STUDIES ON THE ROLE OF POLYGALACTURONASE ISOCENZYMES
IN TOMATO FRUIT SOFTENING AND RIPENING

Thesis submitted for the degree of
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by

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ABSTRACT

Changes in the activity of polygalacturonase during the development and/or ripening of tomato fruit of a wide range of genotypes have been followed. Normal commercial cultivars have three forms of the enzyme; the isoenzyme having the highest molecular weight, PG1, appeared as fruit began to change colour, and its rate of accumulation was reflected in the rate of fruit softening. This early rate of softening was closely related to the eventual degree of softness shown by the fully ripe fruit.

PG1 was also the predominant isoenzyme during the senescence of slow-ripening (Never ripe; Longkeeper, Spanish Winter) genotypes. Trace levels of this isoenzyme were also found in aged ripening-resistant (ripening inhibitor, non-ripening and Alcobaca) tomato genotypes. The negligible degree of softening of these lines was reflected by their low polygalacturonase activity.

Two smaller isoenzymes of polygalacturonase, PG2A and PG2B, appeared as normally ripening fruit turned orange and then red in colour, so that in ripe fruit up to 95% of the total polygalacturonase activity occurred in these forms. Neither the rate of accumulation nor the total activity of PG2 correlated with the extent of fruit softening at this period of ripening, and there appeared to be a supra-abundance of the enzyme.

Loss of cell wall material during ripening was correlated with the degree of fruit softening, but differences in this property shown by fruit from a range of cultivars could not be explained by altered susceptibility of their cell walls to attack by exogenous polygalacturonase extracted from red fruit.
It is hypothesised that PG1 degrades the middle lamella of the tissue leading to rapid fruit softening. The high activity of PG2 produced by fruit during the later stages of ripening is seen as eroding the primary cell wall more extensively, causing a release of soluble wall components, but not producing the increase in loss of fruit firmness that might be expected.

Normal tomato fruit ripening involves autocatalytic ethylene production and exponential polygalacturonase synthesis accompanied by pigment transformations and the adjustment of fruit composition to produce an acceptable flavour. However, in this study of wild species and salt-induced ripening of various genotypes, instances have been found in which low levels of ethylene production and very limited polygalacturonase do eventually result in partial ripening of the fruit.
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Additional Material in Back-Pocket

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   In "Ethylene: Biochemical, Physiological and Applied Aspects".
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ABBREVIATIONS AND SYMBOLS

a  Hunterlab colour specification designating red to green

A  Absorbance (optical)

ΩA  Angstroms

ACC  1-Aminocyclopropane-1-carboxylic acid

ATP  Adenosine-5 triphosphate

b  Hunterlab colour specification designating blue to yellow

C-1, C-4, C-6  Carbon atoms attached at position 1, 4 or 6 of the pyranose ring

C₆H₄  Ethylene

Ca²⁺  Calcium ion

CWM  Cell wall material

cv.  Cultivar of tomato

DEAE-Sephadex  Diethylaminoethyl-Sephadex

E  Ethrel

EC  Number of enzyme in Enzyme Commission's system

EDTA  Ethylenediaminetetra-acetate

F₁  First filial generation

f.wt.  Fresh weight of tissue
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<tr>
<td>g</td>
<td>Relative centrifugal force in terms of gravity</td>
</tr>
<tr>
<td>GA</td>
<td>Galacturonic acid</td>
</tr>
<tr>
<td>GO</td>
<td>Green-orange fruit colour</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole acetic acid</td>
</tr>
<tr>
<td>kat</td>
<td>Katals (moles of galacturonic acid per second)</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MG</td>
<td>Mature green stage of fruit development</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamp</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>n</td>
<td>Individuals in a sample</td>
</tr>
<tr>
<td>nsp</td>
<td>Specific viscosity</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>Sodium sulphate</td>
</tr>
<tr>
<td>ND</td>
<td>Not detectable</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>Ammonium sulphate</td>
</tr>
<tr>
<td>nor</td>
<td>Non-ripening</td>
</tr>
<tr>
<td>Nr</td>
<td>Never-ripe</td>
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0 Orange fruit colour
OG Orange-green fruit colour

PAGE Polyacrylamide gel electrophoresis
PG Polygalacturonase
PG1 Polygalacturonase isoenzyme 1
PG2 Polygalacturonase isoenzymes 2A and 2B
PG2A Polygalacturonase isoenzyme 2A
PG2B Polygalacturonase isoenzyme 2B
PGA Polygalacturonic acid

poly (A) + RNA Polyadenylate ribonucleic acid

r Correlation coefficient
R Red fruit colour
R' Soft, red fruit
rin Ripening inhibitor
RNA Ribonucleic acid

s Second
SDS Sodium dodecyl sulphate

TCA Tricarboxylic acid
Tris 2-Amino-2-hydroxymethylpropane-1,3-diol

μp Specific viscosity
var. Variety of tomato

v/v Volume (mls) per volume (mls)
w/v Weight (grams) per volume (mls)
w/w Weight (grams) per weight (grams)

+ Anode
- Cathode

in PAGE diagrams
ACKNOWLEDGMENTS

I wish to express my gratitude to my supervisor, Dr. G. E. Hobson for his guidance and encouragement, and to Professor J. B. Pridham and Dr. P. Dey of the Biochemistry Department, Royal Holloway College for their valued advice.

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1.1. THE TOMATO

The cultivated tomato (*Lycopersicon esculentum* Mill.), a member of the potato family, Solanaceae, was introduced to Europe from the lower slopes of South America in the 15th Century (Davies and Hobson, 1981). Subsequent breeding and selection have produced many cultivars and genotypes, including some mutants with non- or slow-ripening characteristics (Tigchelaar et al., 1978a). Now the tomato fruit is one of the world's most important horticultural crops, its total world production in tonnes being exceeded only by those of grapes and citrus fruits (1979 data, Davies and Hobson, 1981).

The yellow hypogynous tomato flowers, borne in a forked racemous cyme, are generally self-pollinated, and subsequently produce a berry fruit. After fertilisation, the ovary wall swells by cell division for the first seven to fourteen days, then by cell enlargement to form the fleshy pericarp of the tomato fruit. This consists of outer, radial and inner walls which enclose locular cavities containing the seeds. These develop on placental tissue in the middle of the fruit; as they do so they are surrounded by parenchyma cells, derived from the placenta, which eventually fill the locular cavities and form a jelly-like tissue in ripe fruit. As fruit mature so the parenchyma cells of the pericarp tissue become very large (300 µm diameter) and some separation of cells along the middle lamella occurs (Davies and Hobson, 1981; Crookes and Grierson, 1983). They contain a large central vacuole surrounded by a thin layer of cytoplasm (about 10 µm thick), containing chloroplasts,
mitochondria, Golgi bodies, ribosomes, endoplasmic reticulum, vesicles, microbodies, lipid droplets and nuclei (Harris and Spurr, 1969a; Simpson et al., 1976; Crookes and Grierson, 1983).

