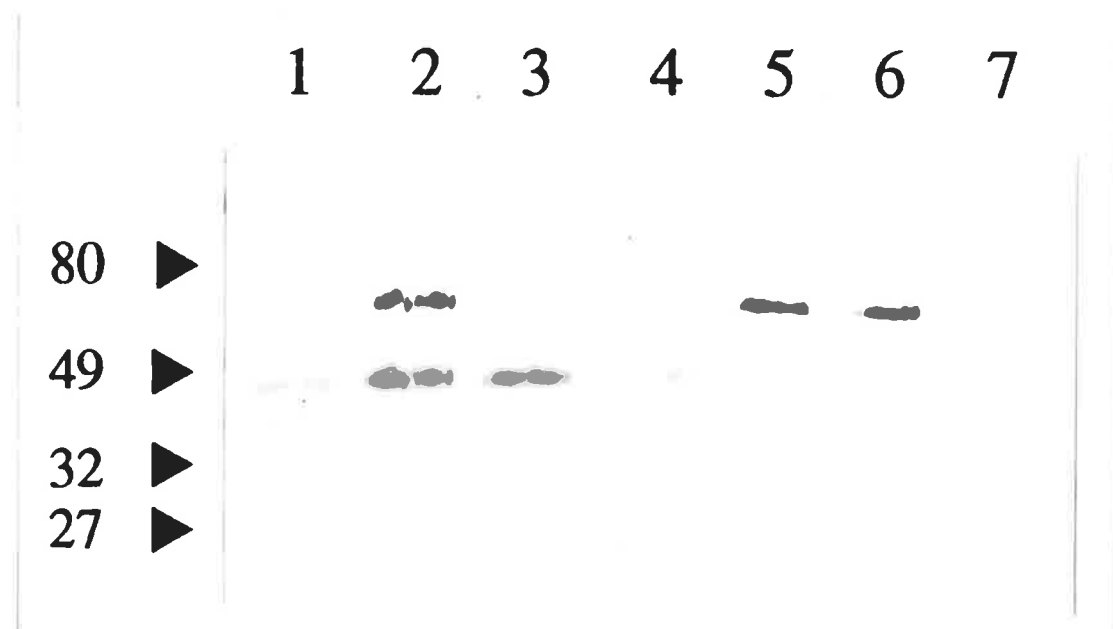


Figure 6.9. Immunoblot of the excised gel bands from an activity stained gel. The excised bands (Figure 6.8) were denatured and re-run on a 10% Tricine gel. The lane numbers indicate the position of the excised band in the activity stained gel (see Figure 6.8).

also low amounts of a 35 kD antigenic protein. Gel slice number 2, which was excised from around the 60 kD mark, contained both the 45 and 60 kD bands.

Cross-reactivity of sugarcane PPO antibody

The cross-reactivity of the sugarcane PPO antibody was tested against bean and grape extracts. The grape and bean antibodies were also tested for their cross-reactivity. The bean PPO antibody which was raised against a 60 kD protein did not recognize the sugarcane protein at all and detected the 40 and 60 kD forms in grape only weakly, with the band intensity about 50 times lower than with the broad bean PPO protein (Figure 6.10). The sugarcane PPO antibody detected the PPO protein in both bean and grape down to 5 ng of protein but the band intensity was again more than 50 times lower than that with the sugarcane PPO protein. The grape PPO antibody also cross-reacted with the PPO protein in bean and sugarcane, but the reactivity was stronger with the bean protein.

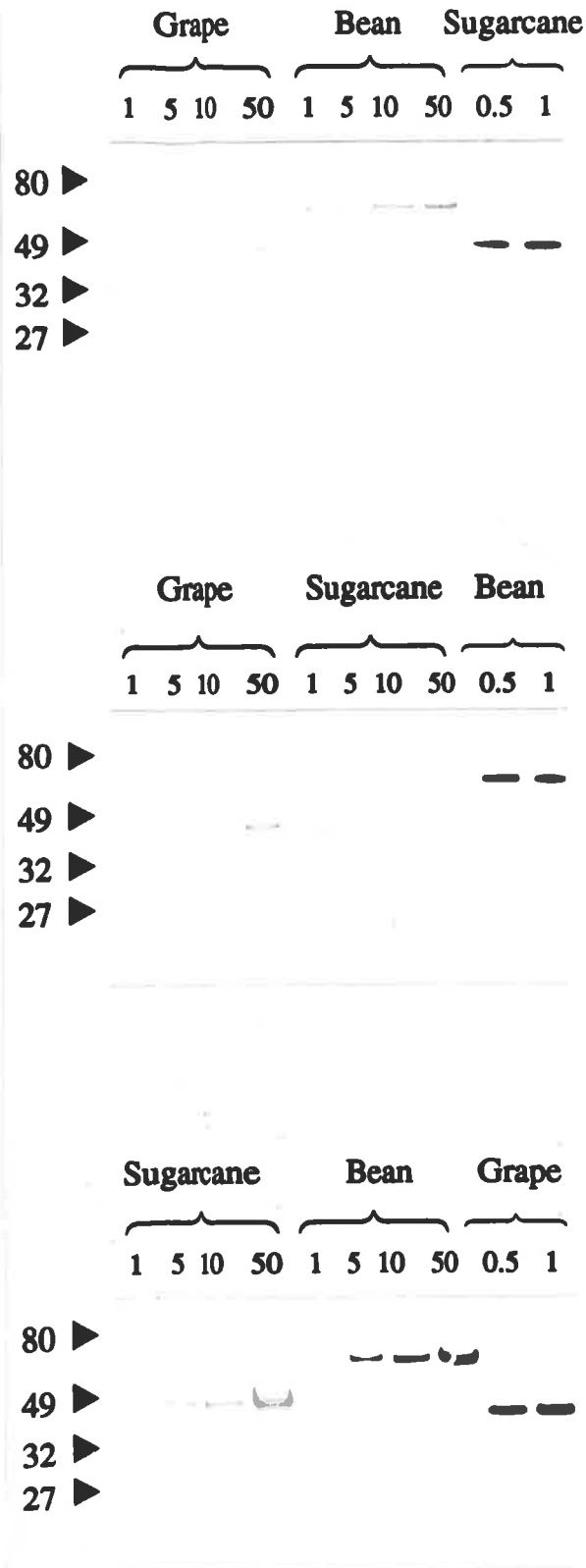


Figure 6.10. Comparison of the cross-reactivity of sugarcane, grape and broad bean antibodies. When tested against the epitope to which the antibody was raised, 0.5 and 1 ng of protein was loaded and 1, 5, 10 and 50 ng of the proteins isolated from the other species. The top blot was probed with the sugarcane PPO antibody, the middle blot with the broad bean PPO antibody and the bottom blot with the grape PPO antibody.

Discussion

In the previous chapter a 45 kD protein, believed to be polyphenol oxidase, was purified from sugarcane. A polyclonal antibody was subsequently raised to this protein. When sugarcane stem and leaf extracts prepared under acidic conditions were separated under fully denaturing conditions, blotted and probed with this antibody, the 45 kD protein band was identified in both extracts, as was the 60 kD PPO protein (Figure 6.1). In contrast, when a range of different tissues were extracted at pH 6.0 and probed with the sugarcane PPO antibody, the predominant band in all extracts was not the 45 kD protein but rather the activity staining 60 kD PPO (Figures 6.2 and 6.3). Attempts to inhibit PPO activity or precipitate the native enzyme with the antibody were unsuccessful (data not shown). This, however, was not unexpected as the antibody was raised against a denatured protein. Driouich *et al.* (1992) could not inhibit laccase activity or precipitate the native form of the enzyme with an antibody raised against the denatured protein.

When subjected to proteases the undenatured 60 kD PPO readily cleaved to the 45 kD peptide without loss of activity. Indeed there was an increase in activity relative to the control (Figure 6.5). Given the amino acid sequence of the peptides shown as in Chapter 5, it would appear the 45 kD peptide is at the N-terminal end of the 60 kD PPO and that cleavage occurs at the C-terminus. Unlike many other plant PPOs the activity of sugarcane PPO does not appear to be latent. However although it was not activated by SDS (Chapter 2), proteolytic cleavage of the 60 kD protein to the 45 kD form increased PPO activity (Figure 6.6). In contrast, broad bean PPO is latent, with activity increased several fold by the presence of SDS (Flurkey, 1986; Moore and Flurkey, 1990). Moore and Flurkey (1990) suggested that a minor conformational change occurs in the presence of SDS which leads to opening or unblocking of the active site. Robinson and Dry (1992) showed that proteolytic cleavage of the 60 kD PPO to the 42 kD form in broad bean was not required for activity, however cleavage at the C-terminus could occur without fully activating the enzyme. They suggested that the proteolytic cleavage may be a prerequisite to activation of PPO, as latency of the enzyme appears to be related to the secondary structure of the N-terminal 45 kD

region of the protein. In sugarcane, although PPO does not appear to be latent it may be that the C-terminus cleavage makes the active site more accessible. As there was no loss of activity with cleavage at the C-terminus it is clear that the cleaved peptide is not involved in catalytic activity of the enzyme.

This C-terminal cleavage could also be induced in sugarcane when the PPO was extracted under acidic conditions (Figure 6.7). As with the proteolytic digestion there was no loss of activity with cleavage (Table 6.1) and the measured activity was higher in the extracts left at pH 4 than in the extracts left at pH 7.5. This increase, however, most likely reflects the greater stability of the enzyme at acid pH than at more alkaline pH (Chapter 4). Interestingly, when the extract was denatured there was no cleavage of the 60 kD protein under acidic conditions. Rathjen and Robinson (1992a) also found in grape that when the 60 kD PPO was denatured it could not be cleaved with proteases. They suggested that the susceptibility of the 60 kD protein to cleavage was structurally conferred, perhaps in the form of a proteolytically-sensitive loop. It is possible a similar situation occurs in sugarcane, that with denaturation of the protein a cleavage sensitive loop in the native protein is lost. An alternative explanation is that an endogenous protease involved in the cleavage at low pH was inactivated by the denaturation. Cleavage however, still occurred under acidic conditions when protease inhibitors were included in the extraction buffer (data not shown). It is possible that the protease inhibitors used were not effective with the specific protease involved in the cleavage.

It appears that the purified 45 kD form arose as a result of the purification procedure when the protein was exposed to chromatography steps involving low pH conditions. The cleavage seems to be an *in vitro* process with no evidence for cleavage in the plant itself. In all cases where steps were taken to prevent proteolysis only the 60 kD form was detected. As discussed in Chapter 5, a 40-45 kD PPO has been isolated from a number of different plant species. It is possible that cleavage to the 40-45 kD forms may have occurred *in vivo* in these species, which suggests that cleavage of the C-terminal peptide in the plant serves some, as yet unknown, function. However, in other instances, where steps have been taken

to inhibit proteolysis during extraction of PPO as in broad bean and tomato (Robinson and Dry, 1992; Shahar *et al.*, 1992) and where adequate levels of reducing agents have been employed as in the isolation of apple and potato trichome PPOs (Kowalski *et al.*, 1992; Murata *et al.*, 1992) a 59-66 kD protein has been purified.

Because it is possible to demonstrate PPO activity in the presence of anionic detergents, partially denaturing activity stained gels have been routinely used in the detection of plant PPOs. In sugarcane, the 60 kD form is readily identified in the partially denaturing system as a discrete band at 60 kD. The 45 kD peptide, however, appears to run as an aggregate when stained for activity, showing apparent molecular masses larger than the expected 45 kD peptide (Figure 6.8) When gel fragments were excised from within this smear and re-run under fully denaturing conditions and probed with the antibody, the 45 kD band was observed (Figure 6.9). In this cleaved form, there appears to be a greater tendency for aggregation with itself and perhaps other proteins.

In many of the sugarcane preparations a band of 35-37 kD was detected by the antibody. Although the identity of this peptide was not fully determined it is possible that it too may be a product of cleavage of the 60 kD PPO. The presence of a similar sized protein excised from the activity stained smear (Figure 6.9) lends further support to the 35 kD being a PPO. In immunoblots of a range of higher plants probed with the broad bean polyclonal antibody Lanker *et al.* (1988) identified, in addition to the proteins with molecular masses of 60-63 and 43-45, a group of antigenic proteins at 34-36 kD. The antigenic proteins at 34-36 kD were detected after denaturing SDS-PAGE with no evidence for them existing in an active state. As yet, no PPO proteins of 30-35 kD MW have been purified and demonstrated to have PPO activity.

In addition to the polyclonal antibody raised to sugarcane, antibodies have now been raised to a number of different plant PPOs including broad bean (Hutcheson *et al.*, 1980), grape (Rathjen and Robinson, 1992a), apple (Murata *et al.*, 1993), tomato (Shahar *et al.*, 1992) and the trichomes of *Solanum berthaultii* (Kowalski *et al.*, 1992). Lanker *et al.* (1988)

showed the broad bean polyclonal anti-PPO to be quite cross-reactive, identifying PPO in eight other species. In apple, among the PPOs of six plant species tested, only pear PPO cross-reacted with the monoclonal apple PPO antibody indicating much greater specificity (Murata *et al.*, 1993). The sugarcane antibody detected both grape and bean PPOs down to 5 ng, the grape PPO antibody was similarly cross-reactive against the other two species while the broad bean antibody was the most specific, not recognising the sugarcane enzyme at all (Figure 6.10). The weak cross-reactivity of the three PPO antibodies seen here suggests that PPOs are not highly conserved in terms of their antigenicity.

As shown in Chapter 3, PPO activity is highest in young, actively growing tissues at the top of the sugarcane stalk, declining on a fresh weight basis down the stem. In all of the sugarcane tissues investigated the 60 kD form was the dominant band identified by the antibody. It was often observed that in tissues with high PPO activity the intensity of the immunostaining band was less than in tissues with lower activity (Figure 6.4). The most likely explanation for this observation is that in those tissues rich in PPO and phenolics, where browning is difficult to control, the PPO protein tans, affecting its antigenicity.

In summary, the evidence indicates that sugarcane PPO exists as a 60 kD protein *in vivo* but that it can be cleaved to the 45 kD form *in vitro* without loss of activity. It seems likely, given the similar observations made by Rathjen and Robinson (1992a) and Robinson and Dry (1992) and those described here, that the PPOs purified from other species as 40 to 45 kD proteins may also result from cleavage of a C-terminal peptide from a larger protein. Whether this cleavage occurs, as for sugarcane PPO as a consequence of extraction, or occurs *in vivo* as found for grape PPO (Rathjen and Robinson, 1992a), the functional role of the C-terminal peptide is yet to be resolved.

CHAPTER 7. ISOLATION OF THE STRUCTURAL GENE ENCODING SUGARCANE POLYPHENOL OXIDASE

Introduction

In Chapter 5 a 45 kD protein believed to be a PPO was purified and an antibody was raised to this protein. Subsequent studies showed that the 60 kD PPO could be cleaved *in vitro* to the 45 kD form (Chapter 6). This suggested that PPO exists as a 60 kD protein *in vivo*. Furthermore, all plant PPO genes isolated encode 56-62 kD mature peptides with no evidence for a gene encoding a 40-45 kD PPO. Isolation of a gene encoding PPO in sugarcane using the antibody raised to the 45 kD form of PPO would confirm its identity. In addition, isolation of the gene is the first step required in attempting to overcome the problem of browning by genetic manipulation. By decreasing expression of the PPO gene and thereby reducing enzymic browning, lighter coloured cane juice, and thus raw sugar could be obtained. With these objectives in mind, this chapter reports on the cloning and characterisation of a sugarcane PPO cDNA .

Materials and Methods

Plant material

For the cDNA library the growing points (first 1-1.5 cm below the apical meristem) of ten glasshouse grown stalks of cultivar Q87 (0.8-2.5 g each) were dissected over liquid nitrogen, frozen immediately in liquid nitrogen and stored at -70°C. For the northern blots, total RNA was extracted from glasshouse grown sugarcane (cultivar Q87), where stem above the point of attachment of leaf D1 was called the growing point (GP) and sections of stem below this point designated as numbered internodes.

Total RNA extraction and mRNA purification

Total RNA was isolated following a modification of a method from Dr Bob Bugos (USDA/HSPA). The frozen tissue (14 g) was ground to a fine powder in a coffee grinder cooled by liquid nitrogen. The powder was slowly added to a stirring beaker containing 45 mL of buffer (0.1 M Tris, (pH 9.0) 0.2 M NaCl, 15 mM EDTA, 0.5 % (w/v) Sarkosyl and 1% (v/v) β -mercaptoethanol), 45 mL phenol (equilibrated with 3 M sodium acetate pH 5.2) and 9 mL of chloroform:*iso*-amyl alcohol (24:1) and left to stir for five minutes. Three molar sodium acetate pH 5.2 (3.8 mL) was added and the extract left on ice for 15 minutes. The homogenate was then centrifuged at 16,000g for 15 minutes at 4°C. The upper aqueous phase was recovered, an equal volume of isopropanol added, and this was incubated at -70°C for 20 minutes. The precipitated RNA was collected by centrifugation at 10,000g for 10 minutes at 4°C, the pellet was washed with 70% (v/v) ethanol and centrifuged again at 10,000g for 5 minutes. The pellets were allowed to air-dry and then resuspended in RNase-free water. Any insoluble material was removed by centrifugation at 10,000g for 5 minutes. Lithium chloride was added to the supernatant to a concentration of 2 M and incubated overnight at 4°C. RNA was pelleted by centrifugation at 12,000g for 15 minutes at 4°C, washed with 70% (v/v) ethanol and resuspended in 600 μ L of RNase free water.