1.2. FRUIT RIPENING

When tomato fruit are at least 80% full size, and the seeds have matured, ripening is initiated. Fruit ripening involves a complex series of morphological, biochemical and physiological reactions which transform the fruit over a period of time from a hard, inedible state to one which is soft, aromatic and edible (Biale, 1964; Hansen, 1966; Sacher, 1973).

The tomato falls into the group of fruit classified as 'climacteric' by Biale (1960) since the morphological and biochemical changes associated with ripening occur over a relatively short period of time, and are accompanied by a rise in respiration, termed the climacteric, and an increase in ethylene production.

The most obvious changes that occur during normal ripening of cultivated tomatoes are those associated with colour, flavour and texture. Mature green tomatoes turn progressively yellow, orange and finally red due to a loss of chlorophyll and synthesis of carotenes and lycopene pigments (Hobson and Davies, 1970). These pigment changes are associated with the conversion of chloroplasts to chromoplasts (Harris and Spurr, 1969a). As chlorophyll is degraded (Ramirez and Tomes, 1964), and lycopene crystals deposited, so the typical granal composition of the chloroplast gives way to the less structured chromoplast (Harris and Spurr, 1969b; Simpson et al., 1976).
As the starch content of chloroplasts declines at the onset of ripening (Yu et al., 1967), so the sugar content of the fruit increases, the predominant sugars being glucose and fructose (Hobson and Davies, 1970).

Fruit acidity increases during the early stages of ripening to a maximum coinciding with incipient colouring, after which it declines (Davies and Hobson, 1981). Thus tomatoes become sweeter and less acid as they ripen. Another important component of tomato flavour is its aroma. Yu et al. (1967; 1968a and b) observed that the content of many amino acids declined during ripening, accompanied by an increase in enzymes capable of producing volatile, aromatic, carbonyl compounds from some of these amino acids.

Softening is an integral part of tomato ripening, rendering the fruit palatable and juicy. When fruit reach maturity, the locular parenchyma cells swell and become jelly-like (Davies and Hobson, 1981). The pericarp tissue also begins to soften due to cell wall breakdown, the mechanisms of which will be discussed later.

These changes in colour, texture and flavour are accompanied by an increase in oxidative metabolism during fruit ripening. Much of the increase in oxygen uptake is due to the respiratory climacteric which accompanies incipient ripening (Rowan et al., 1958; Biale, 1960). Fruit mitochondria remain viable and active throughout ripening, however they appear to have highest activity when isolated from fruit just prior to the climacteric (Hobson, 1969), and their activity decline thereafter (Dickinson and Hanson, 1965). The causes of the respiratory climacteric have not been fully elucidated. Enzymes of glycolysis e.g. phosphofructokinase, have been observed to
increase in activity at the onset of ripening, possibly due to a rise in orthophosphate in the cytoplasm of fruit cells caused by a greater demand for ATP (Chalmers and Rowan, 1971). Studies using many types of fruit tissue (Rowan et al., 1958; Young and Biale, 1967; Biale and Young, 1981) have produced no evidence that the respiratory climacteric is due to the uncoupling of respiration and phosphorylation. In fact Biale and Young (1981) showed that respiration was more tightly coupled in climacteric than pre-climacteric cells.

Respiration is not the only oxygen consuming process to occur during ripening. The conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene requires oxygen, possibly in the form of hydroperoxides (Adams and Yang, 1979; Legge and Thompson, 1983), and tomato lycopene synthesis is stimulated by high oxygen levels rather than by ethylene (Frenkel and Garrison, 1976). The increase in peroxide levels in ripening tomatoes, due in part to the metabolism of cell walls (Mukai, 1981) and of peroxisomes and microbodies, which accompanied ethylene production and fruit softening, led Frenkel (1975, 1978) to suggest that ripening and senescence were oxidative phenomena. He proposed that peroxides might be utilised in the oxidation of sulphhydryl groups, lipids and auxins. The latter role was seen to be particularly important since auxins may play a part in retarding fruit ripening (Dilley, 1969; McGlasson et al., 1978). IAA oxidase/peroxidase systems increase in activity in ripening fruit and the oxidation products of IAA have been shown to hasten ripening (Frenkel, 1972, 1975).

The numerous physiological and biochemical processes occurring during ripening require the activation of enzymes involved in both
anabolic and catabolic events. Thus ripening cannot be viewed merely as degradative changes leading to fruit senescence and death, but a very active and important stage of fruit development. Many of the changes in enzyme activity may be ascribed to increased activation, such as that of phosphofructokinase and enzymes involved in starch hydrolysis in bananas (Young et al., 1975), due to disappearance of inhibitors, mobilisation of calcium from other sites, or changes in membrane organisation. The latter hypothesis was suggested by Sacher (1973), Solomos and Lates (1973) and Poovaiah et al. (1975) who envisaged that alteration in membrane permeability caused by ethylene, led to loss of cell compartmentation, resulting in changes in metabolite distribution and ion fluxes, which in turn activated some enzymes. However, changes in the permeability of the tonoplast and plasmamembrane have not been observed (Vickery and Bruinsma, 1973), although localised changes in these and other membranes, such as those of organelles, may account for some enzyme activation (Hobson, 1979).

Increase in the activity of enzymes may also be due to their synthesis. The view that ripening is a stage in fruit ontogeny reflecting the expression of discrete genetic codes is now widely held (Frenkel, 1972; Grierson et al., 1981; Christoffersen et al., 1982).

Although there are conflicting reports of net protein synthesis in ripening tomatoes (Rowan et al., 1958; Yu et al., 1967), there is evidence that many enzymes and isoenzymes are synthesised during ripening, possibly at the expense of other enzymic proteins (Hobson, 1974; Grierson et al., 1981). Inhibitors of protein synthesis such as cycloheximide and chloramphenicol, prevent ripening of pears,
tomatoes and bananas (Frenkel et al., 1968; Brady et al., 1970; Hobson, 1975). The incorporation of radio-active amino acids into proteins was found to be most active early in the climacteric period of tomatoes, (De Swardt et al., 1973), pears (Frenkel et al., 1968) and apples (Hulme et al., 1971). Hobson (1974) observed that many enzymes showed peak activity, and a maximum number of isoenzymic forms, at this pre-climacteric period.

Some of these changes in enzyme activity have now been traced back to genetic activity at both transcriptional and translational levels. The rate of RNA synthesis has been observed to increase at the onset of avocado (Richmond and Biale, 1967) and pear (Ku and Romani, 1970) ripening. Rattanapanone et al. (1978) observed new mRNA species in the poly(A)+ RNA fraction of ripening tomatoes and recently Grierson and Slater (1984) have identified over fifteen mRNA changes occurring during tomato ripening. They have also purified in vitro translation products from mRNA isolated from unripe and ripe tomatoes and shown that between four and eight new products from mRNA were formed during ripening. One of these new proteins reacted with antibodies raised against tomato polygalacturonase, an enzyme important in wall hydrolysis (Grierson et al., 1981).

Christoffersen et al. (1982) found at least three new mRNA species in ripening avocados, one of which produced a protein which reacted to antibodies raised against cellulase (Tucker et al., 1984). New gene expression was observed to occur only ten hours after ethylene production in avocados (Tucker and Laties, 1984; Tucker et al., 1984).

Small changes in the translational control of protein synthesis at the beginning of tomato, pear and fig ripening were observed by Romani (1975), and these changes were later found to be stimulated by
ethylene treatments (Mettler and Romani, 1976). Thus ripening appears to be genetically regulated at both the transcriptional and translational levels.

1.2.1. Initiation and control of fruit ripening

The metabolic events associated with ripening are normally highly co-ordinated, however they are not immutably linked. Storing tomatoes under controlled atmospheres reduced softening and pigment synthesis but did not affect starch hydrolysis (Goodenough et al., 1982), and gibberellin treatment reduced colour development and softening of tomatoes without affecting respiration (McGlasson et al., 1978). This suggests that ripening events occur along a number of parallel pathways which are loosely linked and co-ordinated in time. The mechanisms by which ripening is initiated and the subsequent metabolic events co-ordinated have not been elucidated, but growth regulators, particularly ethylene, are thought to play an integral role (Dilley, 1969; McGlasson et al., 1978).

In normal ripening tomatoes ethylene and its precursor ACC, are found at low levels in mature green fruit. Both increase just prior to colour development, and ethylene production continues to rise to a peak coincident with the light red colour stage, whilst ACC levels remain fairly constant throughout ripening (McGlasson et al., 1975; Hoffman and Yang, 1980). Ethylene has been implicated in initiating ripening (Lieberman, 1979) since removal of ethylene from the gas phase surrounding fruits retards ripening, whereas an exogenous supply frequently accelerates ripening (Lyons and Pratt, 1964).
Ethylene stimulates fruit respiration (Reid and Pratt, 1970) and increases peroxidase activity (Abeles, 1973), thus possibly stimulating auxin oxidation. It also affects other ripening events such as softening and lycopene synthesis (Jeffery et al., 1984) and possibly mRNA synthesis (Grierson and Slater, 1984; Nichols and Laties, 1984; Tucker et al., 1984). However, there is no evidence as yet to prove that it directly stimulates the activity of any specific enzyme, or that it acts as a cofactor, co-enzyme or uncoupling agent (McGlasson, 1970). In addition, there are some unresolved problems associated with the idea that ethylene is required to initiate ripening. Firstly, there is evidence that fruit stored under hypobaric or low ethylene conditions can ripen, albeit slowly, especially if the oxygen concentration is above atmospheric levels (Stenvers and Bruinsma, 1975). Secondly, whilst some parameters or ripening are influenced by ethylene e.g. pigment development, others such as starch hydrolysis are not (Jeffery et al., 1984). Thirdly, the biosynthesis of ethylene via the methionine pathway itself requires the induction of a key enzyme ACC synthase (Yu et al., 1979; Hoffmann and Yang, 1980). It is possible that ethylene production at the onset of ripening is derived from organic acids and as the concentration of these rises during ripening so does ethylene production, until it reaches levels high enough to trigger its autocatalytic production (De Pooter et al., 1984).

The sensitivity of fruit tissue to ethylene may be the critical factor in the initiation of ripening, rather than the concentration of ethylene itself (Trewavas, 1984). Although tomatoes develop the capacity to ripen about fifteen days after anthesis (McGlasson and
Adato, 1977), fruit remain insensitive to physiological levels of ethylene until 40 to 50 days post-anthesis (Lyons and Pratt, 1964). Sensitivity of fruit to ethylene varies not only with fruit age but is also affected by detachment of fruit from the parent plant. This suggests that the plant produces inhibitors of ripening which it translocates to the fruit, or that the transfer of metabolites or growth factors to or from the fruit is a factor regulating its ripening.

Auxins are generally regarded as ripening retardants (Sacher, 1973), since, as long as adequate penetration of applied auxin into fruit tissue occurred, auxins have been observed to inhibit ripening (Vendrell, 1969; Babbitt et al., 1973; Frenkel and Dyck, 1973). Conversely the oxidation products of auxins have been observed to stimulate ripening (Frenkel et al., 1975). From these results Frenkel hypothesised that the decline of endogenous auxin levels stimulates fruit ripening. However, whilst Abdel-Rahman et al. (1975) found auxin levels to decline prior to ripening, El-Betagy et al. (1976) reported a general increase in acidic auxins throughout fruit development. Thus the role of auxins in tomato ripening is still uncertain.

Likewise, although endogenous levels of gibberellins and cytokinins decline during ripening (Abdel-Rahman, 1977; Desai and Chism, 1978), and applied gibberellins and cytokinins have been shown to delay ripening in tomatoes (Dostal and Leopold, 1967; Babbitt et al., 1973; Bruinsma et al., 1975; Lieberman et al., 1977; Lin, 1978), there have been conflicting reports on their effects (Mizrahi et al., 1975), and the nature of their regulatory role has yet to be ascertained.
McGlasson and Adato (1976) measured abscisic acid levels in developing tomatoes and found that they reached a peak at the mature green stage. Since abscisic acid applied to tomatoes stimulated ripening (Nizrahi et al., 1975; Lin, 1978) and enhanced ethylene production (Lieberman et al., 1977), it was hypothesised that it might play an important role in initiating fruit ripening (McGlasson and Adato, 1976). However, McGlasson et al. (1978) pointed out that because of impurities, results of experiments using applied abscisic acid must be treated with caution, and as yet there is no good evidence for its involvement in fruit ripening. It is likely that the relative amounts of the growth regulators rather than their individual, absolute concentrations regulate ripening (Lin, 1978).