Purification of mRNA was carried out using a PolyAtract mRNA Isolation System (Promega), which utilises a biotinylated oligo(dT) primer able to hybridise to the poly-A⁺ of the mRNA. Following the manufacturer's instructions, 2.7 mg of total RNA in a final volume of 2.43 mL was heated to 65°C. For the annealing reaction, 10 μ L of the biotinylated-Oligo(dT) Probe and 60 μ L of 20xSSC were mixed with the RNA and left to slowly cool to room temperature. The cooled annealing reaction was then added to streptavidin-paramagnetic particles (SA-PMPs) and the mixture incubated at room temperature for ten minutes. The SA-PMPs were captured using a magnetic stand, washed four times with 0.1xSSC, and the mRNA eluted into RNase-free water. An equal volume of

isopropanol and 0.1 volume of 3 M sodium acetate was added and the mixture was left at -20°C overnight. The mRNA was pelleted by centrifugation, washed with 70% ethanol and left to air-dry. The pellet was then resuspended in 50 µL of RNase free water.

cDNA library construction

cDNA synthesis was carried out using the TimeSaver cDNA Kit (Pharmacia). Following the manufacturer's instructions, 3.7 µg of mRNA in a volume of 20 µL was heated to 65°C and immediately chilled on ice. To a First-Strand Reaction Mix, 1 µL of 0.2 M DTT, 1 µL of oligo(dT) and the heat-denatured mRNA was added and incubated for one hour at 37°C. The First Strand Reaction was then added to the Second-Strand Reaction Mix, incubated at 12°C for 30 min and then at 22°C for one hour. The reaction was heated to 65°C for ten minutes and after cooling to room temperature, extracted with 100 µL of phenol/chloroform (1:1). The upper phase containing the cDNAs was then purified on a Sepharose CL-4B spun column previously equilibrated with ligation buffer.

To ligate the *EcoRI/NotI* adaptors, 3.8 µL of *EcoRI/NotI* Adaptors, 30 µL of buffered polyethylene glycol solution, 1 µL 15 mM ATP and 1 µL T4 DNA Ligase were added to the column effluent, mixed and incubated at 16°C for one hour. The reaction was stopped by heating to 65°C for 10 min. The *EcoRI*-ended cDNAs were phosphorylated directly in the ligation mixture by the addition of 1.5 µL of 75 mM ATP and 1 µL of T4 Polynucleotide Kinase and incubated at 37°C for 30 minutes. The reaction was stopped by heating at 65°C for ten minutes, and extracted with phenol/chloroform. The excess and dimerised adaptors were removed using a second spun column. The column effluent containing the cDNAs was precipitated overnight at -20°C in two volumes of ethanol, 0.1 volume of 3 M sodium acetate and 5 µg mussel glycogen. After centrifugation at 30,000g for 30 minutes, the pellet was dried and resuspended in 10 µL of sterile water. The concentration of cDNA was determined using ethidium bromide plates, where the concentration of cDNA was visualized against

known standards. The cDNA was ligated into Lambda ZAP[®]II vector (Stratagene) according to manufacturers instructions and packaged directly into Gigapack[™] II Gold packaging extract (Stratagene). To determine recombination efficiency 1 μ L of the library was plated on *E. coli* SURE[®]Strain (Stratagene) and the number of white plaques counted.

In vivo excision of pBluescript

The ExAssist[™]/SOLR[™] system (Stratagene) was used to excise pBluescript from the Lambda ZAPII vector. The plaque of interest was cored as an agar plug using a sterile pasteur pipette and added to 500 μ L of SM buffer and 20 μ L of chloroform. The phage particles were released by vortexing and incubation overnight at 4°C. Phage stock (100 μ L) was combined with 200 μ L of XL1-Blue cells (OD₆₀₀=1.0) and 1 μ L of Exassist helper phage and the mixture incubated at 37°C for 15 minutes to allow phage adsorption. Three mL of 2xYT media was added and incubated with shaking for a further three hours. The tube was then heated at 70°C for 20 minutes and centrifuged at 4,000g for 15 minutes to pellet the cellular debris. The supernatant containing both helper phage and excised phagemid was used to infect SOLR host cells (OD₆₀₀=1.0). The helper phage contains an amber mutation which prevents its replication in non-suppressing host strains such as SOLR cells. The infected SOLR cells were incubated at 37°C for 15 min and 100 μ L plated on media containing ampicillin to select for bacteria containing pBluescript.

Plating and screening the cDNA library

XL1-Blue cells were grown in LB+0.2% maltose +10mM MgSO₄.7H₂O until they reached an OD₆₀₀ of approximately 0.5. Diluted phage stocks were added to 350 μ L of the prepared cells and incubated for 15 minutes at 37°C. Top agar equilibrated at 50°C was added to the cultures and immediately poured onto NZY plates pre-warmed to 37°C. The plates were incubated at 42°C for three to four hours. The phage stock was plated at a dilution of

approximately 8000 plaques per plate onto 150 mm plates as described above, and incubated upside down at 42°C for three to four hours until cell lysis began to occur. IPTG-treated filters were then placed on the plates and left overnight at 37°C. The filters were screened with the anti-sugarcane PPO as described in Chapter 6, except that *E. coli* extract (Promega) for background reduction was added to the primary antibody at a ratio of 1:100 and incubated at 37°C for 30 minutes prior to screening of the filters .

Putative positives were cored with the 'wide' end of a sterile pasteur pipette into 0.5 mL SM buffer and 10 µL of chloroform, vortexed and incubated overnight at 4°C. Five and 50 µL of a 1:100 dilution of the phage mix from the first round screening was plated as above and screened with the anti-sugarcane PPO. The positives were cored with the 'narrow' end of a sterile pasteur pipette and incubated in SM buffer and chloroform. For the third round screening 1 µL of a 1:100 and 1:1000 dilution was plated. Positives were cored and excised following the ExAssist™/SOLR™ protocol as described above.

Bacterial growth media

NZY Broth: 0.5% NaCl, 0.2% MgSO₄·7H₂O, 0.5% Yeast extract, 1% casein hydrolysate, pH 7.5

2X YT Broth: 1% NaCl, 1% Yeast extract, 1.6% Bacto-Tryptone

LB Broth: 1% NaCl, 1% Bactotryptone, 0.5% Yeast Extract

SM Buffer: 0.2% MgSO₄·7H₂O, 50 mM Tris-HCl, 0.01% gelatin, 100 mM NaCl, pH 7.5

Top Agar: NZY Broth with 0.7% agar

NZY Plates: NZY Broth with 1.5% Agar

LB plates: LB Broth with 1.5% Agar

2X YT Broth: 1% NaCl, 1% Yeast extract, 1.6% Bacto-Tryptone

Extraction of plasmid DNA

Plasmid DNA was isolated using either the Boiling miniprep method described in Holmes and Quigley (1981) or using the QIAGEN plasmid kit (Diagen) according to the manufacturer's instructions.

Northern Blot analysis

RNA was extracted from various tissues as described above. Total RNA samples were resolved in a 1.2% agarose gel containing 8% formaldehyde in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) as described by Sambrook (1989). RNA markers (Promega) were loaded to allow sizing of the transcript after staining with ethidium bromide. Total RNA (10 µg per lane) was mixed with three volumes of load buffer (500 µL deionised formamide, 190 µL formaldehyde(40%), 100 µL x10 Northern buffer and 0.2% bromophenol blue), heated at 65°C for ten minutes and chilled on ice prior to loading. Following electrophoresis the separated RNA was transferred overnight onto Zeta-Probe membrane (Biorad) by alkaline capillary transfer using 50 mM NaOH. The membrane was then rinsed in 2xSSC (150 mM NaCl, 150 mM sodium citrate pH 7.0) and allowed to air-dry.

A 900 bp *Bam*HI/*Hind* II cut fragment of the SUGPPO1 clone was used as a probe. The digested fragment was run on a 2% Nusieve (FMC Bioproducts) gel and the gel fragment excised using a sterile scalpel blade and purified using a QIAEX kit (Diagen) according to the manufacturer's instructions. The DNA (200 ng) was labelled using a GIGAprime DNA labelling kit (Bresatec) using 50 µCi of α -³²P-dATP according to the manufacturer's instructions. After incubation at 37°C for 30 minutes, 1 µL of EDTA, 15 µL of TE and 5 µL of Blue Dextran were added. Labelled DNA was separated from unincorporated nucleotides using 1 mL Sephadex G-50 columns equilibrated with TE, the labelled DNA eluting from the column with the Blue Dextran.

Hybridization was carried out in a Hybaid™ mini hybridisation oven. The membranes were initially incubated in 5 mL of prehybridisation solution (250 mM Na₂HPO₄ (pH 7.0), 7% SDS, 1 mM EDTA) at 65°C for at least one hour. The DNA probe was denatured at 100°C for five minutes and cooled on ice, before addition to the prehybridisation mix. Hybridisation was carried out overnight at 65°C. Following hybridisation the membrane was washed for 15 minutes at room temperature in 2xSSC, 0.1% SDS followed by two washes in 0.1xSSC, 0.1% SDS for ten minutes at 65°C.

Subcloning

Specific DNA fragments were separated after digestion on a 1.5% Nusieve low melting point agarose gel. Excised gel slices were melted at 68°C for ten minutes and placed at 37°C. To 7 µL of molten agarose was added 4 µL of sterile water (at 37°C), 1.5 µL of Boehringer 10x Ligase Buffer (660 mM Tris-HCl, 50 mM MgCl₂, 10 mM DTT, 10 mM ATP, pH 7.5), 1.5 µL Boehringer T4 DNA Ligase and 1 µL of vector (pBluescript SK⁺ or pGEM3Zf), mixed and left at 37°C for 30 seconds, before incubation at room temperature overnight. Ligation mixtures were transformed into *E. coli* strain DH5α by electroporation. The ligation mixes were heated at 68°C for five minutes and then placed at 37°C. To 1 µL of the ligation, 40 µL of competent cells was added, mixed and transferred into a pre-chilled 0.1 cm electroporation cuvette (Biorad). The cuvette was pulsed at 1.8 kV and then 800 µL of LB added to the cuvette to remove the transformed cells. The cells were incubated at 37°C for one hour and then plated onto solid media, with ampicillin selection.

DNA sequencing

Double-stranded plasmid DNA templates were sequenced using the di-deoxy chain termination method (Sanger *et al.*, 1977). The sequences were read using an Applied Biosystems 373A DNA sequencer. SUGPPO1 was fully sequenced using subclones and

oligonucleotide primers as shown in Figure 7.2. The oligonucleotides were synthesised by Bresatec Ltd and purified on OPC columns (Applied Biosystems) following the manufacturer's instructions.

Results

PCR

Degenerate oligonucleotide primers designed to conserved regions identified in sequenced plant PPO genes have been used successfully to amplify and isolate PPO genes from a range of plant species including lettuce, potato, bean, apple and grape (Dr Simon Robinson, CSIRO Division of Horticulture, unpublished data). A similar approach was tested using sugarcane mRNA from growing point tissue. First-strand cDNA was synthesised and PCR was carried out with a number of different degenerate primers. None of the primer combinations produced a DNA species of the predicted size. To check the quality of the first-strand cDNA, degenerate primers designed to conserved regions of various plant invertases (Dr. Chris Davies, CSIRO Division of Horticulture, unpublished data) were used to amplify an internal fragment (570 bp) of sugarcane invertase. This fragment was cloned and sequenced and shown to encode sugarcane invertase (data not shown). It appeared therefore that the cDNA was intact, but that the PPO oligonucleotide primers were unable to anneal to the sugarcane PPO transcript. As a result, a different strategy for cloning PPO was utilised, involving antibody screening of a cDNA expression library.

Screening the cDNA library

The λ ZAPII expression library constructed from Q87 growing point mRNA was screened with the sugarcane PPO antibody. Approximately 40,000 primary plaques were screened and 14 plaques remained positive after the tertiary screen. *Eco*RI restriction digestion of

phagemid DNA from the purified phage clones showed the inserts to range in size from 600bp to 4.0 kb. However, eight of the 14 clones had inserts of around 2.0 kb which approximated the predicted size of a full-length PPO cDNA. Two of the clones (1A and 6A) had inserts of 4 and 3.5 kb respectively. To determine the relatedness of the clones, α -³²P labelled clone 8A was used to probe a blot of a *Hind* II restriction digest of the remaining clones. All of the clones, except 3A, cross-hybridised strongly with the probe at high stringency (data not shown). Subsequent restriction endonuclease mapping of the clones showed the relatedness of all but one of the clones (3A) and suggested that clones 1A and 6A were concatemeric. Of the other clones, only 11A differed significantly, having no *Kpn*I sites (compare with SUGPPO1; see Figure 7.1) and an additional *Hind* II site. Furthermore, digestion of 11A with *Rsa*I gave a very different restriction pattern to that seen with the other clones (data not shown). One clone SUGPPO1 (2209 bp) was fully sequenced and partial nucleotide sequence was obtained from the 5' end of many of the other clones, identified by the bold arrows (see Figure 7.1). Comparison of the limited 5' sequence of these clones with the SUGPPO1 sequence and restriction endonuclease mapping (Figure 7.1) indicated a number of the clones (7A, 8A, 9A, 14A and 13A) were shorter versions of the full length (SUGPPO1) clone. Other sequenced clones (4A, 5A and 10A) aligned with SUGPPO1 and showed similar restriction endonuclease sites but were longer at the 3' end, indicating either a 3' attachment of unrelated DNA or the presence of a long poly-A⁺ tail. The limited nucleotide sequence obtained for 11A confirmed that it is quite different to SUGPPO1.

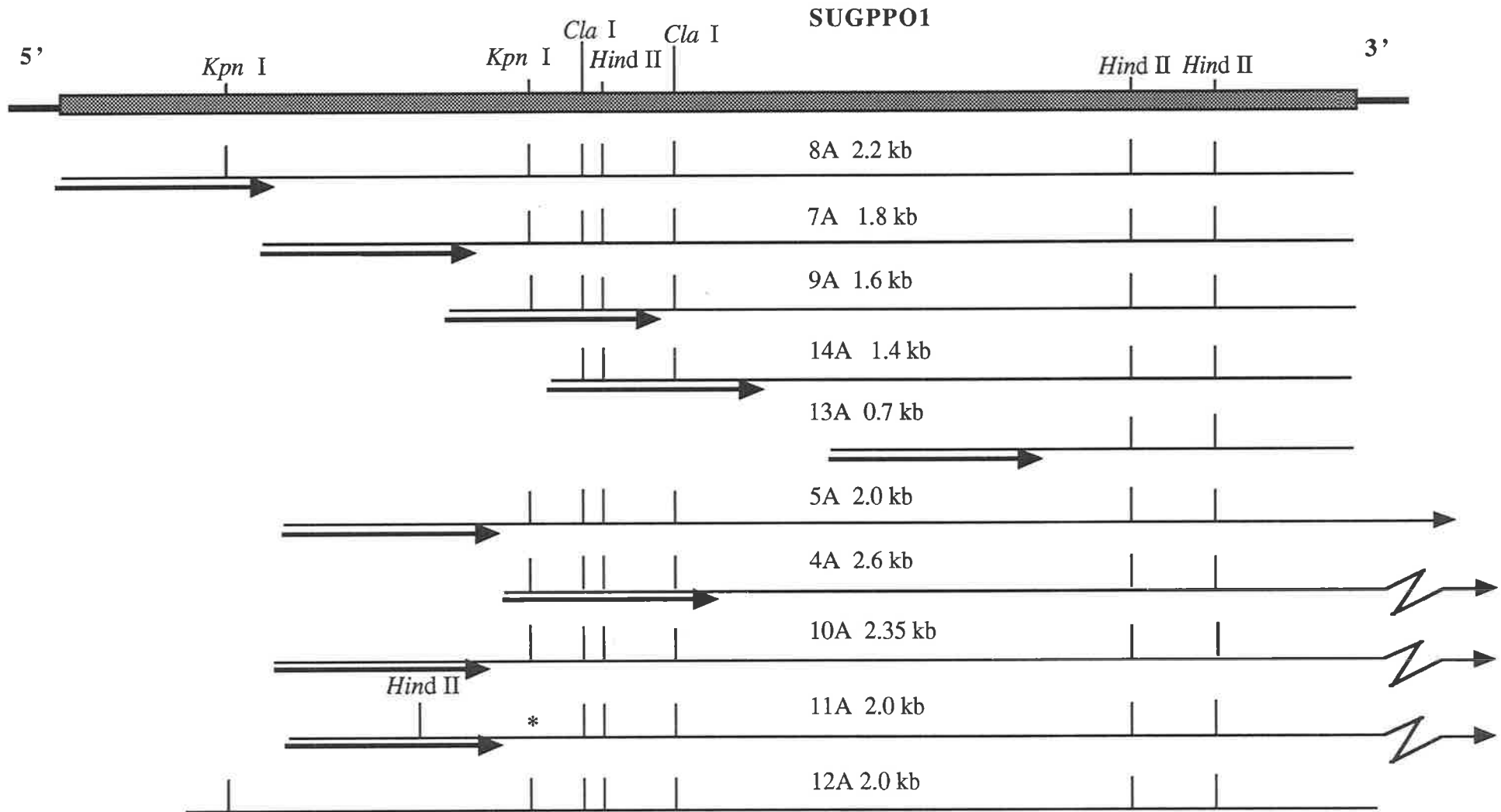


Figure 7.1 Comparison of sugarcane PPO clones using restriction mapping. The limited 5' sequence obtained for the various clones are indicated by the bold arrows and the inserts are positioned relative to sequence obtained for SUGPPO1. Note the presence of an extra *Hind* II site and the absence of a *Kpn* I site (*) in clone 11A. 1A and 6A were not sequenced because of the large size of the inserts. Sequence was not obtained for 12A (2.0 kb) however restriction mapping suggested it was similar to SUGPPO1.

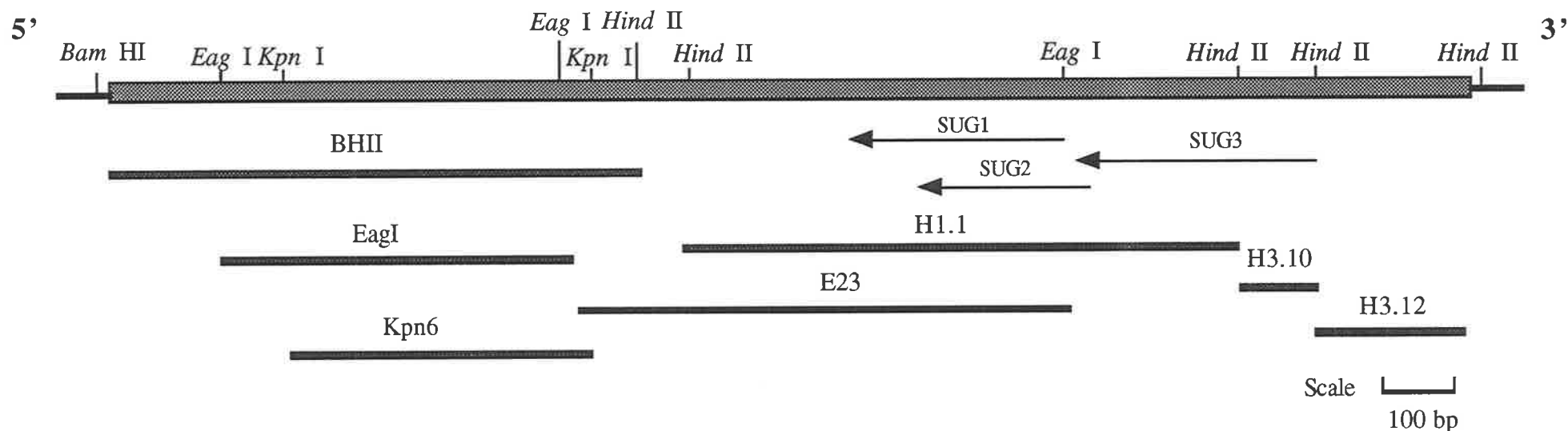


Figure 7.2 Subcloning strategy for sequencing SUGPPO1

This figure shows a restriction map of the SUGPPO cDNA (2209 bp). The sites were used in the construction of subclones as shown above. Sequence obtained using the oligonucleotides SUG1, SUG2 and SUG3 are also indicated. *Bam* HI and *Hind* II sites in the polylinker sequence of the cloning vector were used in the construction of the subclones.

The ten subclones generated were as follows:

- pBHII 870 bp (approx.) *Bam* HI/*Hind* II fragment in pBluescript SK+
- pEagI 580 bp *Eag* I fragment in pBluescript SK+
- pKpn6 505 bp *Kpn* I fragment in pGEM-3Z
- pH1.1 888 bp *Hind* II fragment in pBluescript SK+
- pE23 792 bp *Eag* I fragment in pBluescript SK+
- pH3.10 131 bp *Hind* II fragment in pBluescript SK+
- pH3.12 250 bp (approx.) *Hind* II fragment in pBluescript SK+

Characterisation of SUGPPO1

The sequencing strategy of SUGPPO1 is shown in Figure 7.2, which indicates the subclones that were made and the oligonucleotide primers that were used. Figure 7.3 shows the nucleotide and deduced amino acid sequences of the 2209 bp SUGPPO1. Consistent with genes isolated from other monocots (Campbell and Gowri, 1990) sugarcane PPO has a high G+C content (66%). In comparison the G+C content of the potato tuber cDNA is only 42%. Analysis of the nucleotide sequence indicated the presence of two methionine residues which could potentially act as the site of initiation of translation. In the alignment of the sugarcane PPO protein sequence with other plant PPO protein sequences (Figure 7.4) the amino acids M*S are highly conserved at the start of the protein. In both grape and potato tuber PPO, the start sequence is MAS, which is consistent with the sequence following the second methionine in the translation of SUGPPO1. The open reading frame encodes a protein of 615 amino acids with a calculated molecular mass of 67066. From the N-terminal amino acid sequence of the 60 and 45 kD sugarcane PPO proteins (ADSS, Chapter 5), a putative transit peptide was identified upstream of the mature PPO protein. This peptide encodes 80 residues with a predicted molecular weight of 8063 and isoelectric point of 11.79. The mature PPO protein, therefore, is 535 residues in length with a predicted molecular weight of 59021 and an isoelectric point of 6.96.

*

1 TCAAATCCAGACCGCTGCAAATATCACGTAGTCTCGTAGACATGAACGGCTCCATGGCG
M N G S M A

60 AGCGCCTGCGCCACCTCCAGCCCCCTCGTCTCGGGCGCCCTCTGCATGCCCGTCCAAGAAG
S A C A T S S P L V S A P S A C P S K K

120 ACCTCCGCCGCCAGGTTCCGGCGCCGACGTTCTCGTGCAGGGCCACCGGCGGGAGGC
T S A A R F R R R T F S C R A T G G G G

180 GGCGGCCGGGCAACAATGACGGCTTCTTGTGGCTGCCTCGGCGGGACGTGATGCTCAGC
G G R G N N D G F L W L P R R D V M L S

240 GGCCTGAGCGGTGTCGCCGCCGGGCTCGCCTGGTACCCGGGCCTCGCGTCTGGTGC GGAT
G L S G V A A G L A W Y P G L A S G A D

300 TCGTCTGCTTCGCCGTGCACGACGGCGGACAAGGTGAACGAGAAGATCTTGCTGTGCGCG
S S S S P C T T A D K V N E K I L L C A

360 GACACGGACAAGCAGAAGCCCTGCCCTCTTGTGTGCGCCGGCGGCCCCCGTGGACTTCACG
D T D K Q K P C P L V S P A A P V D F T

420 CCGGAAGGCAAGGTGACGCGCGTCCGGCAGCCCGTGCATCTCCTGAGCCGGAATACCAG
P E G K V T R V R Q P V H L L S R E Y Q

480 GAGAAGTACAAGGAGGCCGTCCGGGAAGATGAAGGCGCTGCCGGAGTCGAATCCGCTGAGC
E K Y K E A V G K M K A L P E S N P L S

540 TTCAAGGCGCAGGCGGCCATCCACCAGGCTTACTGCGACA ACTACTACAAGTACCACAAG
F K A Q A A I H Q A Y C D N Y Y K Y H K

600 TCATCCGGCTCGACGGTGGCCAAGGACGACCCGGCGTTCGACGTGCACTACTCGTGGATC
S S G S T V A K D D P A F D V H Y S W I

660 TTCGCGCCGTGGCACCGCATGTACATCTACTTCTACGAGCGTGCCCTCGGCGACCTCATC
F A P W H R M Y I Y F Y E R A L G D L I

720 GGCGACAAAACCTTCGCGCTGCCGTTCTGGAGCTGGGACGCGCCGGCCGGCATGGTGGTA
G D K T F A L P F W S W D A P A G M V V

780 CCGGCTCTCTTCAAGGAAGCCTTTGCCAACCCGCTGTACGACCCCAACCGGAACACGGCG
P A L F K E A F A N P L Y D P N R N T A

840 AACCTCAACGCGCTGGTTCGACCTAGACTACCTCAGCCACAGGGATGCGAAGCCCATCGAT
N L N A L V D L D Y L S H R D A K P I D

900 TTCAAGGGCCAAAGGACGAAAAATACAAGGA ACTTGTTAACAAGAACCTGTGCACCGTA
F K G P K D E K Y K E L V N K N L C T V

960 TACAACCAGCAAGTGCCTAAGGGCCCGGAGTCGTTCTGGGCGAGAAGTTCTGCACCGCC
Y N Q Q V R K G P E S F L G E K F C T A

1020 ATCGATGGCACCAGCGGCATGGGTTTCGCTGGAGCGGATGGCGCACACCGCCATGCACGTC
I D G T S G M G S L E R M A H T A M H V

1080 TGGTTCGGCAAGGCGGGCGGAAGCCTTGCACGCGCCGCGCCGGCGGGCGTCTGAGCCAC
W V G K A G A K P C D A A A G G V L S H

```

1140  AAGGATAATGGCGCGTTCAACTGCAACAACGACATGGGGTTCCTGGGGTCGGTCGGGAAC
      K D N G A F N C N N D M G F L G S V G N
1200  GACCCACTCTTCTACTCACACCACTCCAACGTGGACCGCATGTGGCACCTCTGGTCCACC
      D P L F Y S H H S N V D R M W H L W S T
1260  AGGATGGGCGGCGGGCAGGGCATCACGGACCCGGACTGGCTGGACGCCAGCTTCGTCTTC
      R M G G G Q G I T D P D W L D A S F V F
1320  TACGACGACGTCAAGAGCCCCGCGAAGGTGCGCATCAAGTTCCGCGACGTGCGTGGACACG
      Y D D V K S P R K V R I K F R D V V D T
1380  CGCGACCTCGGCTACACGTACGACCCCCGAGTACGACAGGGACCTGCCGTGGCTGCGGCCC
      R D L G Y T Y D P E Y D R D L P W L R P
1440  AAGATCACGACGCTGGTGCCCCACGGCAAGGACAGCGGCGGCGGCGGCGAGGTCGTGCG
      K I T T L V P H G K D S G G A A A R S S
1500  GCGGCGCCCGCGGTGTTCCCGCTCGCTCTGACCAAGGGCCAGGTCGTGGAGGTGCCGGCC
      A A P P V F P L A L T K G Q V V E V P A
1560  GTGGCCGTGCCGGCCAGGGAGGCGGGGAAGGAACAGCTGCTGGTGATCGACGGCATCGAC
      V A V P A R E A G K E Q L L V I D G I D
1620  TTCGACCCGCGAGGCGAACAACAAGTTCGACGTGCCATCAACCTGCCCGCGGACAAGGCG
      F D P Q A N N K F D V A I N L P A D K A
1680  CTGCTGGTGGGGCCGAGTACAAGGAGTACCCGGGAGCTTCGCCGTGCTGCCGGGCTCC
      L L V G P Q Y K E Y A G S F A V V P G S
1740  GGGGCCGCGAGACGCGGAAGGGGAAGGTCTCCCTCTGCATCACCGACGTGCTGTACGAC
      G A G E T R K G K V S L C I T D V L Y D
1800  CTCGACGCGGAGGACGATAGCACCGTCGACGTCTTATCGTGCCGCGCACCAATGCAAAG
      L D A E D D S T V D V L I V P R T N A K
1860  GTGACGATCAACGTTCCGCCCCACCATCAAGAACCGCAAGTAGCTAAGCTAGCTACTGGAG
      V T I N V R P T I K N R K *
1920  TAGTACTCGGTTCGCGACGATTGTGTGGTCAATGACGTGACCATCGTTTCATGCACAGCT
1980  GATTGCGACACCGGGTGTCCGCAAGAATAAACTTATTGTAGTACCAGCTGATTGCATTGA
2040  GTGGTTTATATTAGGAAAGAGCCCATGGTAGCTGCTGTTGGAGCAGGCCTTGGTCCGGGT
2100  GCAGAATCAGTTCGAGACTGCATTCCAAATTGTATTTCAGACCTTGTGGCCAATGATTTG
2160  GCCGCTGTTTCTCAGTAATGGAGTTCCACTTCCATGTTAAAAAAAAAAAAA  2209

```

Figure 7.3. Nucleotide and deduced amino acid sequence of SUGPPO1. The putative site of initiation of translation is indicated by (*). The N-terminal amino acid sequence of the purified 45 kD sugarcane PPO protein is underlined indicating the start of the mature peptide.

Alignment of SUGPPO1 with deduced amino acid sequences of PPO cDNAs isolated from grape berry (Dry and Robinson, 1994), bean leaf (Cary *et al.*, 1992), apple (Boss *et al.*, 1995), tomato leaf (Shahar *et al.*, 1992) and potato tuber (Dr Peter Thygesen, CSIRO Division of Horticulture, unpublished data) is shown in Figure 7.4. Within the putative transit peptides there is little homology other than a region between residue numbers 81 and 94. The greatest sequence homology was present in the two putative copper binding domains designated CuA and CuB with the histidine residues highly conserved (Figure 7.5). The CuA domain is highly conserved across the species whereas the CuB domain is not (Steffens *et al.*, 1994). In the CuA domain sugarcane PPO is the only plant PPO to have a tryptophan (W) instead of a phenylalanine (F) preceding the second histidine residue. In this instance, sugarcane PPO is like the tyrosinases in that a tryptophan (W) at this position is highly conserved in tyrosinases (Steffens *et al.*, 1994). In the CuB domain both the sugarcane and potato tuber proteins have a large insertion between the two groups of histidine residues which decreases the overall consensus. The greatest sequence conservation in the CuB domain is around the third histidine residue (Figure 7.5).

	1	Transit peptide	70
potato leaf	...SSSSTTTIPLCTNKSLSSSFTTNNSSFLS...	KPSQLFLHGRRNQSF...	KVSCNANNVGEHDKNL
tomato leaf	MSSSSSITTTPLCTNKSLSSSFTTNNSSLLS...	KPSQLFLHGRRNQSF...	KVSCNANN...VDKNP
potato tuber	...MASLCNSS...STSLKTPFTSSSTLSLSS...	TPKPSQLFIHGKRNQMF...	KVSCKVTNNNGDQONQV
apple	...MTSLSP.PVVTTPTVNPATKPLS...	PFSQNNQVSLLTTPKRSFAR.	KVSKATNNDQNDQAQS
grape	...MASL.PWSLTTSTAIANTTINISAFPPSPFLQ	RASHVPVARNRSRRFAPS	KVSCNSANGDPNSDSTS
sugarcane	...MASACAT.....	SSPLVSAPSACPSKKTSAARFR	RRTFSCRATGGGGGGRGNN
Consensus	-----		
	71	Mature peptide	140
potato leaf	D....T..VDRRNVLLGLGGLYGAAN...LAPL.	ASASPIPPDLKSC.	GVAHVTEGVDVT.YSCCPPV
tomato leaf	D....A..VDRRNVLLGLGGLYGAAN...LAPL.	ATAAPIPPDLKSC.	GTAHVKEGVDVI.YSCCPPV
potato tuber	E....TNSVDRRNVLLGLGGLYGVAN...AIPLAASA	APPPDLSSC.	SIARINENQVVP.YSCCAPK
apple	KL.....DRRNVLLGLGGLYGVAG..MGTD	PFAPFAKPIAPPVSKC.	GPADLPQGAVPT..NCCPPP
bean	ELSNIVG...HRRNVLIGLGGIYG..T..LATN	PSALASPIPPDLKSCVPPSDLP	SGTTPPNINCCPPY
grape	DVRETSSGKLDRRNVLLGIGGLYGAAGGLGATK	PLAFGAPIQAPDISK.	GTATVPDGVPTP..NCCPPV
sugarcane	D....GFLWLPRRDVM..LSGLSGVAAGLAWY	PGLASGADSSS...	SPECTADKVNEKILLCADTKQKP
Consensus	-----RR-V-----G--G-----A-----C-----		
	141		210
potato leaf	PDDIDSVPYKFPF.MTKLRIRPPAHAADDEEYVAKY	QLATSRMRELDK.	DSFDPLGFKQQANIHCAYCNG
tomato leaf	PDDIDSVPYKFPS.MTKLRIRPPAHAADDEEYVAKY	QLATSRMRELDK.	DPFDPLGFKQQANIHCAYCNG
potato tuber	PDDMEKVPYKFPF.MTKLRVRQPAHEANEYI	AKYNLAISRMDLDTQPLNPI	GFKQQANIHCAYCNG
apple	STKIID...FKLPAPA.KLRIRPPAHAVDQAYR	KYKAMELMKALPDD...	DPRSFKQQAIVHCAYCDG
bean	STKITD...FKFPSNQ.PLRVRQAAHLVDNE	FLEKYYKATELMKALPSN...	DPRNFTQQANIHCAYCDG
grape	TTKIID...FQLPSSGSPMRTRPAAHLV	SKEYLAKYKKAIELOKALPDD...	DPRSFKQQANVHCTYCQG
sugarcane	CPLVSPAAPVDFTPPEKQVTRVRQPVHLLS	REYQEKYEAVGKMKALPES...	NPLSFKAQAAIHQAYCDN
Consensus	-----R-R--H-----KY--A-----P--F-QQA--H--YC--		
	211	<----- CuA ----->	280
potato leaf	AYK.....VGG...KELQVHFSWLF	FPFHRWLYFYERILGSLINDPTFAL	PYWNWDHPKGMRIIPMF
tomato leaf	AYK.....VGG...KELQVHFSWLF	FPFHRWLYFYERILGSLINDPTFAL	PYWNWDHPKGMRIIPMF
potato tuber	AYR.....IGG...KELQVHNSWLF	FPFHRWLYFYHERIVGKFIDDP	TFALPYWNWDHPKGMRFAMY
apple	AYD.....QVGFPELELQIHNSWLF	FPFHRYYLYFFEKILGKLINDPTFAL	PFWNWDSPAGMPLPAIY
bean	AYS.....QIGFPDLKLQVHGSWLF	FPFHRWLYFYERILGSLINDPTFAL	PFWNWDAPDGMQLPTIY
grape	AYD.....QVGYTDELELQVHASWLF	FLPFHRYLYFNERILAKLIDDP	TFALPYAWNDNPDGMYMPTIY
sugarcane	YYKYHKSSGSTVAKDDPAFDVHYSWIF	APWHRMYIYFYERALGDLIGDK	TFALPFWSWADPAGMVVPALF
Consensus	-Y-----H-SW-F-P-HR-Y-YF-ER-----I-D-TFALP-W-WD-P-GM--P---		
	281		350
potato leaf	DRE.GSSLYDDKRNQNRNGTIIDLGHFGQEVDTPQ	QIMTNNLTLMYRQMTNAPCPSQ
tomato leaf	DRE.GSSLYDEKRNQNRNGTIIDLGHFGKEVDTPQ	QIMTNNLTLMYRQMTNAPCPSQ
potato tuber	DRE.GTSLFDVTRDQSHRNGAVIDLGF	FGNEVETTQL.....	QLMSNNLTLMYRQMTNAPCPRM
apple	ADP.KSPLYDKLRSANHPPTLVLDL	YNGTEDNVSKE.....	TTINANLKIYRQMVSNKNAKL
bean	ADK.ASPLYDELRNASHQPPTLIDL	NFCDIGSDIDRN.....	ELIKTNLSIMYRQVYVSNKTSRL
grape	ASS.PSSLYDEKRNKHLPPPTVIDLDY	DGTEPTIPDD.....	ELKTDNLAIMYKQIVSGATTPKL
sugarcane	KEAFANPLYDPNRNTANLNA.LVDLDYL	SHRDAKPIDFKGPKDEKYL	VNKNLCTVYNQQVRKGPES..
Consensus	-----LYD-----DL-----NL---Y-Q-----		
	351	<----- CuB ----->	420
potato leaf	FFGAAAYPLGTEPSPGMGTIENIPHTPV	HIWTGD.....	SPRQK.....NGENMGNFYSAGLD
tomato leaf	FFGAAAYPLGSEPSPGQGTIENIPHTPV	HIWTGD.....	KPRQK.....NGEDMGNFYSAGLD
potato tuber	FFGGPYDLGVNTELP.GTIENIPHG	VHIVHSWGTVRGSLPNGAIS.....	NGENMGHFYSAGLD
apple	FFGNPYRAGDEPDPPGGGSI	EGTPHAPVHLWTGD.....	NTQP.....NFEDMGNFYSAGR
bean	FLGNPYRAGDAEPQAGSI	ENVPHAPVHTWTGD.....	NTQT.....NIEDMGIFYSAARD
grape	FLGYPYRAGDAIDPGAGTLEHAP	HNIIVHKWTGL.....	ADKP.....S.EDMGNFYTAGRD
sugarcane	FLGEKFCTAIDGTSGMGS	LERMAHTAMHVWVGKAGAK	PCDAAAGGVLVSHKDN
Consensus	FFG-----G-IE--PH--H-W-G-----MG-F-----D		