Factors other than growth regulators may play a part in the initiation and regulation of ripening. Tigchelaar et al. (1978a) suggested that enzymes degrading the walls of fruit cells, such as polygalacturonase, released enzymic proteins which were involved in ripening metabolism. This was supported by evidence from Strand et al. (1976) and Hobson et al. (1983b) who found that polygalacturonase released proteins, some with enzymic activity from cell walls. Degradation of the complex polysaccharides of the cell wall during ripening into oligosaccharides with regulatory functions might also affect ripening according to a recent hypothesis by Albersheim et al. (1983). However, Grierson and Tucker (1983) found that polygalacturonase was not synthesised until after the rise in ethylene production by tomato fruit, thus could not initiate ripening. However, it is possible that the rate of cell wall degradation could influence the subsequent rate of fruit ripening once the process has been initiated,
either directly by the release of regulatory compounds, e.g. enzymes, or indirectly by influencing the cellular environment and plasmalemma integrity.

Thus studies of cell wall metabolism during fruit ripening may not only further knowledge of fruit softening, itself a process of importance in commercial fruit handling and storage, but of other ripening events as well. Hence the structure of the cell wall and its changes during fruit ripening will be discussed further.

1.3. CELL WALL STRUCTURE AND METABOLISM DURING RIPENING

The softening of tomato fruit is one of the major changes occurring during ripening. The softness and texture of tomato fruit tissue is affected by fruit structure, cellular anatomy, water relations of cells and composition of the cell walls.

1.3.1. Composition of cell walls

The plant cell wall is a dynamic structure; during fruit growth, maturation and ripening, the shape, composition and properties of the wall are constantly changing in response to the environment of the cell and its activities (Selvendran, 1983). Its structure first emerges as cells of meristematic tissue divide and become separated by a newly synthesised partition, the cell plate. Once this plate is fully formed the cells that it separates deposit wall constituents on either side of it, while it remains as a layer of intercellular cement called the middle lamella (Selvendran, 1983).
The structure of the middle lamella has not been studied as extensively as that of the primary cell wall. However, it is thought to be an amorphous region composed largely of pectic substances (Albersheim and Killias, 1963). This has been confirmed by the fact that pectolytic enzymes cause dissolution of the middle lamella (Crookes and Grierson, 1983) and cell separation (Cormack, 1956). These pectic polysaccharides are thought to be less highly substituted i.e. have fewer rhamnose residues and neutral sugar side chains, than the primary wall pectins (Knee, 1975). Thus the chains are able to pack together in parallel, each pair of residues enclosing a calcium ion in 'egg-box' junction zones (Rees, 1969; Grant et al., 1973), forming insoluble calcium pectate. Treatment of cells with chelating agents, such as EDTA dissolved the middle lamella layer (Letham, 1958; Ginsburg, 1961; Linehan and Hughes, 1969), suggesting that cohesion of the middle lamella depends on ionic rather than covalent bonds.

The primary cell wall has a higher degree of organisation than the middle lamella, consisting of polysaccharides such as pectic substances, hemicelluloses, cellulose and glycoproteins. The cellulose microfibrils being embedded in an amorphous matrix of pectic and hemicellulose polysaccharides and hydroxyproline rich glycoprotein (Northcote, 1972).

Water is an important component of cell walls (Selvendran, 1983). Polysaccharides at normal humidities contain eight to ten percent water as water of hydration. This may influence the conformation of the matrix polysaccharides, the strength of

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interpolysaccharide associations, the permeability of the wall, and enzymic reactions likely to occur within the cell wall, since these reactions are dependent on the presence of solvated metallic ions as well as low molecular weight substances. The quantity of water within the wall matrix can be controlled to some extent by the proportions of the polymers, as pectic polysaccharides are able to bind more water than hemicelluloses (Bartley and Knee, 1982). Chemical analysis of angiosperm primary cell wall has shown that it is composed of 30-60% cellulose, 15-45% pectic polysaccharides, 15-22% hemicellulose, and 10-15% glycoproteins. Recent work by Selvendran (1983) suggests that it also contains 5-10% polyphenolic compound in the form of polysaccharide-protein-polyphenol complexes. The relative proportions of cell wall materials depends very much on the type of tissue under consideration.

Fibrils of cellulose are considered to be the structural backbone of the cell wall. The polymer is composed of β-glucose residues glycosidically linked to each other from C-1 of one residue to C-4 of the adjacent residue (Selvendran, 1983). Its degree of polymerisation ranges from 8000 to 12000, which corresponds to a chain length of 40000 to 60000 Å and a molecular weight of more than a million (Selvendran, 1983). Interchain associations are stabilised by hydrogen bonds, thus the molecules are arranged in a highly ordered manner parallel to each other, usually in groups of 100 or more to form a strong fibril. These fibrils are further organised into groups of ten to twenty to form microfibrils, which vary from 10-25 nm in breadth depending on species. Recent evidence suggests that the microfibrils have the same polarity and are oriented in the plane of the wall (Lamport and Epstein, 1983).
It is thought cellulose chains pass repeatedly through highly ordered crystalline regions, just described, and amorphous regions of a low degree of order; other linear carbohydrate molecules may be incorporated into the amorphous region. Since the molecules in the crystalline region are held by hydrogen-bonds, these regions are difficult to penetrate by reagents, whilst the amorphous regions are more reactive. The proportion of cellulose in these two forms is probably of importance in explaining differences in properties of cellulose in different cells (Selvemran, 1983).

The primary cell walls of dicotyledonous plants have a relatively high content of pectic polysaccharides (20–30%) (Darvill et al., 1980), which form complex colloidal mixtures capable of forming gels. The dominant structural feature of these pectins is a linear chain of \( \alpha(1-4)-D\)-galacturonan molecules, which have a C-1 conformation, and form axial-axial glycosidic bonds, giving the pectic polymer chain a threefold screw axis (Pilnik and Voragen, 1970; Aspinall, 1980). The carboxyl groups of these galacturonic acids may be partly esterified by methyl, or occasionally acetyl, groups and partly or completely neutralised by one or more bases (McCready and McComb, 1954; Worth, 1967).