	421		490
potato leaf	PIFYCH H ANVDRMWDEWKLIGGK.RRDLSNKDWLNSEFFFYDENRNP..YRVKVRDCLDSKKMGFSYAPM		
tomato leaf	PIFYCH H ANVDRMWNEWKLIGGK.RRDLTDKDWLNSEFFFYDENRNP..YRVKVRDCLDSKKMGFDYAPM		
potato tuber	PVFFCH H SNVDRMWSEWKATGGK.RTDITHKDWLNSEFFFYDENENP..YRVKVRDCLDTKKMGYDYKPI		
apple	PIFFA H SNVDRMWSIWKTLGGK.RTDLTDSDWLDSGFLFYNENAEL..YRVKVRDCLDTKNLGYVYQDV		
bean	PIFYSH H SNVDRWLWIWKTLLGGK.KHDFTDKDWLSEGFLFYDENKNL..VRVNVKDSLDDIDKLGAYAYQDV		
grape	PIFFGH H ANVDRMWNIWKTIGGKNRKFDTDTDWLDATEFVFFYDENKQL..VKVKVSDCVDTSKLRYQYQDI		
sugarcane	PLFYSH H SNVDRMHWLWSTRMGGGQ.GITDPDWLDASFVFFYDDVKS PRKVR IKFRD VVDTRDLGYTYDP.		
Consensus	P-F-- H H-NVDR-W--W-----DWL---F-FY-----R---D-----		
	491	C-terminal cleavage	560
potato leaf	PTPWRNFKPI.....RKTTAGKVNTASIAPVTKVFLAK.....LDRAISFSITRPA.S		
tomato leaf	PTPWRNFKPI.....RKSSSGKVNTASIAPVSKVFLAK.....LDRAISFSITRPA.S		
potato tuber	ATPWRNFKPL.....TKASAGKVNTASLPPASNVFLAK.....LDKAISFSINRPT.S		
apple	DIPWLSKPTPRRAKVALSKVAKKLGVAHA AVASSKVVAGTE.....FPISLGSKISTVVKRPKQK		
bean	PIPWEKAKPVPRRTKV...QKLVEVEVNDGNLRKSP TIFL V RQ Q SPRK YVTFPLVLNKNVSAIVKRPK.K		
grape	PIPWL...PKNTKAKAKTTTKSSKSGVAK AAELPKTT ISSIGD.....FPKALNSVIRVEVPRPK.K		
sugarcane	..EYDRDLPWLRPKITTLVPHGKDSGGAA ARSSAAPPVFLALTKGQVVEVPAVAVP		
Consensus	-----P-----L-----		
	561		630
potato leaf	SRTTQEKNEQEELTFNKVAYDDTKYVRFDVFLNVDKT.VNADELDKAEFAGSYTSLPHVH.GSNTNHVT		
tomato leaf	SRTTQEKNEQEELTFNKISYDDRNRYVRFDVFLNVDKT.VNADELDKAEFAGSYTSLPHVH.GSNTNHVT		
potato tuber	SRTQEKNAQEEMLTFSSIRYDNRGYIRFDVFSNVDDN.VNANELDKAEFAGSYTSLPHVHRAGETNHIA		
apple	KRSKKAKEDEEEILVIEGIEFDRDVAVKFDVYVN.DVD.DLPSGPDKTEFAGSFVSVPHS.HKHK.KKMN		
bean	LRSKKEKEEEEEVLVIEGIEFYMNIAIKFDVYIN.DED.D.KVGAGNTEFAGSFVNI PHSAHGHNKKII		
grape	SRSKKEKEDEEEVLLIKGIELDRENFVKFDVYIN.DED.YSVSRPKNSEFAGSFVNVPHK..HMKEMKTK		
sugarcane	AR...EAGKEQLLVIDGIDFDPQANNKFDVA INL PADKALLVGPQYKEYAGSFAVVP...GSGAGETR		
Consensus	-R-----E--L-----FDV--N-----E-AGS-----		
	631		679
potato leaf	SVTFKLAITELLEDNGLLED EDTIAVTLV PKVGGEGVSIESVEIKLEDC.		
tomato leaf	SLTFKLAITELLEDIGLEDED TIAVTLV PKAGGEEVSIESVEIKLEDC.		
potato tuber	TVDFQLAITELLEDIGLEDED TIAVTLV PKRGEGISIEGATISLADC*		
apple	T.ILRGLTDLLEEIEAEDDSDVVVTLV PKFGA..VKIGGIKIEFAS..		
bean	T.SLRGLITDLLEDLHVEGDDNIVVTLV PKCGSGQVKINNVEIVFED*.		
grape	T.NLRFAINELLEDLGAEDDESIVITIVPRAGDDVTIGGIEIEFVSD*		
sugarcane	KGKVS LCITDVLYDLDAEDDSTVDVLI VPR TNAKVTINVRPTIKNRK..		
Consensus	-----I---L-D---E-DD---V--VP-----		

Figure 7.4. Comparison of deduced amino acid sequences of cDNA clones encoding plant PPO proteins. The multiple alignment of sequences from bean (Cary *et al.*, 1992), grape (Dry and Robinson, 1994), potato leaf (Hunt *et al.*, 1993), tomato leaf (Newman *et al.*, 1993), apple (Boss *et al.*, 1995) and potato tuber (Dr. Peter Thygesen, unpublished data) was carried out with the program PILEUP from the GCG Sequence analysis software package. In the consensus, an upper case letter indicates total conservation. Gaps introduced to maximise homology are indicated by (.). The histidine residues thought to be involved in copper binding are shown in bold. The N-terminal sequence of the cleaved C-terminal peptides of bean and grape are shown in bold and the proposed region of C-terminal cleavage in the sugarcane sequence is underlined.

CuA	
potato leaf	VHFSWLFFPF HR WYLYFYERILG
tomato leaf	VHFSWLFFPF HR WYLYFYERILG
potato tuber	VHNSWLFFPF HR WYLYFHERIVG
apple	IHNSWLFFPF HR YLYFFEKILG
bean	VHGSWLFFPF HR WYLYFYERILG
grape	VHASWLFLPF HR YLYFNERILA
sugarcane	VHYSWIFAPW HR MYLYFYERALG
consensus	.H.SW.F.P HR .Y.YF.E.... W
CuB	
potato leaf	PHTPV H IWTGD.....sprQk.....NGEnMGNFYSAGLDPIFYCH H aNVDRMW
tomato leaf	PHTPV H IWTGD.....kprQk.....NGEDMGNFYSAGLDPIFYCH H aNVDRMW
potato tuber	PHgPV H IWsGtvrgstlpngais.....NGEnMGhFYSAGLDPVFFCH H SNVDRMW
apple	PHaPV H lWTGD.....ntQp.....NfEDMGNFYSAGrDPIFFa H HSNVDRMW
bean	PHaPV H tWTGD.....ntQt.....NiEDMGiFYSAarDPIFYs H HSNVDRMW
grape	PHniV H kWTG1.....adkp.....s.EDMGNFYtAGrDPIFFg H HaNVDRMW
sugarcane	aHTam H VWvGkagakpcdaaagv1shkdnga fncnnDMGfLgSvGnDPIFYs H HSNVDRMW
consensus	.H...H.W.G.....MG.....DP.F.. H H.NVDRMW

Figure 7.5. Homology between the putative copper-binding domains of PPO amino acid sequences shown in Figure 7.4. Histidine residues believed to be involved in copper binding are shown in bold.

A dendrogram illustrating the relatedness of the translated sugarcane SUGPPO1 to various plant PPO proteins and the related tyrosinases is shown in Figure 7.6. The PPO proteins isolated from the Solanaceae family are closely related and form one group. Within the Solanaceae there are a number of subgroups which are not species specific, thus the tomato_d protein is more similar to the potato tuber protein than it is to the tomato_b protein. The grape, bean and apple proteins form another group, distinct from the Solanaceae, with the apple and bean proteins more closely related than the grape protein. Of the sequenced plant PPOs, sugarcane is the most different, forming its own subgroup.

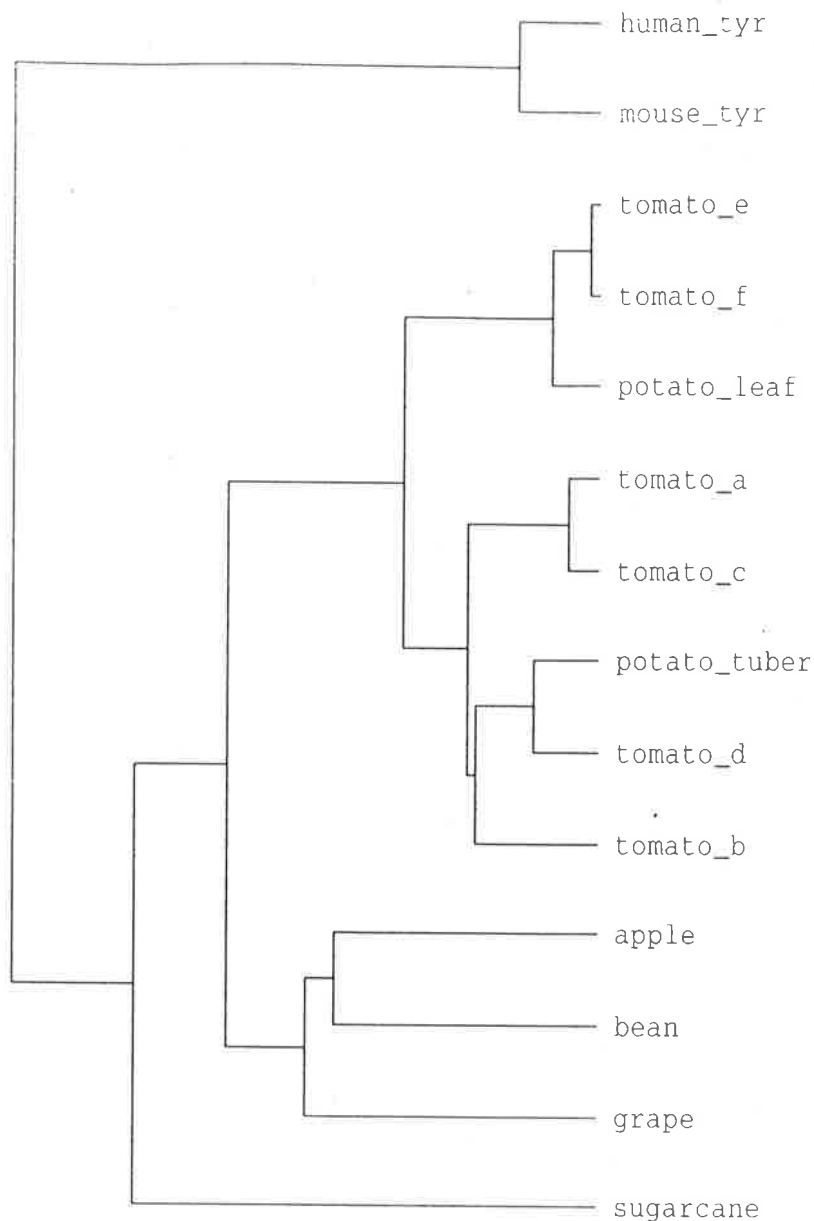


Figure 7.6 Dendrogram showing the relatedness of a number of plant PPO and tyrosinase proteins to the SUGPPO1 protein. The multiple alignment was done using the program PILEUP from the GCG Sequence analysis software package and displayed using FIGURE. The tyrosinase sequences were obtained from the Swiss-Prot Database under the accessions TYRO_MOUSE and TYRO_HUMAN. The apple, bean, grape, potato leaf and tomato (a, b, c, d, e and f) are published sequences (Boss *et al.*, 1995; Cary *et al.*, 1992; Dry and Robinson, 1994; Hunt *et al.*, 1993; Newman *et al.*, 1993) respectively. The potato tuber sequence is unpublished (Dr. Peter Thygesen, unpublished data).

Figure 7.7a and 7.7b show hydropathicity plots of the putative transit sequence and the entire precursor PPO protein. Transit peptides are characterised by high amounts of the hydroxy amino acids, serine (about 20%) and threonine and the lack of negatively charged amino acids (Douwe de Boer and Weisbeek, 1991). In the SUGPPO1 putative transit peptide 15 out of the 80 residues are either serine or threonine and there are only two negatively charged residues. In the amino terminal portion of the transit peptide there is a high proportion of hydrophilic amino acids, typical of stromal protein precursors. Before the site of cleavage of the transit peptide, there is a hydrophobic domain of about 22 amino acids (Figures 7.7a and 7.7b) This hydrophobic domain is preceded by two arginine residues (Figure 7.3) typical of the lumen targeting of transit peptides (Douwe de Boer and Weisbeek, 1991). The Kyte-Doolittle analysis of the complete PPO precursor protein also shows the carboxyl-terminal 100 residues to be quite hydrophobic (Figure 7.7b).

Expression of PPO mRNA in sugarcane

Northern blot analysis of sugarcane RNA probed with the 900 bp *Bam* HI/*Hind* II fragment of SUGPPO1 identified a transcript of approximately 2.2 kb (Figure 7.8). PPO mRNA was detected in the tissues designated as growing point, and in the immature stem immediately below it. The same transcript was also detected in young leaf roll and in the RNA from which the cDNA library was made. Expression was highest in the growing point tissues. There was no detectable message in the older stem and leaf RNA even after five days of exposure to X-ray film.