Methoxyl content of fruit cell wall pectin varies according to the type of fruit, from 0.2% in strawberries to 9–11% in apples (Worth, 1967). The polygalacturonic acids in cell walls have been classified according to their degree of methylation into two major groups, (1) the pectic acids, those polygalacturonic acids essentially free of methoxyl groups, the salts of which are termed pectates, and (2) the pectinic acids, those acids with more than a negligible methoxyl
content, the salts of these being called pectinates (Joesburg, 1973).
The distribution of methyl ester groups along polygalacturonic acid
chains is still unknown, but the degree of methylation is known to
affect the aggregation and intermolecular bonding of pectins.

Low-methoxyl pectic acid may form calcium or magnesium
cross-bridges with itself, or with other polysaccharides with acidic
groups. Single calcium links have little effect on the strength of
the polygalacturonic acid chain, but the buckled conformation of
the polygalacturonic chain leaves spaces for the insertion of several
divalent cations, which create a strong bond (Rees, 1969; Grant et al.,
1973; Jarvis, 1984). Whilst the existence of such bonding in middle
lamella pectins has been shown, the extent to which it occurs in the
cell wall is unknown.

In addition to the galacturonosyl residues, pectic polysaccharides
are characterised by the presence of rhamnosyl, arabinosyl, xylosyl and
galatosyl residues (Barrett and Northcote, 1965; Talmadge et al., 1973).
Of these, L-rhamnose appears to be unique, since in certain areas of
the pectin polymer it is found linked in the galacturonan chain to
form a distinct polymer, called Rhamnogalacturonan 1 (Barrett and
Northcote, 1965; Talmadge et al., 1973; McNeil et al., 1980). It
accounts for at least 23% of wall pectic polymers (McNeil et al., 1980).
These two types of residue are thought to be arranged in an orderly
fashion as rhamnosyl-(1-4)-galacturonosyl-(1-2)-rhamnosyl units
(Talmadge et al., 1973; McNeil et al., 1980), to form a Z-shaped
molecule. This polymer appears to alternate with a homogalacturonan
chain containing about eight galacturonic acid residues. These two
types of polymer give rise to the two major fractions obtained by
chemical and enzymic hydrolysis of cell wall pectins (Barrett and
The rhamnogalacturonan polymer is also rich in neutral sugar residues, 50% of which are thought to be attached to the C-4 of the rhamnosyl residue to form a Y-shaped branch point (Knee et al., 1975; McNeil et al., 1980, 1982; de Vries et al., 1982; Thibault, 1983). Most of these residues are either branched arabinan or linear galactan molecules, and some xyloglucan residues have been found attached to the galactan side chains (Talmadge et al., 1973), but over seven different types of glucosyl side chains have been found attached to rhamnogalacturonan (McNeil et al., 1982). Thus rhamnogalacturonan 1 can be viewed as a complex family of polysaccharides, all with a similar backbone but possessing many different side chains.

Darville et al. (1978) have identified another pectic polymer, which they call rhamnogalacturonan 2. It also appears to be a complex polysaccharide containing up to ten different monosaccharides. This pectin contributes to three percent of the wall polysaccharides, but its structure and function are unknown.

Since neutral sugar side chains appear to be attached only to the rhamnogalacturonan chain, the pectin polymers of the wall have "hairy" and "smooth" regions, reflecting the alternate homogalacturonan (smooth) and rhamnogalacturonan (hairy due to neutral sugars) chains in the backbone (de Vries et al., 1982; Thibault, 1983).

Recently, phenols such as ferulic and coumaric acids have been found linked to the neutral sugar side chains of pectins in cell walls (Fry, 1983; Selvendran, 1983). Fry (1983) has suggested that these phenols could be oxidised by wall-located peroxidases and the resulting oxidative coupling could cause the cross-linking of pectin molecules via di-ferulate bridges.
Xyloglucans are the predominant hemicellulosic polysaccharides in walls of parenchymatous cells (Bauer et al., 1973; Knee et al., 1975; Selvendran, 1983). They consist of a \( \beta-(1-4) \)-linked glucan backbone with frequent xylosyl side chains attached to C-6 of the glucosyl residues (Bauer et al., 1973). The xylose residues may be extended by the addition of \( \beta-D \)-galactopyranose, L-arabinofuranose or \( \beta-L \)-fucopyranosyl(1-2)-\( \beta-D \)-galactopyranose residues (Selvendran, 1983). In primary cell walls, xyloglucan is hydrogen bonded to cellulose (Selvendran, 1983), and Bauer et al. (1973) calculated that there is enough xyloglucan in sycamore cell walls to encapsulate all the cellulose fibrils with a monolayer of xyloglucan. Darvill et al. (1980) have identified a second, complex hemicellulose called glucuronolcarabinoxylan, which comprises up to five percent of the wall polysaccharides.

The primary cell walls of dicots contain between three and six percent w/w protein, mainly as glycoproteins. These have been classified into two groups — those rich in, and those poor in hydroxyproline (Selvendran, 1983). The relative amounts of each type present in the cell wall varies with tissue. Whilst most of the protein in walls is regarded as being structural (Lamport, 1970), some of it has enzymic activity (Hobson et al., 1983b).

It is the hydroxyproline-rich fraction, called extensin, that is thought to play an important part in the structural integrity of the cell wall. The backbone of extensin is formed of repetitive, rod-like pentapeptide groups, composed of one serine residue linked to four hydroxyproline residues (Talmadge et al., 1973; Lamport and Epstein, 1983). Arabinosyl residues wrap around and hydrogen bond
with the polypeptide backbone, through proline C-4 hydroxyl groups, thereby stabilising the whole structure. Although extensin is very insoluble, thus difficult to characterise, soluble, monomer precursor proteins have now been isolated (Cooper and Varner, 1983; Smith et al., 1984). Two of these have been analysed and together they were found to have an amino acid composition similar to that of extensin (Smith et al., 1984). Cooper and Varner (1983) observed that the precursor molecules could be made insoluble by an isodityrosine complex. The crosslinked amino acid isodityrosine has been isolated from cell wall hydrolysates and from tryptic peptides of extensin (Fry, 1983; Epstein and Lamport, 1984) and has been found to form intramolecular linkages between extensin molecules (Epstein and Lamport, 1984). Thus it is suggested that the precursor proteins are crosslinked in muro by isodityrosine to form a heteromultimeric extensin network (Smith et al., 1984).

1.3.2. Interconnections of wall polymers

The primary cell wall of higher plants is very thin, yet it has two seemingly incompatible features; plasticity and high tensile strength. To account for these properties, various interconnections between wall polymers have been proposed.

Cellulose fibrils are thought to be the structural backbone of the wall, and appear to be held together by hydrogen bonds. Cellulose and hemicellulose polymers are also thought to be crosslinked by hydrogen bonds (Darvill et al., 1980). The hemicellulose molecules may also bind to each other to form aggregates and gels which in turn could crosslink to other polymers (Bauer et al., 1973; Darvill et al., 1978). There is good evidence that pectic polysaccharides and neutral
Sugars covalently bond to form a complex pectin network (Talmadge et al., 1973), and that pectin microfibrils may be formed through calcium (Grant et al., 1973) or phenol (Fry, 1983) crosslinks.

Talmadge et al. (1973) were the first to propose a model for primary cell wall structure (Plate 1.1). They suggested that the hemicellulose-coated cellulose microfibrils were covalently attached to the arabinogalactan side chains of pectin molecules. However, later experiments have not confirmed this linkage (Darville et al., 1980). Certainly xylose and glucose molecules have been found attached to pectins (Knee et al., 1975) but the nature of the linkages remains unknown.

There is much evidence to suggest that wall glycoproteins are attached to pectic polysaccharides (Ginsburg, 1961; Talmadge et al., 1973; Selvedran, 1983), but the nature of these links are unknown, except that phenols may be involved as bridging links (Fry, 1982, 1983; Selvedran, 1983). Talmadge et al. (1973) produced evidence that protein-pectin links might be formed between the arabinogalactan sidechains of the pectin molecules and the serine residues of extensin. However, such covalent attachments have not been verified (Darville et al., 1980).

Recently a new model for primary wall structure has been hypothesised by Lamport and Epstein (1983), (Plate 1.2), based on evidence of the existence of a bipheryl crosslinking amino acid, isodityrosine, in cell walls and its action in crosslinking extensin (Fry, 1982, 1983; Smith et al., 1984; Epstein and Lamport, 1984). They suggest that the cell wall "is a woven structure consisting of two concatenated polymers: cellulose microfibrils penetrating the mesh of an extensin net, suspended in a hydrophylic pectin-
Plate 1.1. Model of cell wall structure from Albersheim (1976) based on the data of Keegstra et al. (1973) and Talmadge et al. (1973). The circled areas are representative wall fractions released by degradative enzymes.

Perspective view (A) represents an extensin-rich primary cell wall.

The view in cross-section (B) shows how two isoxytyrosine-crosslinked strands of extensin might define the size and shape of a cellulose microfibril.
EXTENSIN-CELLULOSE NETWORK

A. Perspective view

- Cellulose microfibril
- Crosslinked extensin
- Monomeric extensin
- 100Å
- Cell wall
- Protoplast
- Plasma membrane

B. Cross section view

- Cellulose microfibril
- Extensin
- Tosylated tyrosine crosslink
- Chemical structures:
  - HO-CH2-CHNH2-CO2H
  - CH2-CHNH2-CO2H
  - CH2-CHNH2-CO2H
  - CH2-CHNH2-CO2H
hemicellulose gel". They produced evidence to show that the extensin mesh, crosslinked by isodityrosine, is of the right dimensions to encompass the cellulose microfibrils. These are oriented parallel to the cell plasmalemma whilst the extensin and pectin molecules are inserted perpendicular to the long axis of the wall. The isodityrosine linkages are seen as points of restraint around the cellulose fibrils, aided by hemicellulose bonded to cellulose acting as steric restraints preventing microfibrillar slippage through the extensin net (Lamport and Epstein, 1983). Hemicellulose turnover would remove these restraints, hence allowing cell extension and growth. This model accounts for both the strength and plasticity of the cell wall and is consistent with evidence of wall polymer associations (Talmadge et al., 1973; Knee et al., 1975; Selveniran, 1983). However, further work is required to determine its accuracy.

1.3.3. Changes in fruit cell walls during ripening

The softening of fruits as they ripen is due largely to changes in the amount and structure of cell wall polymers.

Ultrastructural studies show that at the onset of ripening, the electron dense middle lamella loses its structure, and in apples and tomatoes the fibrillar content of the outer primary wall becomes less organised (Ben Arie et al., 1979; Crookes and Grierson, 1983). In pears and avocados more extensive dissolution of the cell wall occurs (Pesis et al., 1978; Ben Arie et al., 1979; Platt-Alcida et al., 1980). Results of chemical analysis of ripening fruit walls substantiate these studies. No evidence for the degradation of cellulose or hemicellulose has been obtained from studies of pears,
The significance of this loss of neutral sugar residues to fruit softening in uncertain. Wallner and Walker (1975) proposed that loss of these sugars could affect the stability of the pectin-cellulose crosslinks in the wall thus causing softening. However, the non-ripening tomato mutant rin, has been observed to have a decline in galactose content during development yet it softens hardly at all (Gross and Wallner, 1979). Wallner and Bloom (1977) found that exogenous polygalacturonase applied to cell walls from unripe tomato fruit caused solubilisation of the walls to an extent observed in normal ripening but did not cause a decrease in wall galactose content. It has, therefore, been proposed that throughout fruit development there is a turnover of sugars in the cell wall, and that loss of galactose during ripening represents a decline in the rate of synthesis rather than an increase in degradation (Gross and Wallner, 1979; Lackey et al., 1980), or an inability of the tissue to metabolise the galactose in the cell wall (Gross, 1983). Thus, although changes in neutral sugars occur during ripening it is thought that they are not closely associated with textural changes, unlike the alterations in pectin composition (Van Buren, 1979; Bartley and Knee, 1982).

Ripening tomato fruit also have been found to lose cell wall protein during the later stages of ripening, much of which has enzymic activity (Hobson, 1964, 1983b) and thus may influence ripening.