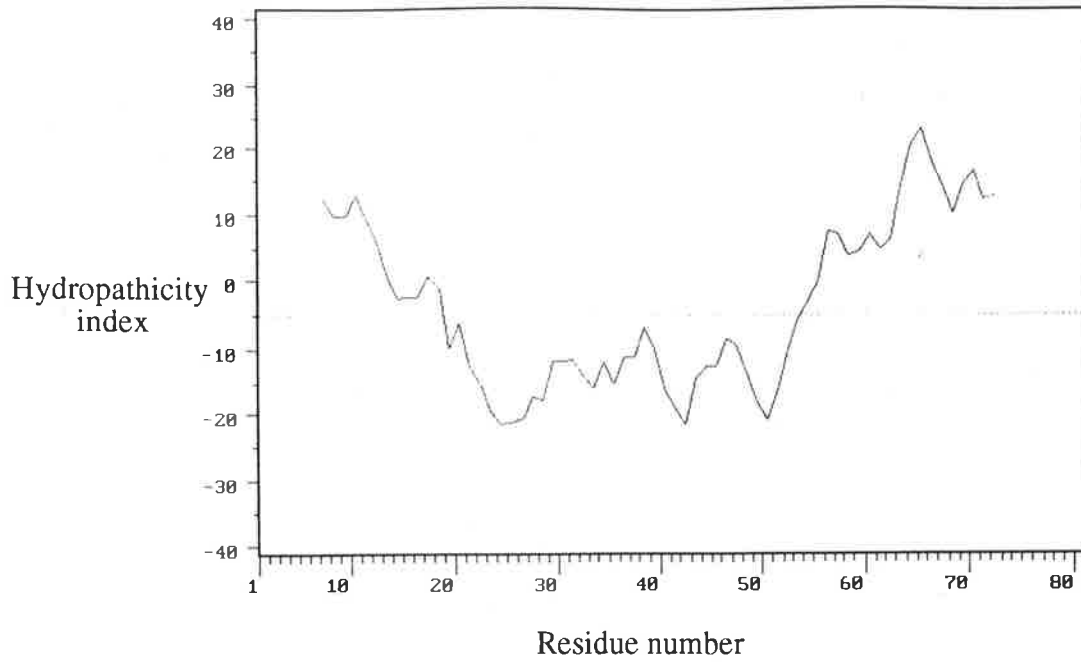


Figure 7.7a. Hydropathicity (Kyte and Doolittle) plot of the sugarcane putative transit peptide.

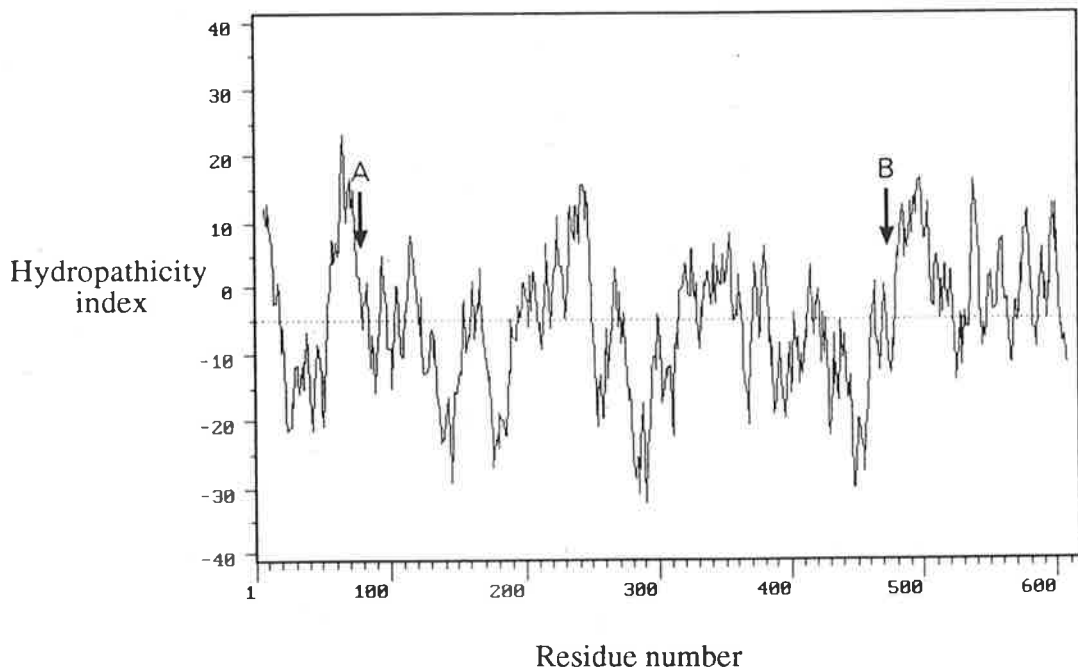


Figure 7.7b. Hydropathicity (Kyte and Doolittle) plot of the sugarcane precursor PPO protein. Arrows indicate (A) the site of cleavage of the transit peptide to yield the mature PPO protein and (B) the proposed site of cleavage of the C-terminal extension *in vitro*.

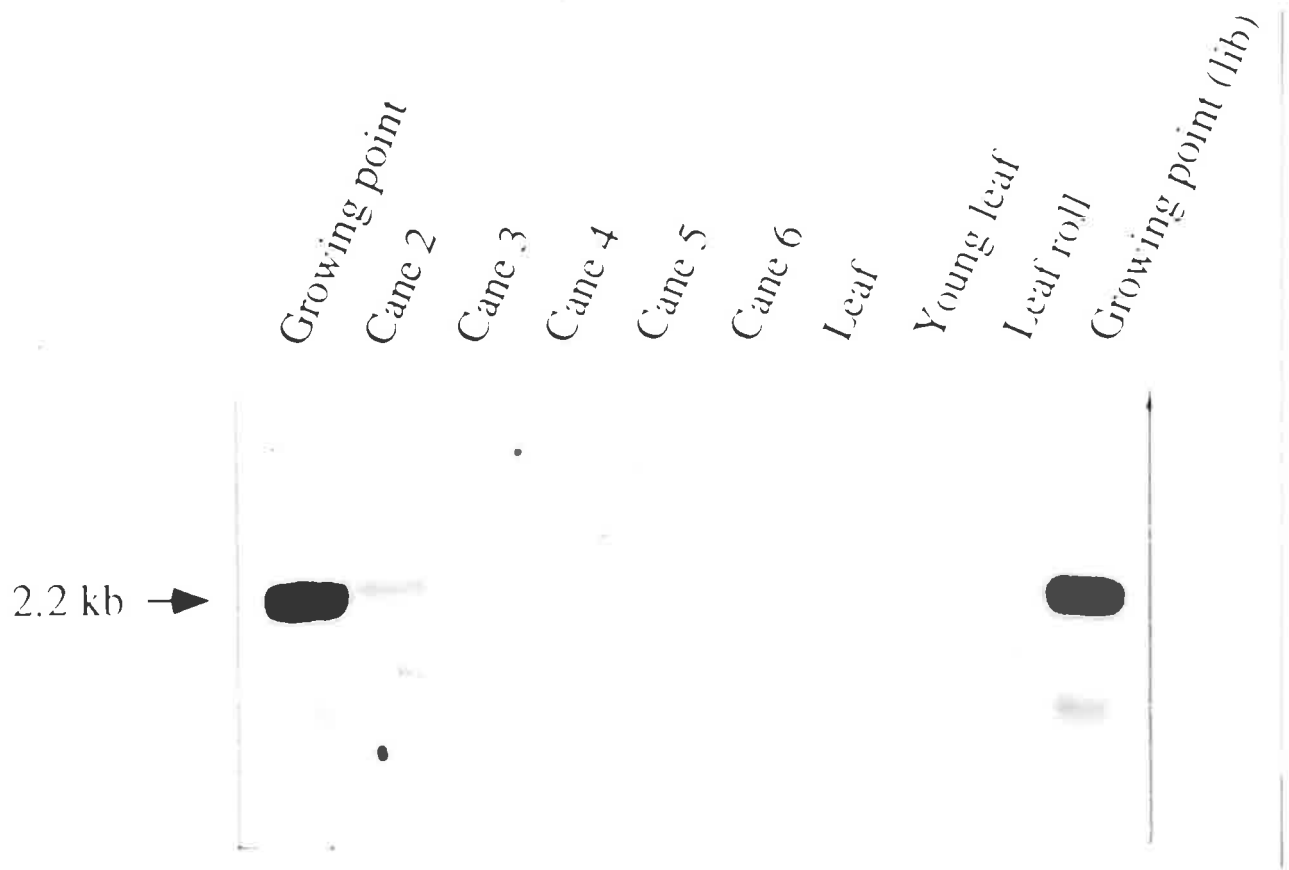


Figure 7.8. Northern analysis of PPO expression in different sugarcane tissues. Total RNA (10 μ g) from each tissue was gel-fractionated, blotted and probed with a radiolabelled 900 bp *Bam* H1/*Hind* II fragment of SUGPPO1. The blot was washed at high stringency and exposed to X-ray film for 24 hours. The size of the band was determined from RNA standards (Promega). Tissue 1-1.5 cm below the apical meristem was designated as growing point, the node and internode below the attachment of the D1 leaf as cane 2, internode 4 as cane 3, internode 6 as cane 4, internode 8 as cane 5, young furled green leaf as young leaf and the first 5 cm of leaf roll above the apical meristem as leaf roll. RNA which was used to make the cDNA library (10 μ g) was also loaded.

Discussion

cDNAs isolated from a sugarcane cDNA expression library with the antibody raised to the 45 kD protein were confirmed to encode PPO by comparison with known PPO sequences. The antibody appears to be highly specific as 13 of the 14 clones isolated contained either partial, full length or concatameric clones of PPO. Without sequencing all of the clones fully, it would seem there are at least two different PPO genes. Based on the limited nucleotide sequence and restriction mapping, clone 11A appears to be quite different to SUGPPO1.

SUGPPO1 encodes a protein with a predicted mature molecular mass of 59 kD plus a putative transit peptide of 8 kD. This is consistent with plant PPO genes cloned to date, which all encode mature proteins of 56-62 kD with 8-12 kD putative transit peptides (Shahar *et al.*, 1992; Cary *et al.*, 1992; Hunt *et al.*, 1993; Newman *et al.*, 1993; Dry and Robinson, 1994; Boss *et al.*, 1995). Cellular localisation studies indicate that PPO is plastid associated (Vaughn and Duke, 1981a; Vaughn *et al.*, 1988; Lax and Vaughn, 1991). All plant PPO genes encode plastidic transit peptides with hydrophobic C-terminal regions which could direct transport of the mature protein to the thylakoid lumen. More recently the import, targeting and processing of a tomato PPO by isolated plastids has been demonstrated (Sommer *et al.*, 1994), showing the *in vitro* transcribed and translated precursor to be directed to the thylakoid lumen in two steps, giving rise to a 59 kD mature form. The cleavage site of the putative sugarcane PPO transit peptide was identified from the N-terminal sequence of the purified 60 and 45 kD proteins (see Chapter 5). Comparison of seven putative transit peptide sequences from different PPO proteins reveals little conservation apart from a region at residues 81-94, just upstream of the cleavage site (Figure 7.4). The hydrophobic C-terminal region of the sugarcane PPO transit peptide identified in

the hydropathy plot (Figures 7.7 and 7.7b) is consistent with a role in directing transport of the mature protein to the thylakoid lumen.

The strongest regions of homology across the plant PPO proteins appear to be in the two copper binding domains (Figure 7.5) with the CuA site more highly conserved than the CuB site. The histidine residues are totally conserved in both domains and are thought to be involved in copper coordination. Site-directed mutagenesis of histidine residues in the CuA and CuB binding regions of *Streptomyces glaucensens* tyrosinase resulted in synthesis of a protein with no detectable tyrosinase activity and decreased affinity for copper (Jackman *et al.*, 1991). Experimental evidence for copper coordination by the conserved histidine residues of CuA and CuB in plant PPOs is not yet available.

Figure 7.6 clearly illustrates that the PPO protein encoded by SUGPPO1 is very different to the plant PPO protein sequences previously identified. As sugarcane is both the first C₄ plant and monocot for which PPO sequence has been obtained it is not surprising that it is quite different to the other sequenced plant PPOs and the tyrosinases. There may well be specific residues conserved in PPOs isolated from C₄ plants and/or monocots.

Numerous attempts to amplify PPO from sugarcane cDNA using degenerate oligonucleotide primers were unsuccessful. Amplification of invertase from the same cDNA (data not shown) confirmed that the problem was not with the cDNA but with the primers. Comparison of the nucleotide sequence of SUGPPO1 with the primer sequences revealed sequence differences which must have destabilised the annealing reaction sufficiently to inhibit the generation of any PPO specific PCR product. If PCR is to be used in the future to isolate PPO from other monocots and/or C₄ plants these important sequence differences observed in sugarcane PPO need to be incorporated into the primer design.

In Chapter 6 it was demonstrated that the 60 kD sugarcane PPO was susceptible to *in vitro* cleavage to the 45 kD form. Similarly, in bean leaves (Robinson and Dry, 1992), PPO exists as an active 60 kD protein susceptible to *in vitro* cleavage at the C-terminal end, yielding an active 42 kD protein. In Sultana (*Vitis vinifera* L.), there appears to be an inactive 60 kD form which is cleaved *in vivo* to an active 40 kD protein. In a variegated mutant of Sultana (Bruce's Sport) *in vivo* cleavage of the 60 kD protein does not occur in the variegated (white) regions (Rathjen and Robinson, 1992a). As in bean, however, the 60 kD protein from grape can be cleaved *in vitro* to the 40 kD form. N-terminal amino acid sequence analysis of the C-terminal cleavage peptides from Bruce's Sport PPO (AELPKTTISSIGDF; Dry and Robinson 1994) and bean PPO (VRQQSPR; Robinson and Dry 1992) locates the respective cleavage sites at equivalent positions (amino acid 464) within both proteins (Dry and Robinson, 1994). With respect to sugarcane PPO, in the absence of the N-terminal sequence of the C-terminal cleavage peptide or any apparent structural motif to locate it, it is impossible to determine the exact position of cleavage. If it is speculated however, that the sugarcane PPO protein is cleaved at a similar relative position to that of bean and grape (see Figure 7.4) the cleaved peptide would have a molecular weight of approximately 45 kD and a pI of 7.3, which is in close agreement with the apparent molecular weight of the purified PPO protein (Chapter 5). The predicted pI of the mature sugarcane PPO protein is 6.96 but the proposed site of cleavage of the C-terminus predicts that the pI of the cleaved peptide is 7.3. This predicted difference in pI is significant as it would explain the purification step, where separation of the 60 and 45 kD proteins on the Q-Sepharose column (equilibrated at pH 7.5) was achieved (Chapter 5). The 45 kD form would carry more of a negative charge than the 60 kD PPO at this pH, binding weakly, with most of the 60 kD protein not binding to the column at all. As there has not yet been a gene isolated that encodes a 45 kD PPO protein it is likely that the various reports of 45 kD PPO proteins represent similar cleaved forms of a larger protein.

Historically, PPO has often been described as tightly bound to thylakoids but has also been observed in plastid envelopes or the thylakoid lumen (Kowalski *et al.*, 1992; Mayer, 1987; Vaughn *et al.*, 1988). The hydrophobic domain close to the C-terminus of the mature SUGPPO1 protein (Figure 7.7b) is consistent with membrane association of the enzyme. In Chapter 4, however, the sugarcane PPO protein was demonstrated to be either very soluble or readily released from membranes during extraction. In the tomato gene family only three of the seven genes encode mature polypeptides with sufficient hydrophobic character to suggest the possibility of their being membrane associated (Newman *et al.*, 1993). Sommer *et al.* (1994) therefore suggest that some of the reports of PPO tightly bound to the thylakoid membrane could thus be due to post-import modification or crosslinking reactions during the isolation of the proteins.

SUGPPO1 mRNA was only detected in very young tissues, the growing point, the immature stem tissue immediately below it and young leaf roll tissue. Most reports show expression of PPO mRNA to be high in early developmental stages, declining with tissue age. In grapevine (Dry and Robinson, 1994) the highest levels of expression were in young developing tissues of berries and leaves. Similar developmental patterns of PPO expression were also found in the floral organs of tomato (Shahar *et al.*, 1992) and vegetative organs of potato (Hunt *et al.*, 1993). In contrast, in broad bean leaves, translatable message is present throughout leaf development with more translatable message reported in the intermediate leaves than either the young or mature leaves (Cary *et al.*, 1992). Whilst the expression of sugarcane PPO mRNA declines rapidly with tissue age, the sugarcane PPO protein was detected in immunoblots throughout development (see Chapter 6) suggesting that the protein is very stable. This supports the conclusions of Dry and Robinson (1994) who proposed a relatively slow turnover of the PPO protein in the grape berry and (Hunt *et al.*, 1993) regarding the apparent stability of PPO protein in potato tissues.

Isolation of SUGPPO1 represents the first step towards using genetic engineering to modify PPO expression in sugarcane. Down-regulation of PPO expression could significantly decrease the colour of sugarcane juice. Conversely, as PPO has been implicated in plant defence, overexpression of PPO may potentially minimise pest damage by antinutritive effects on plant protein (Steffens *et al.*, 1994).

Given the complex genetics of sugarcane, before attempting to modify gene expression in sugarcane the number of genes present and their similarity needs to be established. At this point there would appear to be at least two different PPO genes present in sugarcane. In tomato, seven PPO genes have been isolated (Newman *et al.*, 1993) and at least six are thought to be present in the closely related potato plant (Thygesen *et al.*, 1994). In grapevine, however, Southern analysis revealed a banding pattern consistent with a single PPO gene (Dry and Robinson, 1994). The expression of the genes present also needs to be investigated, to determine whether they are spatially or temporally expressed as in tomato (Newman *et al.*, 1993).