The events leading to these cell wall changes are not yet fully understood. However, enzymic degradation of the pectic polymers is without doubt one of the major causes of wall degradation in ripening fruit tissue (Pilnik and Voragen, 1970; Knee and Bartley, 1981); and
the enzyme implicated in this process in tomatoes is an endo-
polygalacturonase (poly(1,4-\( \alpha \)-D-galacturonid)glycane-hydrolase,
EC 3.2.1.15). This enzyme hydrolyses the 1,4-\( \alpha \)-galacturonosyl linkages
of pectic acid. Although both exo- and endo-polygalacturonase occur
in some fruit e.g. peaches, the predominant enzyme in tomatoes is an
endo-polygalacturonase (Pressey and Avants, 1971; Hunter and Elkan,
1974). Pressey and Avants (1971) first suggested that polygalacturonase
exists in at least two isoenzymic forms. These were later separated
and characterised (Pressey and Avants, 1973; Tucker et al., 1980;
Grierson et al., 1981; Ali and Brady, 1982). The largest isoenzyme,
PG1, was found to have a molecular weight of about 80 000 to 115 000
and the smaller PG2 isoenzyme, of 42 000 to 46 000. This isoenzyme
was later resolved into two components, PG2A and PG2B (Rexova-Benkova
et al., 1977; Ali and Brady, 1982).

Polygalacturonase protein and enzymic activity have not been
found in mature green tomatoes (Tucker and Grierson, 1982), but the
enzyme begins to accumulate, as PG1, during the early stages of
ripening; PG2 isoenzymes only appear in normal ripening tomatoes as
they turn an orange colour (Pressey and Avants, 1973; Tucker et al.,
1980). The amounts of PG2 isoenzymes increase rapidly as fruit turn
red and are the predominant form of polygalacturonase in ripe tomatoes
(Tucker et al., 1980).

The role of polygalacturonase isoenzymes in tomato softening
and cell wall degradation is still speculative. Although loss of
firmness and degradation of wall pectins have been associated with
polygalacturonase activity (Poda, 1957; Hobson, 1964; Pilnik and Voragen,
1970; Themmen et al., 1982) the correlation between fruit firmness
and polygalacturonase activity has not always been consistent (Hobson,
The fact that PG2 isoenzymes are the predominant forms of polygalacturonase in soft, ripe tomato fruits and that slow-ripening genotypes, such as Never-ripe, contain only PG1 activity, led Tucker et al. (1980) to suggest that PG2 played an important role in fruit softening. However, they produced no further evidence to support this idea.

Tigchelaar et al. (1978a) proposed that polygalacturonase might be involved in the initiating events of ripening and could regulate the rate of ripening by controlling the release of enzymes from the cell wall. Since polygalacturonase first appears as PG1, this isoenzyme might therefore play an important role in these events. However, Grierson and Tucker (1983) have shown that ethylene production precedes that of polygalacturonase by at least 24 hours, thus discounting the idea that PG1 might initiate ripening. However, it may regulate the subsequent rate of softening and other ripening processes by its effects on cell walls.

Whether PG1 is able to initiate cell wall degradation in vivo, as polygalacturonase has been found to do in vitro (Wallner and Bloom, 1977), or whether it requires the modification of cell wall structure, either by pectinmethylesterase (an enzyme which demethoxylates pectins to pectic acid), or by glycosidase enzymes (which might remove the neutral sugar side chains from rhamnogalacturonan) is unknown. Also whether the loss of calcium from cell walls during ripening is a pre-requisite for PG1 action or a consequence of its action is unclear.
1.4. AIMS OF THIS THESIS

Although the isoenzymes of polygalacturonase have been and are being studied and characterised in vitro in some detail (Pressey and Avants, 1973; Tucker et al., 1980; Brady et al., 1982; Moshrefi and Luh, 1983), studies of their accumulation and effect on fruit softening and wall degradation were limited until very recently when Brady et al. (1983) published a study on the accumulation of isoenzymes in non-ripening mutant hybrid tomatoes.

The aim of this thesis is to investigate the relationship between the accumulation of polygalacturonase isoenzymes, softening and other ripening events in tomato fruit, and their possible roles in vivo by examining:

(a) The accumulation of PG isoenzymes in ripening tomato fruit of several genotypes which have inherently different rates of softening and ripening. There is much variation in the rate and degree of softening among fruit of commercially grown tomato cultivars (Hobson, 1965; Gormley and Egan, 1978). This was utilised in an examination of the contribution made by polygalacturonase isoenzymes to fruit softening during normal ripening.

In addition, several non-ripening mutant genes exist which have been produced in backgrounds nearly isogenic with normal tomato cultivars such as Ailsa Craig, by breeding programmes conducted by Mr. Peter Grimby of Glasshouse Crops Research Institute. These mutant non-ripening tomatoes and their hybrids with normal ripening cultivars, produce fruit of similar morphology but different rates of ripening and softening. Some of these mutant genotypes were used in a further
study of the roles of polygalacturonase isoenzymes in fruit ripening and softening.

Wild tomato species display a variety of ripening characteristics (Grumet et al., 1981), and some of these species provided material for a study of the interaction of polygalacturonase activity with other ripening events.

(b) The degradation in vitro of cell walls and tomato tissue discs by purified polygalacturonase isoenzyme extracts. Although the action of the isoenzymes against defined substrates, such as polygalacturonic acid, has been well studied (Pressey and Avants, 1971, 1973; Ali and Brady, 1982; Noshrefi and Luh, 1983), less is known of their action against natural substrates such as fruit cell walls. Only two studies comparing the activity of PG1 and PG2 isoenzymes against either cell wall preparations (Pressey and Avants, 1982a) or pericarp tissue (Crookes and Grierson, 1983) have been published. Thus in this work the activity of PG1 and PG2 against both cell wall preparations and tomato pericarp tissue discs was examined.

(c) The effect of salt treatment on non-ripening mutants. Salt solutions have been reported to overcome the inhibition of ripening in mutant genotypes such as non-ripening (nor) (Mizrahi et al., 1982). However, whether salt treatment is able to overcome the genetic block to polygalacturonase production has not been ascertained. Thus the polygalacturonase content of salt-treated non-ripening mutant fruit of several genotypes was examined to establish whether its activity could be stimulated by salt treatment, and whether it was necessary for ripening events other than fruit softening.
2.1. INTRODUCTION

One of the first researchers to extract and study tomato polygalacturonase was Foda (1957), and his methods for extraction and assay of enzyme activity were later improved by Hobson (1963, 1964). However, although tomato polygalacturonase was suspected of having different isoenzymes (Patel and Phaff, 1960), it was not resolved into its isoenzymic forms until 1973 when Pressey and Avants (1973) separated two enzymes, having apparent molecular weights of 84,000 and 44,000, using ion exchange and gel filtration techniques. The larger isoenzyme was called PG1, and the smaller, PG2.