CHAPTER 8 . GENERAL DISCUSSION

The formation of brown colourants in sugarcane juice is a complex process, colourants being both derived from the plant and formed during the processing of sugarcane. The data presented in Chapter 2 indicates that enzymic activity does contribute significantly to colour formation in juice. Inhibition of PPO activity using specific inhibitors such as SHAM, removal of oxygen or heat inactivation, all led to decreased juice colour, thereby demonstrating that PPO, and not peroxidase, was the major contributor to enzymic browning in sugarcane.

The extent of the browning reaction in sugarcane juice is not determined by PPO activity alone, as demonstrated by the difference in browning of juice extracted from Q87 and Q96 despite their similar PPO activities (Chapter 3). Other factors such as the type and availability of the substrate (Chapter 2), the reaction conditions (Chapter 4) and the presence of other cellular components such as amino acids with which the PPO-formed quinones can react, also determine the extent of the browning reaction. Despite this, inhibition of PPO does result in cane juice of lower colour (Chapters 2 and 3).

It remains to be determined whether the reduction in juice colour observed when PPO activity is decreased can be translated into lighter-coloured raw sugar in commercial production. The previous studies of Smith (1976) and Tu (1977) indicate that inhibition of enzymic browning does result in decreased juice colour and lighter-coloured raw sugar and that the level of high molecular weight colourants in the raw sugar is also reduced. The question of whether inhibiting enzymic browning would produce lighter-coloured raw sugar could be answered by comparing the colour of raw sugar produced from untreated and PPO-inhibited juice. For this to be successful, PPO would need to be inhibited prior to or immediately after disruption of the cane as the formation of colourants is rapid once disintegration of the cane occurs. Furthermore, the quinones produced by PPO can

subsequently react with other cellular components including amino acids, flavonoids and other phenolics, to produce brown colourants in non-enzymic reactions.

From an industry perspective there are a number of approaches which could lead to lower juice colour and, ultimately, lighter-coloured raw sugar. The first approach would be to inhibit enzymic browning during the processing of sugarcane. The data presented in Chapters 2 and 4 suggest a number of potential methods of achieving this. The use of chemical inhibitors such as SHAM has been shown to be effective in inhibiting PPO activity (Chapter 2) and in reducing browning. However, the use of chemical inhibition on a commercial scale is unlikely to be economically feasible because of the high volume and relatively low value of the product. In addition, it may be undesirable to use many of these chemicals in a food product where the emphasis is on sugar as a 'natural' product. Alternatively, heat could be used to inactivate the enzyme, since excess heat is produced by the mill process and PPO is heat-labile (Chapter 4). The cane could be shredded and immediately steamed at the time of disruption, prior to crushing. The effectiveness of this approach would be determined by the speed at which the enzyme is inactivated once the cane is disrupted. The feasibility of commercial heat treatment of cane would need to be evaluated by engineering studies since the scale of such an operation may not make it commercially viable. A further option to control PPO activity could be to alter the pH of the sugarcane juice. PPO is less active and less stable at high pH (Chapter 4). When Tu (1977) raised the pH of cane juice by adding lime he observed a marked decrease in the colour of both juice and raw sugar. Alternatively, the pH of the juice could be lowered and NaCl, which inhibits PPO at relatively low concentrations at low pH, could be added (Chapter 4). This is also perhaps undesirable as the salt would need to be removed at a later stage.

The second approach to decreasing enzymic browning in cane juice would be to select for low-browning phenotypes. Clearly there is a genetic basis to juice colour, with the cultivars Q87 and H56-742 producing juice of much lighter colour than Q96, whilst clones

of *Erianthus arundinaceus* were generally found to produce juice of much higher colour than commercial clones (Chapter 3).

In a breeding program, plants could be readily screened for low browning by extracting cane juice, incubating for a set period of time and then measuring juice colour. There are, however, many other traits such as soluble sugar content, low fibre and disease resistance which have much higher priority in sugarcane breeding programs. In a large scale breeding program, adding another selection criterion to the breeding program would be labour intensive and expensive.

With the development of genetic engineering techniques it is now feasible to directly engineer existing sugarcane varieties to produce low-browning phenotypes. The advantage of this technology is that low-browning sugarcane plants could be produced directly from existing commercial cultivars. Stable transformation of sugarcane and regeneration of transgenic plants following microprojectile bombardment of embryogenic callus, has recently been demonstrated (Bower and Birch, 1992).

The strong correlation found between cane juice colour and the level of total phenolics in the cane (Chapter 2), might suggest targeting of the phenolic substrates. However, given the heterogeneity of these phenolic compounds, and the complex secondary metabolic pathways in which they are involved, genetic manipulation to reduce phenolic levels could have potentially deleterious effects.

Targeting of PPO, however, has already proved successful in potatoes (Bachem *et al.*, 1994 ; Dr. Peter Thygesen, CSIRO Division of Horticulture, unpublished data) where antisense inhibition of PPO gene expression decreased PPO activity in potato tubers and inhibited the formation of blackspot bruises following mechanical damage. Similarly, transformation of sugarcane with antisense constructs of PPO should decrease expression of the endogenous genes and inhibit synthesis of PPO in sugarcane tissues.

As discussed in Chapter 7, before attempting any genetic manipulation of sugarcane PPO, the number of genes present and their similarity needs to be established. Although only one of the PPO cDNAs isolated (SUGPPO1) was fully sequenced, nine of the other clones appear to be the same or very similar genes (Chapter 7). At least one of the other cDNAs (11A) is clearly different to SUGPPO1. Further sequencing of the other cDNAs will be required to establish the relationship between these clones. Once the number of PPO genes present in sugarcane is established, their temporal and spatial expression patterns need to be investigated. The expression of SUGPPO1 is developmentally regulated, and PPO mRNA was detected only in young tissues (Chapter 7). There may, however be, other tissue-specific PPO genes the expression of which is induced by wounding or stress.

At present one of the greatest limitations to the successful expression of introduced genes in sugarcane is the availability of effective monocot promoters. Various promoters, including rice actin, maize ubiquitin and 'Emu' are currently being assessed for the effectiveness and stability of expression in transgenic sugarcane plants (Elliot *et al.*, 1994). Isolation of the specific PPO promoter in sugarcane would increase the likelihood of effective expression of the PPO constructs. The isolation of a strong, early development promoter such as the PPO promoter may also be useful for manipulating expression of other genes in sugarcane. Bachem *et al.* (1994), using both CaMV 35S and patatin promoter constructs, showed that using the appropriate promoter to express antisense PPO was very important. The patatin constructs were less successful and the authors suggested that the temporal expression pattern conferred by this promoter does not coincide precisely with the onset of endogenous PPO gene expression in developing tubers. Thus early expression of endogenous PPO, together with its long half-life, may allow enough protein to be accumulated to give high PPO activities in antisense lines with this promoter.

Purification of the PPO protein from sugarcane and subsequent cloning of the gene has provided a great deal of information about this enzyme. Initial purification of the PPO

protein isolated low amounts of a 60 kD protein which could be stained for activity. Subsequent modification of the purification protocol increased the proportion of the 45 kD protein band at the expense of the 60 kD protein band. The many reports in the literature of a purified 45 kD PPO (see Chapter 5) suggested that the purified 45 kD protein from sugarcane was the predominant form and that the 60 and 45 kD bands represented two different proteins. However, when various sugarcane tissue extracts were probed with the antibody raised to the 45 kD protein, the predominant band was at 60 kD and not 45 kD. Limited N-terminal amino acid sequence suggested that the 60 kD protein was cleaved at the C-terminus to give rise to the 45 kD protein. Subsequent studies showed the 60 kD PPO could be cleaved *in vitro* to the 45 kD form without loss of activity by incubating the extracts at low pH or by digestion with certain proteases (Chapter 6). This C-terminal cleavage has been observed with PPO isolated from both broad bean (Robinson and Dry, 1992) and grape (Rathjen and Robinson, 1992a) and with a tyrosinase protein from *Neurospora crassa*. In *Neurospora crassa* the mature protein is a 46 kD protein but is synthesised as a larger precursor with a 24 kD C-terminal extension. The precursor is inactive until it is cleaved *in vivo* (Kupper *et al.*, 1989). The authors suggest the C-terminal extension could function to shield the active site of the enzyme and so prevent its activity. This is supported by work with the variegated grapevine mutant, Bruce's Sport which indicates that C-terminal processing of the 60 kD PPO protein may be required for enzyme activation (Rathjen and Robinson, 1992a). Dry and Robinson (1994) suggested that regulation of PPO in this way may be important to prevent potentially deleterious effects occurring in the cytoplasm during biogenesis and transport of the protein to the chloroplast compartment. However sugarcane PPO, like broad bean PPO, does not require cleavage of the C-terminal extension for activation of the enzyme (Robinson and Dry, 1992).

In the tomato PPO gene family three of the seven genes encode proteins with C-terminal hydrophobic structures consistent with membrane association (Newman *et al.*, 1993). However if the protein was only released from this association following cleavage, the 45 kD form would only ever be observed *in vitro* and this is not the case (Shahar *et al.*, 1992).

Alternatively, the harsh measures involved in extraction may release the protein without cleavage. Although the function of this C-terminal extension has yet to be determined the fact that such a large peptide can be cleaved from PPO without loss of activity suggests it must have some other role. This may be in the correct folding of the protein or in the correct insertion of the two copper atoms once the protein has reached the thylakoid lumen. Sommer *et al.* (1994) showed that low concentrations of Cu^{2+} inhibited the import of the precursor PPO protein into the chloroplast. In the absence of a crystal structure to visualise how the C-terminal extension relates to the rest of the protein, this remains speculative.

If, as speculated in Chapter 7, the C-terminal peptide of sugarcane PPO is cleaved at a similar position to that of grape and broad bean PPO, the deduced pI of the 45 kD form is sufficiently different to that of the 60 kD protein to explain the separation of the two forms on the Q-sepharose column at pH 7.5. Further, the presence of the two proteins could explain the biphasic heat inactivation of sugarcane PPO (Chapter 4) if one of the proteins was less heat stable. The unusual inhibition of the enzyme with SDS may also be explained by the presence of the two forms. PPO activity was decreased by 70% with the addition of only 0.1% (w/v) SDS, but at higher concentrations there was little effect on PPO activity (Figure 4.1). It is possible that the predominant form was totally inhibited by the SDS and the other not at all.

Isolation of cDNAs encoding sugarcane PPO with the antibody raised to the 45 kD protein confirmed the identity of the 45 kD protein and showed the antibody to be specific for PPO. Sequencing of a full-length PPO cDNA (SUGPPO1) confirmed that it encoded a mature protein of 59 kD, not 45 kD. From the limited N-terminal sequence of the purified 45 kD protein, an 8 kD putative transit peptide was identified. The hydrophobic C-terminal region of this putative transit peptide is consistent with a role in directing transport of the mature protein to the thylakoid lumen (Figures 7.7a and 7.7b) as demonstrated by Sommer *et al.* (1994).

The deduced protein sequence of SUGPPO1 is clearly very different to the sequences derived from other plant PPOs and the tyrosinases (Figure 7.6). However, all of the plant PPO cDNAs isolated to date have been from dicots. Genes encoding PPO have not been isolated from other monocots or C₄ plants. It would be interesting to see whether there are conserved regions between PPO from sugarcane and from other monocots or C₄ plants.

There are a number of other very interesting experiments yet to be done. One of the most important would be to investigate the localisation of PPO in sugarcane at both the cellular and subcellular level. Sugarcane PPO is interesting, firstly because unlike other PPOs it is readily soluble, and secondly because it is present in largely non-photosynthetic stem tissues. Previously most studies indicated that PPO was membrane-bound in plastids of non-senescing tissues (Mayer, 1987). However, the *in vitro* uptake studies by Sommer *et al.* (1994) indicate that PPO is soluble in the thylakoid lumen. The authors suggest that as a significant portion of endogenous tomato PPO was found soluble in the lumen that a similar scenario occurs *in vivo*. Reaction products of PPO were observed in the lumen, and the enzyme was suggested to be located on the luminal face of the thylakoid membrane (Shomer *et al.*, 1979; Sherman *et al.*, 1990). Thus in the chloroplast, PPO would seem to be localised in the thylakoid lumen, however the location of PPO in non-photosynthetic plastids is yet to be determined.

The sugarcane PPO antibody makes localisation of the protein possible. As discussed in Chapter 3, one can only speculate on where sugarcane PPO is localised in stem tissue. From the browning of the internode and node sections, it would seem PPO is localised in cells around the vascular bundles and below the rind (Chapter 3). At a subcellular level it is possibly localised in a non-photosynthetic plastid such as a leucoplast. In other non-photosynthetic plastid classes, PPO has generally been associated with membrane-bound vesicles within the plastid (Vaughn and Duke, 1984). Localisation of PPO in sugarcane leaves would also be interesting given the localisation of PPO in the leaves of the C₄ plant,

Sorghum. In *Sorghum*, PPO is present in the plastids of mesophyll cells but absent in bundle sheath cells (Vaughn and Duke, 1981b). The significance of this localisation is particularly interesting as it may be related to its function.

The use of *in situ* hybridisation techniques would allow determination of the cell types in which PPO is expressed, and at what point in development gene expression occurs in a given tissue. Once the number of genes present is established, then *in situ* hybridisation could be used to determine in what cells they are expressed by using gene-specific probes.

PPO activity was found to be highest in immature stem tissue and especially high in the meristematic tissue. Activity was also higher in young leaves than in older leaves and in the nodes compared with the internodes, with activity on a fresh weight basis decreasing with tissue age. This high activity in early developmental stages is consistent with the proposal that it is involved in defence against predation (Mayer and Harel, 1981). Cellular damage and the breakdown of compartmentation which occurs following pathogen invasion, allows the mixing of PPO with its substrates which are normally contained in the vacuole. The bitter and unpalatable tannins formed as a result of the action of PPO discourages further invasion by the pathogen. Sugarcane meristematic tissues are soft and vulnerable, as is the immature stem, and are important for the continued growth of the plant. Once tissue has matured, cells become lignified which provides some physical means of protection, and PPO may no longer be as important a form of defence. PPO activity however, is often high in peripheral tissues. The skin or peel of fruit often has higher PPO activity than the flesh as observed in apples (Harel *et al.*, 1964) and grapes (Rathjen and Robinson, 1992b). Whilst the function of PPO is still unclear, there would seem to be growing evidence associating PPO with defence against pathogens. Recently, enhanced expression of PPO mRNA was detected in apple fruit six hours after wounding (Boss *et al.*, 1995). The authors suggested that wound-induction of PPO occurs in cells adjacent to the wound. The increased levels of polyphenolics produced in the cells surrounding the site of wounding as a result of PPO activity may prevent further spread of

a pathogen. They also suggested that the elevated expression of PPO could cause an alteration in the concentration of oxygen and oxygen free radicals in the chloroplast. Reactive oxygen species have been implicated as endogenous signals in plant defence responses (Chen *et al.*, 1993)

It has only been in the last three years that plant PPO genes have been cloned and sequenced. With the isolation of these PPO genes, genetic manipulation of its expression is now possible. The production of PPO-null (Bachem *et al.*, 1994) and PPO-overexpressing transgenic plants (Steffens *et al.*, 1994) provides another avenue for investigating the much asked question as to the function of PPO. However any attempt to modify PPO expression, either to down-regulate or over-express, must take into account the possibility that PPO possesses vital functions which, to date, have been overlooked. As discussed in Chapter 1, a number of hypotheses suggest involvement of PPO in a number of processes unrelated to phenolic oxidation, particularly with regard to photosynthesis and regulation of plastidic oxygen levels. Transgenic plants exhibiting modified PPO expression provide a means to determine *in vivo* PPO function. For example if chloroplast PPO has a role in oxygen buffering, antisense transgenics exposed to high light should exhibit chlorophyll photobleaching (Steffens *et al.*, 1994).

Given this uncertainty of PPO function *in vivo*, reducing PPO expression could lead to unexpected and perhaps deleterious phenotypes. As mentioned earlier, antisense inhibition of PPO gene expression inhibited enzymic browning in potato tubers, thereby stopping the discoloration which usually occurs after bruising (Bachem *et al.*, 1994). However, no aberrant phenotypes associated with transformed potato plants with reduced PPO activity were observed. Future field trials of these plants and assessment of their resistance to diseases and pests may provide a better insight into the *in vivo* function of PPO.

In summary, the results presented in this thesis suggest that enzymic browning contributes significantly to colour in sugarcane juice and that PPO is the predominant enzyme

involved. The sugarcane PPO protein has been purified, a polyclonal antibody prepared, and the gene encoding this enzyme has been isolated and characterised. This will provide tools to further investigate the localisation of PPO in sugarcane tissues and additional work on the expression of PPO genes and their promoters could be undertaken.

The availability of a transformation system for sugarcane makes it feasible to use direct genetic modification to produce low PPO transgenic sugarcane plants. This would be of potential commercial benefit as it would allow the role of PPO in raw sugar colour to be determined directly and be a test of the potential to use this technology to produce low browning commercial sugarcane plants. Of equal importance, it may be a valuable way of determining the role of PPO in plants, which remains the major unanswered question in this area of plant research.

References

- Abbattista Gentile, I., Ferraris, L. and Matta, A. (1988). Variations of phenoloxidase activities as a consequence of stresses that induce resistance to *Fusarium* wilt of tomato. *Journal of Phytopathology*. **122**: 45-53.
- Alexander, A.G. (1966a). Oxidizing enzymes of sugarcane: Peroxidase. *Journal of Agriculture of University of Puerto Rico*. **50(1)**: 36-52.
- Alexander, A.G. (1966b). The oxidizing enzymes of sugarcane: Tyrosine (Polyphenol oxidase). *Journal of Agriculture of University of Puerto Rico*. **50(1)**: 36-52.
- Allan, A.C. and Walker, J.R.L. (1988). The selective inhibition of catechol oxidases by salicylhydroxamic acid. *Phytochemistry*. **27(10)**: 3075-3076.
- Anderson, J.W. (1968). Extraction of enzymes and subcellular organelles from plant tissues. *Phytochemistry*. **7**: 1973-1988.
- Angleton, E.L. and Flurkey, W.H. (1984). Activation and alteration of plant and fungal polyphenoloxidase isoenzymes in sodium dodecyl sulfate electrophoresis. *Phytochemistry*. **23(12)**: 2723-2725.
- Artschwager, E. (1925). Anatomy of the vegetative organs of sugar cane. *Journal of Agricultural Research*. **30**: 197-221.
- Bachem, C.W.B., Speckmann, G.-J., van der Linde, P.C.G., Verheggen, F.T.M., Hunt, M.D., Steffens, J.C. and Zabeau, M. (1994). Antisense expression of polyphenol oxidase genes inhibits enzymatic expression browning in potato tubers. *Bio/Technology*. **12**: 1101-1105.
- Baldry, C.W., Bucke, C., Coombs, J. and Gross, D. (1970). Phenols, Phenoloxidase, and Photosynthetic Activity of Chloroplasts isolated from sugar cane and spinach. *Planta*. **94**: 107-123.
- Bao, W., O'Malley, D.M., Whetten, R. and Sederoff, R.R. (1993). A laccase associated with lignification in Loblolly pine xylem. *Science*. **260**: 672-674.

- Batistuti, J.B. and Lourenço, E.J. (1985). Isolation and purification of polyphenol oxidase from a new variety of potato. *Food chemistry*. **18**: 251-263.
- Ben-Shalom, N., Kahn, V., Harel, E. and Mayer, A.M. (1977). Catechol oxidase from green olives: properties and partial purification. *Phytochemistry*. **16**: 1153-1158.
- Benjamin, N.D. and Montgomery, M.W. (1973). Polyphenol oxidase of Royal Ann cherries: purification and characterization. *Journal of Food Science*. **38**: 799-806.
- Bligny, R. and Douce, R. (1983). Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cells. Purification and properties of the enzyme. *Biochemical Journal*. **209**: 489-496.
- Boss, P.K., Gardner, R.C., Janssen, B.-J. and Ross, G.S. (1995). An apple polyphenol oxidase cDNA is upregulated in wounded tissues. *Plant Molecular Biology*. **In Press**:
- Bouthyette, P.Y., Eannetta, N., Hannigan, K.J. and Gregory, P. (1987). *Solanum berthaultii* trichomes contain unique polyphenoloxidases and a peroxidase. *Phytochemistry*. **26(11)**: 2949-2954.
- Bower, R. and Birch, R.G. (1992). Transgenic sugarcane plants via microprojectile bombardment. *The Plant Journal*. **2(3)**: 409-416.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248-254.
- Bruinsma, J. (1963). The quantitative analysis of chlorophylls *a* and *b* in plant extracts. *Phytochemistry and Photobiology (Chloroplast Metabolism Symposium)*. **2**: 241-249.
- Bryant, B.E., Fernelius, W.C. and Douglas, E. (1953). Formation constants of metal complexes of tropolone and its derivatives. I. Tropolone. *Journal of the American Chemical Society*. **39(6)**: 1043-1046.
- Burnette, F.S. (1977). Peroxidase and its relationship to food flavour and quality: a review. *Journal of Food Science*. **42**: 1-6.
- Campbell, W.H. and Gowri, G. (1990). Codon usage in higher plants, green algae and cyanobacteria. *Plant Physiology*. **92**: 1-11.

Cary, J.W., Lax, A.R. and Flurkey, W.H. (1992). Cloning and characterisation of cDNAs coding for *Vicia Faba* polyphenol oxidase. *Plant Molecular Biology*. **20**: 245-253.

Chen, Z., Silva, H. and Klessig, D.F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science*. **262**: 1883-1886.

Clarke, M.L., Stringer, M.B., Futo, V. and Sumpter, E.A. (1990). The significance of sugarcane variety in relation to raw sugar colour . BSES, Qld.

Coombs, J. and Baldry, C.W. (1978). Formation of colour in cane juice by enzyme catalyzed reactions. Part 1. Mechanism and chemical inhibition. *International Sugar Journal*. **80**: 291-294.

Coombs, J., Baldry, C.W., Bucke, C. and Long, S.P. (1974). *o*-Diphenol: oxygen oxidoreductase from leaves of sugarcane. *Phytochemistry*. **13**: 2703-2708.

Coombs, J., Goodacre, B.C. and Vane, G.W. (1980). Process advantages and disadvantages associated with the inhibition of enzyme-catalyzed color formation in sugarcane. *International society of sugar cane technologists*. **17**: 2098-2117.

Czaninski, Y. and Catesson, A.M. (1974). Polyphenoloxidases (plants). In M.A. Hayat (Ed.) *Electron microscopy of enzymes*. Vol 2. Van Nostrand Reinhold: New York. pp 66-78

Daniels, J. and Roach, B.T. (1987). Taxonomy and evolution. In D.J. Heinz (Ed.) *Sugarcane improvement through breeding*. Elsevier: New York. pp 7-84

Delhaize, E., Loneragan, J.F. and Webb, J. (1985). Development of three copper metalloenzymes in clover leaves. *Plant Physiology*. **78**: 4-7.

Dijkstra, L. and Walker, J.R.L. (1991). Enzymic browning in apricots (*Prunus armeniaca*). *Journal of the Science of Food and Agriculture*. **54**: 229-234.

Dixon, M. and Webb, E.C. (1958). *Enzymes*. (Fifth ed.). Longmans, Green and Co Ltd: London.

Douwe de Boer, A. and Weisbeek, P.J. (1991). Chloroplast protein topogenesis: import, sorting and assembly. *Biochimica et Biophysica Acta*. **1071**: 221-253.

- Driouich, A., Lainé, A.-C., Vian, B. and Faye, L. (1992). Characterization and localization of laccase forms in stem and cell cultures of sycamore. *The Plant Journal*. **2**(1): 13-24.
- Dry, I.B. and Robinson, S.P. (1994). Molecular cloning and characterisation of grape berry polyphenol oxidase. *Plant Molecular Biology*. **26**: 495-502.
- Duke, S.O. and Vaughn, K.C. (1982). Lack of involvement of polyphenol oxidase in ortho-hydroxylation of phenolic compounds in mung bean seedlings. *Physiologia Plantarum*. **54**: 381-385.
- Egley, G.H., Paul, R.N., Jr., Vaughn, K.C. and Duke, S.O. (1983). Role of peroxidase in the development of water-impermeable seed coats in *Sida spinosa* L. *Planta*. **157**: 224-232.
- Egley, G.H., Paul, R.N.J., Duke, S.O. and Vaughn, K.C. (1985). Peroxidase involvement in lignification in water-impermeable seed coats of weedy leguminous and malvaceous species. *Plant Cell and Environment*. **8**: 253-260.
- Elliot, A.R., Bower, R. and Birch, R.G. (1994). Transformation of commercial sugarcane cultivars and analysis of constitutive monocot promoter elements. (Ed.), *Australian Society of Plant Physiologists* Brisbane.
- Farber, L. and Carpenter, F.G. (1971). Identification of cane pigments that persist into refined sugar. *Proceedings of the International Society of Sugar Cane Technologists*. : 1589-1600.
- Felton, G.W., Donato, K., Del Vecchio, R.J. and Duffey, S.S. (1989). Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *Journal of Chemical Ecology*. **15**(12): 2667-2694.
- Flurkey, W.H. (1985). *In vitro* biosynthesis of *Vicia faba* polyphenoloxidase. *Plant Physiology*. **79**: 564-567.
- Flurkey, W.H. (1986). Polyphenoloxidase in higher plants. Immunological detection and analysis of *in vitro* products. *Plant Physiology*. **81**: 614-618.
- Flurkey, W.H. (1989). Polypeptide composition and amino-terminal sequence of broad bean polyphenoloxidase. *Plant Physiology*. **91**: 481-483.

Flurkey, W.H. and Jen, J.J. (1978). Peroxidase and polyphenol oxidase activities in developing peaches. *Journal of Food Science*. **43**: 1826-1831.

Flurkey, W.H. and Jen, J.J. (1980). Purification of peach polyphenol oxidase in the presence of added protease inhibitors. *Journal of Food Biochemistry*. **4**: 29-41.

Fujita, S. and Tono, T. (1988). Purification and some properties of polyphenoloxidase in eggplant (*Solanum melongena*). *Journal of the Science of Food and Agriculture*. **46**: 115-123.

Galeazzi, M.A.M., Sgarbieri, V.C. and Constantinides, S.M. (1981). Isolation, purification and physicochemical characterization of polyphenoloxidases (PPO) from a dwarf variety of banana (*Musa cavendishii*, L). *Journal of Food Science*. **46**: 150-155.

Ganesa, C., Fox, M.T. and Flurkey, W.H. (1992). Microheterogeneity in purified broad bean polyphenol oxidase. *Plant Physiology*. **98**: 472-479.

Golan, A., Kahn, V. and Sodovski, A.Y. (1977). Relationship between polyphenols and browning in avocado mesocarp. Comparison between the Fuerte and Lerman cultivars. *Journal of Agricultural and Food Chemistry*. **25(6)**: 1253.

Golbeck, J.H. and Cammarata, K.V. (1981). Spinach thylakoid polyphenol oxidase. Isolation, activation, and properties of the native chloroplast enzyme. *Plant Physiology*. **67**: 977-984.

Goodacre, B.C. and Coombs, J. (1978). Formation of colour in cane juice by enzyme catalysed reactions Part II. Distribution of enzyme and colour precursors. *International Sugar Journal*. **80**: 323-327.

Goodacre, B.C., Hutson, J. and Coombs, J. (1980). Enzyme catalyzed formation of colour in cane juice. Part 3. Inactivation of *o*-diphenol oxidase by heating intact cane. Distribution of enzyme and colour precursors. *International Sugar Journal*. **82**: 11-14.

Gross, D. and Coombs, J. (1971). Chlorogenic and caffeic acids in sugarcane. *International Sugar Journal*. **73**: 100.

Gross, D. and Coombs, J. (1976a). Enzymic color formation in beet and cane juices. *International Sugar Journal*. **78**: 106-109.

- Gross, D. and Coombs, J. (1976b). Enzymic colour formation in beet and cane juices. *International Sugar Journal*. **78**: 106-109.
- Harborne, J.B. (1984). *Phytochemical Methods*. Chapman and Hall: London.
- Harel, E. and Mayer, A.M. (1968). Interconversion of sub-units of catechol oxidase from apple chloroplasts. *Phytochemistry*. **7**: 199-204.
- Harel, E. and Mayer, A.M. (1971). Partial purification and properties of catechol oxidases in grapes. *Phytochemistry*. **10**: 17-22.
- Harel, E., Mayer, A.M. and Lehman, E. (1973). Multiple forms of *Vitis vinifera* catechol oxidase. *Phytochemistry*. **12**: 2649-2654.
- Harel, E., Mayer, A.M. and Shain, Y. (1964). Catechol oxidases from apples, their properties, subcellular location and inhibition. *Physiologia Plantarum*. **17**: 921-.
- Harel, E., Mayer, A.M. and Shain, Y. (1965). Purification and multiplicity of catechol oxidase from apple chloroplasts. *Phytochemistry*. **4**: 783-790.
- Harel, E., Mayer, A.M. and Shain, Y. (1966). Catechol oxidases, endogenous substrates and browning in developing apples. *Journal of the Science of Food and Agriculture*. **34**: 987-991.
- Hawker, J.S., Buttrose, M.S., Soeffky, A. and Possingham, J.V. (1972). A simple method for demonstrating macroscopically the location of polyphenolic compounds in grape berries. *Vitis*. **11**: 189-192.
- Henry, E.W. (1976). The ultrastructural localization of polyphenol oxidase in chloroplasts of *Brassica napus* cv. Zephyr. *Zeitschrift für Pflanzenphysiologie*. **78(5)**: 446-452.
- Holmes, D.S. and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry*. **114**: 193.
- Hunt, M.D., Eannetta, N.T., Haifeng, Y., Newman, S.M. and Steffens, J.C. (1993). cDNA cloning and expression of potato polyphenol oxidase. *Plant Molecular Biology*. **21**: 59-68.

- Hutcheson, S.W., Buchanan, B.B. and Montalbini, P. (1980). Polyphenol oxidation by *Vicia faba* chloroplast membranes. Studies on the latent membrane-bound polyphenol oxidase and on the mechanism of photochemical polyphenol oxidation. *Plant Physiology*. **66**: 1150-1154.
- Ingebrigtsen, J., Kang, B. and Flurkey, W.H. (1989). Tyrosinase activity and isoenzymes in developing mushrooms. *Journal of Food Science*. **54(1)**: 128-131.
- Interesse, F.S., Ruggiero, P., D'Avella, G. and Lamparelli, F. (1983). Characterization of wheat *o*-diphenolase isoenzyme. *Phytochemistry*. **22(9)**: 1885-1889.
- Jackman, M.P., Hajnal, A. and Lerch, K. (1991). Albino mutants of *Streptomyces glaucensens* tyrosinase. *Biochemistry Journal*. **274**: 707-713.
- Janovitz-Klapp, A.H., Richard, F.C., Goupy, P.M. and Nicolas, J.J. (1990). Inhibition studies on apple polyphenol oxidase. *Journal of Agricultural and Food Chemistry*. **38**: 926-931.
- Jimenez, P.O. and Samaniego, R.L. (1981). Enzymic browning in cane juice. *Crystallizer*. **4**: 10-11.
- Joel, D.M., Marbach, I. and Mayer, A.M. (1978). Laccase in Anacardiaceae. *Phytochemistry*. **17**: 796-797.
- Kahn, V. (1977). Latency properties of polyphenol oxidase in two avocado cultivars differing in their rate of browning. *Journal of the Science of Food and Agriculture*. **28**: 233-239.
- Kahn, V. (1985). Tropolone - a compound that can aid in differentiating between tyrosinase and peroxidase. *Phytochemistry*. **24(5)**: 915-920.
- Kahn, V. and Andrawis, A. (1985). Inhibition of mushroom tyrosinase by tropolone. *Phytochemistry*. **24(5)**: 905-908.
- Kenten, R.H. (1957). Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 1. Activation by acid and alkali. *Biochemical Journal*. **67**: 300-307.

- Kenten, R.H. (1958). Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 1. Activation by anionic wetting agents. *Biochemical Journal*. **68**: 244-251.
- Kidron, M., Harel, E. and Mayer, A.M. (1978). Catechol oxidase activity in grapes and wine. *American Journal of Enology and Viticulture*. **29(1)**: 30-35.
- King, R.S. and Flurkey, W.H. (1987). Effects of limited proteolysis on broad bean polyphenoloxidase. *Journal of the Science of Food and Agriculture*. **41**: 231-240.
- Kort, M.J. (1979). Colour in the sugar industry. In G.G. Birch and K.J. Parker (Ed.) *Sugar: Science and Technology*. Applied Science Publishers: London. pp 97-130
- Kowalski, S.P., Bamberg, J., Tingey, W.M. and Steffens, J.C. (1990). Insect resistance in the wild potato *Solanum berthaultii* : inheritance of glandular trichome polyphenol oxidase. *Journal of Heredity*. **81**: 475-478.
- Kowalski, S.P., Eannetta, N.T., Hirzel, A.T. and Steffens, J.C. (1992). Purification and characterization of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. *Plant Physiology*. **100**: 677-684.
- Kupper, U., Niedermann, D.M., Travaglini, G. and Lerch, K. (1989). Isolation and characterization of the tyrosinase gene from *Neurospora crassa*. *The Journal of Biological Chemistry*. **264(29)**: 17250-17258.
- Lagrimini, L.M. (1991). Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. *Plant Physiology*. **96**: 577-583.
- Lam, Y.-L. and Ho, K.-K. (1990). Purification of polyphenol oxidase from orchid leaves. *Plant Physiology and Biochemistry*. **28(2)**: 209-213.
- Lanker, T., Flurkey, W.H. and Hughes, J.P. (1988). Cross-reactivity of polyclonal and monoclonal antibodies to polyphenoloxidase in higher plants. *Phytochemistry*. **27(12)**: 3731-3734.
- Lanker, T., King, T.G., Arnold, S.W. and Flurkey, W.H. (1987). Active, inactive and *in vitro* synthesized forms of polyphenoloxidase during leaf development. *Physiologia Plantarum*. **69**: 323-329.

- Lax, A.R., Vaughn, K.C. and Templeton, G.E. (1984). Nuclear inheritance of polyphenol oxidase in *Nicotiana*. *Journal of Heredity*. **75**: 285-287.
- Lax, A.R. and Vaughn, V.C. (1991). Colocalization of polyphenol oxidase and photosystem II proteins. *Plant Physiology*. **96**: 26-31.
- Lee, C.Y., Smith, N.L. and Pennesi, A.P. (1983). Polyphenoloxidase from DeChaunac grapes. *Journal of the Science of Food and Agriculture*. **34**: 987-991.
- Lehman, E., Harel, E. and Mayer, A.M. (1974). Copper content and other characteristics of purified peach laccase. *Phytochemistry*. **13**: 1713-1717.
- Lerch, K. (1981). Copper monooxygenases: tyrosinase and dopamine β -monooxygenase. In H. Sigel (Ed.) *Copper proteins*. Vol 13. Metal ions in biological systems. Marcel Dekker: New York.
- Lerch, K. (1987). Monophenol monooxygenase from *Neurospora crassa*. In S. Kaufman (Ed.) *Metabolism of aromatic amino acids and amines*. Vol 142. Methods in enzymology. S.P. Colowick and N.O. Kaplan (Ed.) Academic Press: Orlando. pp 165-170
- Lerner, H.R. and Mayer, A.M. (1975). Stokes' radius changes of solubilized grape catechol oxidase. *Phytochemistry*. **14**: 1955-1957.
- Lerner, H.R., Mayer, A.M. and Harel, E. (1972). Evidence for conformational changes in grape catechol oxidase. *Phytochemistry*. **11**: 2415-2421.
- Lieberi, R. and Biehl, B. (1978). Activation of latent phenolase from spinach chloroplasts by ageing and by frost. *Phytochemistry*. **17**: 1427-1429.
- Lourenço, E.J., Neves, V.A. and Da Silva, M.A. (1992). Polyphenol oxidase from sweet potato: purification and properties. *Journal of Agricultural and Food Chemistry*. **40**: 2369-2373.
- Marbach, I. and Mayer, A.M. (1975). Changes in catechol oxidase and permeability to water in seed coats of *Pisum elatius* during seed development and maturation. *Plant Physiology*. **56**: 93-96.
- Mayer, A.M. (1987). Polyphenol oxidases in plants - recent progress. *Phytochemistry*. **26(1)**: 11-20.

- Mayer, A.M. and Harel, E. (1979). Polyphenol oxidases in plants. *Phytochemistry*. **18**: 193-215.
- Mayer, A.M. and Harel, E. (1991). Phenoloxidases and their significance in fruit and vegetables. In P.F. Fox (Ed.) *Food enzymology*. Elsevier Science Publishers: New York. pp 373-398
- Mayer, E.M. and Harel, E. (1981). Polyphenol oxidases in fruits - changes during ripening. In J. Friend and M.C. Rhodes (Ed.) *Recent advances in the biochemistry of fruit and vegetables*. Vol 19. Annual Proceedings of the Phytochemical Society of Europe. Academic Press: London. pp 161-180
- Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science*. **211**: 1437-1438.
- Meyer, H.-U. and Biehl, B. (1981). Activation of latent phenolase during spinach leaf senescence. *Phytochemistry*. **20(5)**: 955-959.
- Moore, B.M. and Flurkey, W.H. (1990). Sodium dodecyl sulfate activation of a plant polyphenoloxidase. *The Journal of Biological Chemistry*. **265(9)**: 4892-4898.
- Moore, B.M., Kang, B. and Flurkey, W.H. (1988). Histochemical and immunochemical localization of tyrosine in whole tissue sections of mushrooms. *Phytochemistry*. **27(12)**: 3735-3737.
- Moore, P.H. (1987). Anatomy and Morphology. In D.J. Heinz (Ed.) *Sugarcane improvement through breeding*. Elsevier Science Publishers B.V.: Amsterdam. pp 85-142
- Mukherjee, S.K. (1957). Origin and distribution of *Saccharum*. *Botanical Gazette*. **119**: 55-61.
- Murata, M., Kurokami, C. and Homma, S. (1992). Purification and some properties of chlorogenic acid oxidase from apple (*Malus pumila*). *Bioscience, Biotechnology and Biochemistry*. **56**: 1705-1710.

- Murata, M., Kurokami, C., Homma, S. and Matsushashi, C. (1993). Immunochemical and immunohistochemical study of apple chlorogenic acid oxidase. *Journal of Agricultural and Food Chemistry*. **41**: 1385-1390.
- Nakamura, K., Amano, Y. and Kagami, M. (1983). Purification and some properties of a polyphenol oxidase from Koshu grapes. *American Journal of Enology and Viticulture*. **34(2)**: 122-127.
- Newman, S.M., Eannetta, N.T., Yu, H., Prince, J.P., de Vincente, M.C., Tanksley, S.D. and Steffens, J.C. (1993). Organisation of the tomato polyphenol oxidase gene family. *Plant Molecular Biology*. **21**: 1035-1051.
- Okamoto, A., Imagawa, H., Arai, Y. and Ozawa, T. (1988). Partial purification and some properties of polyphenoloxidases from sago palm. *Agricultural and Biological Chemistry*. **52(9)**: 2215-2222.
- Olah, A.F. and Mueller, W.C. (1981). Ultrastructural localization of oxidative and peroxidative activities in a carrot suspension cell culture. *Protoplasma*. **106**: 231-248.
- Park, E.Y. and Luh, B.S. (1985). Polyphenol oxidase of kiwifruit. *Journal of Food Science*. **50**: 678-684.
- Paton, N.H. and Duong, M. (1990). Phenolic colorants in sugar cane: Role of chlorogenic acid and flavonoids in enzymic browning and colour in cane juice (113). CSR.
- Paton, N.H., Duong, M. and Bartlett, M.W. (1991). Phenolic colorants in sugar cane; role of phenolics in milling and in storage of raw sugar . Report on SRDC project CSR4S.
- Paton, N.H. and McCowage, R.J. (1987). Colour forming mechanisms during mill processing. *Proceedings of Australian Society of Sugar Cane Technologists*. : 11-20.
- Peñafiel, R., Galindo, J.D., Solano, F., Pedreño, E., Iborra, J.L. and Lozano, J.A. (1984). Kinetic study of the interaction between frog epidermis tyrosinase and chloride. *Biochimica et Biophysica Acta*. **788**: 327-332.
- Pierpoint, W.S. (1966). The enzymic oxidation of chlorogenic acid and some reactions of the quinone produced. *Biochemistry Journal*. **98**: 567-580.

- Ploug, M., Jensen, A.L. and Barkholt, V. (1989). Determination of amino acid compositions and NH₂-terminal sequences of peptides electroblotted onto PVDF membranes from tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis: application to peptide mapping of human complement component C3. *Analytical Biochemistry*. **181**: 33-39.
- Radler, E. and Torokfalvy, E. (1973). The affinity for oxygen of polyphenoloxidase in grapes. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*. **152**: 38-41.
- Rathjen, A.H. and Robinson, S.P. (1992a). Aberrant processing of polyphenol oxidase in a grapevine mutant. *Plant Physiology*. **99**: 1619-1625.
- Rathjen, A.H. and Robinson, S.P. (1992b). Characterisation of a variegated grapevine mutant showing reduced polyphenol oxidase activity. *Australian Journal of Plant Physiology*. **19(1)**: 43-54.
- Raymond, J., Rakariyatham, N. and Azanza, J.L. (1993). Purification and some properties of polyphenoloxidase from sunflower seeds. *Phytochemistry*. **34(4)**: 927-931.
- Robb, D.A., Mapson, L.W. and Swain, T. (1965). On the heterogeneity of the tyrosinase of broad bean (*Vicia faba* L.). *Phytochemistry*. **4**: 731-740.
- Robb, D.A., Swain, T. and Mapson, L.W. (1966). Substrates and inhibitors of the activated tyrosinase of broad bean (*Vicia faba* L.). *Phytochemistry*. **5**: 665-675.
- Robinson, S.P. and Dry, I.B. (1992). Broad bean leaf polyphenol oxidase is a 60 kiloDalton protein susceptible to proteolytic cleavage. *Plant Physiology*. **99**: 317-323.
- Robinson, S.P., Loveys, B.R. and Chacko, E.K. (1993). Polyphenol oxidase enzymes in the sap and skin of mango fruit. *Australian Journal of Plant Physiology*. **20**: 99-107.
- Rouet-Mayer, M. and Philippon, J. (1986). Inhibition of catechol oxidases from apples by sodium chloride. *Phytochemistry*. **25(12)**: 2717-2719.
- Rouet-Mayer, M.A., Ralambosoa, J. and Philippon, J. (1990). Roles of σ -quinones and their polymers in the enzymic browning of apples. *Phytochemistry*. **29(2)**: 435-440.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning, a laboratory manual*. Cold Spring Harbour Laboratory Press: Cold Spring Harbour, New York.

- Sánchez-Ferrer, A., Laveda, F. and García-Carmona, F. (1993). Partial purification of soluble potato polyphenol oxidase by partitioning in an aqueous two-phase system. *Journal of Agricultural and Food Chemistry*. **41**: 1219-1224.
- Sanger, J., Nicklen, S. and Coulsen, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings Of the National Academy of Science USA*. **74**: 5463-5467.
- Sapis, J.C., Macheix, J.J. and Cordonnier, R.E. (1983). The browning capacity of grapes. II. Browning potential and polyphenol oxidase activities in different mature grape varieties. *American Journal of Enology and Viticulture*. **34(3)**: 157-162.
- Satô, M. and Hasegawa, M. (1976). The latency of spinach chloroplast phenolase. *Phytochemistry*. **15**: 61-65.
- Schägger, H. and Von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*. **166**: 368-379.
- Sciancalepore, V. and Longone, V. (1984). Polyphenol oxidase activity and browning in green olives. *Journal of Agricultural and Food Chemistry*. **32**: 320-321.
- See, Y.P. and Jackowski, G. (1989). Estimating molecular weights of polypeptides by SDS gel electrophoresis. In T.E. Creighton (Ed.) *Protein structure, A practical approach*. IRL Press: Oxford.
- Shahar, T., Hennig, N., Gutfinger, T., Hareven, D. and Lifschitz, E. (1992). The tomato 66.3-kD polyphenoloxidase gene: molecular identification and developmental expression. *Plant Cell*. **4**: 135-147.
- Sherman, T.D., Vaughn, K.C. and Duke, S.O. (1990). A limited survey of the phylogenetic distribution of polyphenol oxidase. *Phytochemistry*. **30(8)**: 2499-2506.
- Shomer, I., Ben-Shalom, N., Harel, E. and Mayer, A.M. (1979). The intracellular location of catechol oxidase in the olive fruit. *Annals of Botany*. **44**: 261-263.

- Simpson, R.J., Moritz, R.L., Begg, G.S., Rubira, M.R. and Nice, E.C. (1989). Micropreparative procedures for high sensitivity sequencing of peptides and proteins. *Analytical Biochemistry*. **177**: 221-236.
- Smith, N.H. (1976). Inhibition of enzymatic browning in cane sugar processing. *International Sugar Journal*. **78**: 259-263.
- Smith, P. and Paton, N.H. (1985). Sugarcane flavonoids. *Sugar Technology Reviews*. **12(2-3)**: 117-142.
- Smith, P., Paton, N.H., Delaney, H.R. and Ames, R. (1981). Colour studies in milling. *Proceedings of Australian Society of Sugar cane Technologists*. : 71-80.
- Söderhäll, I., Bergenstråhle, A. and Söderhäll, K. (1990). Purification and some properties of a *Daucus carota* Lectin which enhances the activation of prophenoloxidase by CaCl₂. *Plant Physiology*. **93**: 657-661.
- Sommer, A., Ne'eman, E., Steffens, J.C., Mayer, A.M. and Harel, E. (1994). Import, targeting, and processing of a plant polyphenol oxidase. *Plant Physiology*. **105**: 1301-1311.
- SRDC. (1992). 5 Year Plan .
- SRDC. (1994). Annual Research and Development Program 1994/95 . Sugar Research and Development Corporation.
- Steffens, J.C., Harel, E. and Hunt, M.D. (1994). Polyphenol oxidase. In B.E. Ellis, G.W. Kuroki and H.A. Stafford (Ed.) *Genetic engineering of plant secondary metabolism*. Vol 28. Recent Advances in Phytochemistry. Plenum Press: New York. pp 275-312
- Takahama, U. and Oniki, T. (1991). Participation of peroxidase in the metabolism of 3,4-dihydroxyphenylalanine and hydrogen peroxide in vacuoles of *Vicia faba* L. mesophyll cells. *Plant and Cell Physiology*. **32(6)**: 745-754.
- Thomas, P. and Janave, M.T. (1986). Isoelectric focusing evidence for banana isoenzyme with mono and diphenolase activity. *Journal of Food Science*. **51(2)**: 384-387.

- Thygesen, P.W., Dry, I.B. and Robinson, S.P. (1994). Polyphenol oxidase in potato tubers. In W.R. Belknap, M.E. Vayda and W.D. Park (Ed.) *The molecular and cellular biology of the potato*. CAB International: Wallingford. pp 151-159
- Tolbert, N.E. (1973). Activation of polyphenol oxidase of chloroplasts. *Plant Physiology*. **51**: 234-244.
- Tu, C.C. (1977). Effect of cane liming on sugar refinability. *Report-Hawaiian Sugar Technologists*. : 105-108.
- Tu, C.C., Kondo, A. and Sloane, G.E. (1977). The role of high and low molecular weight colorants in sugar color. *The Sugar Journal*. **40**: 9-12.
- Valero, E. and García-Carmona, F. (1992). Hysteresis and cooperative behavior of a latent plant polyphenoloxidase. *Plant Physiology*. **98**: 774-776.
- Valero, E., García-Moreno, M., Varón, R. and García-Carmona, F. (1991). Time-dependent inhibition of grape polyphenol oxidase by tropolone. *Journal of Agricultural and Food Chemistry*. **39**: 1043-1046.
- Valero, E., Varón, R. and García-Carmona, F. (1988). Characterization of polyphenol oxidase from Airen grapes. *Journal of Food Science*. **53(5)**: 1482-1485.
- Vámos-Vigyázó, L. (1981). Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Critical Reviews in Food Science and Nutrition*. **15**: 49-127.
- van Dillewijn, C. (1952). *Botany of sugarcane*. Chronica Botanica Co.: Waltham, Moss.
- van Huystee, R.B. (1987). Some molecular aspects of plant peroxidase biosynthetic studies. *Annual Review of Plant Physiology*. **38**: 205-217.
- van Lelyveld, L.J. and Bower, J.P. (1984). Enzyme reactions leading to avocado fruit mesocarp discoloration. *Journal of Horticultural Science*. **59(2)**: 257-263.
- Vaughan, P.F.T., Eason, R., Paton, J.Y. and Ritchie, G.A. (1975). Molecular weight and amino acid composition of purified spinach beet phenolase. *Phytochemistry*. **14**: 2383-2386.

- Vaughn, K.C. and Duke, S.O. (1981a). Tentoxin-induced loss of plastidic polyphenol oxidase. *Physiologia Plantarum*. **53**: 421-428.
- Vaughn, K.C. and Duke, S.O. (1981b). Tissue localization of polyphenol oxidase in *Sorghum*. *Protoplasma*. **108**: 319-327.
- Vaughn, K.C. and Duke, S.O. (1982). Tentoxin effects on *Sorghum*: the role of polyphenol oxidase. *Protoplasma*. **110**: 48-53.
- Vaughn, K.C. and Duke, S.O. (1984a). Function of polyphenol oxidase in higher plants. *Physiologia Plantarum*. **60**: 106-112.
- Vaughn, K.C. and Duke, S.O. (1984b). Tentoxin stops the processing of polyphenol oxidase into an active protein. *Physiologia Plantarum*. **60**: 257-261.
- Vaughn, K.C., Lax, A.R. and Duke, S.O. (1988). Polyphenol oxidase: the chloroplast oxidase with no established function. *Physiologia Plantarum*. **72**: 659-665.
- Vaughn, K.C., Miller, P.D. and Wilson, K.G. (1981). Ultrastructural localization of polyphenol oxidase in *Aegopodium podagraria* L. *Cytobios*. **31**: 27-36.
- Walker, J.R.L. (1964). Studies on the enzymic browning of apples. II. Properties of apple polyphenoloxidase. *Australian Journal of Biological Sciences*. **17**: 360-371.
- Walker, J.R.L. and McCallion, R.F. (1980). The selective inhibition of *ortho*- and *para*-diphenol oxidases. *Phytochemistry* **19**: 373-377.
- Williams, C.A., Harborne, J.B. and Smith, P. (1974). The taxonomic significance of leaf flavonoids in *Sacchaum* and related genera. *Phytochemistry*. **13**: 1141-1149.
- Williams, R.G. and Rijven, A.H.G.C. (1970). The physiology of growth in subterranean clover.II. The dynamics of leaf growth. *Australian Journal of Botany*. **18**: 146-166.
- Wissemann, K.W. and Lee, C.Y. (1981). Characterization of polyphenoloxidase from Ravat 51 and Niagara grapes. *Journal of Food Science*. **46**: 506-508, 514.
- Zawistowski, J., Biliaderis, C.G. and Murray, E.D. (1988). Isolation and some properties of an acidic fraction of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosus* L.). *Journal of Food Biochemistry*. **12**: 23-35

Bucheli, C.S. & Robinson, P. (1994). Contribution of enzymic browning to color in sugarcane juice. *Journal of Agricultural and Food Chemistry*, 42(2), 257-261

NOTE:

This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1021/jf00038a006>