These results were confirmed by Tucker et al. (1980) using similar methods, although they estimated that PG1 had a molecular weight of 110,000. However, the characteristics of this isoenzyme were so similar to those of PG1 separated by Pressey and Avants (1973), that the two were thought to be the same enzyme.

The existence of a third isoenzyme was first shown by Rexová-Benková et al. (1977) using a column of crosslinked pectic acid. However, its molecular weight and enzymic characteristics were not studied. Later, after my own work had started, Ali and Brady (1982) reported having resolved the PG2 isoenzyme into two components, PG2A, with a molecular weight of 43,000, and PG2B, with a molecular weight of about 45,000.
Since any examination of the role of polygalacturonase in fruit softening requires quantitative extraction of the enzyme in forms which reflect the in vivo isoenzymic content, it was necessary to investigate methods for enzyme extraction and analysis of the isoenzyme content of these extracts. In addition, purified samples of PG1 and PG2 were required, for studies of their possible physiological roles in cell wall degradation.

2.2. MATERIALS AND METHODS

2.2.1. Methods for the extraction of tomato polygalacturonase

Three methods for the extraction of polygalacturonase (PG) were investigated to determine the optimum conditions for isoenzyme extraction. Since Hobson (1964) reported finding no polygalacturonase activity in the locular tissue and very little in the placental tissue of ripe tomatoes, only the pericarp tissue was used in these experiments. The tissue was blotted to remove excess cell sap and frozen at -20°C for no more than four months prior to extraction. Hobson (1963) reported finding no loss of activity in fruit tissue stored in this way for up to six months.

The methods for polygalacturonase extraction investigated were:

(a) Hobson (1963, 1964): Frozen tomato pericarp tissue (30 g) was macerated with 2.25 g sodium chloride/disodium EDTA (10:1 w/w). The homogenate was brought to pH 6.0 with 1 M sodium hydroxide. The mixture was left 30 minutes at 0°C and then centrifuged at 2 000 g for seven minutes. The liquid layer was decanted and filtered through muslin and Whatman No. 541 filter paper with suction. The filtrate
was kept at 0°C. To the pulp, 20 ml of 7.5% solution of sodium chloride/disodium EDTA (10:1 w/w) was added, and the solid material extracted in the same way. After the third extraction, the filtrates were combined to form the crude extract. Cold (-20°C) absolute alcohol was added to the filtrate in the ratio 5:1 (v/v alcohol:filtrate) and left overnight at -20°C. Following centrifugation, for seven minutes at 2,400 g, the precipitate was freeze-dried for 40 minutes. The precipitated enzyme was brought back into solution by adding 5 to 10 ml 0.15 M sodium chloride to the solid material, according to expected activity, and centrifuging until clear.

(b) Tucker et al. (1980): Tomato pericarp tissue (30 g) was macerated with an equivalent volume of cold distilled water to an homogenate, which was then centrifuged at 2,400 g for ten minutes. The pellet was resuspended in 30 ml 1 M sodium chloride, the pH adjusted to 6.0 with 1 M sodium hydroxide, and the suspension stirred for three hours at 0°C. After further centrifugation at 2,400 g for ten minutes, the supernatant was filtered through muslin and Whatman No. 541 filter paper. The filtrate was made 75% saturated with respect to ammonium sulphate and left overnight at 0°C. The resulting precipitate was centrifuged at 0°C for 20 minutes at 10,000 g and resuspended in 5 to 10 ml 0.15 M sodium chloride.

(c) Ali and Brady (1982): Tomato pericarp tissue (30 g) was homogenised at 4°C in 50 ml 0.017 M Tris, 5 mM 2-mercaptoethanol pH 10.5. After centrifuging at 15,000 g for 15 minutes, the pellet was incubated for one hour in 20 ml 1.7 M sodium chloride, 50 mM sodium citrate, 15 mM disodium EDTA pH 5.5. The resulting slurry was centrifuged at 15,000 g for 30 minutes. The supernatant, or crude extract, was subject to
ammonium sulphate fractionation in the 40-80% saturation range and, after centrifuging at 10 000 g for 20 minutes, the precipitate was dissolved in 5 to 10 ml 0.125 M sodium acetate pH 6.0.

2.2.2. Assay of polygalacturonase activity

Two types of method for measuring polygalacturonase activity were investigated. One chemically measures the increase in reducing power of the substrate (polygalacturonic acid) due to its hydrolysis by polygalacturonase. The second measures changes in the viscosity of the substrate.

Two chemical methods were examined:

(a) Hobson (1964): The assay medium comprised 0.125 g polygalacturonic acid (Sigma, Grade 3), 1 ml saturated (at 20°C) sodium chloride and 0.04 M potassium phthalate buffer pH 4.25 made up to 50 ml. Enzyme extract (between 0.1 and 1 ml, depending on activity) was incubated with 7.5 to 8.4 ml of substrate at 30°C for 20 to 60 minutes, again depending on enzyme activity. The increase in reducing power of the substrate brought about by the action of the enzyme was measured by mixing 1 ml samples (two replicate samples were taken every 10 or 15 minutes) with 2 ml saturated (at 20°C) picric acid solution and 1 ml 20% (w/v) sodium carbonate. The mixture was heated at 100°C for one hour, cooled, made up to 10 ml with industrial ethanol and kept at 0°C overnight. After centrifugation to remove excess substrate, the optical density of the supernatant was measured at 540 nm. A standard curve was constructed using 0 to 0.8 mg per ml galacturonic acid.
(b) Gross (1982): The assay medium was the same as for (a). The released reducing groups were measured by adding 0.4 ml of the reaction mixture to 2 ml cold 100 mM borate buffer, pH 9.9 and 0.4 ml of 1% 2-cyanoacetamide. The samples were mixed and immersed in a boiling water bath for ten minutes. After equilibration to 25°C, the absorbance of the samples at 276 nm was measured. A standard curve was constructed using 0 to 250 nm galacturonic acid.

Enzyme activity was expressed as the increase in galacturonic acid residues in moles per second (katals).

Viscometric assay of polygalacturonase activity

Decrease in viscosity was measured in a reaction mixture containing 16 ml of 2% (w/v) polygalacturonic acid in 0.5 M sodium acetate pH 4.25, 2 ml 0.19 M ammonium chloride and 2 ml enzyme solution. Flow time was measured in a standardised Oswald viscometer bath (Gallenkamp Ltd) at 30°C.

2.2.3. Identification and separation of polygalacturonase isoenzymes

(a) Separation of PG1 and PG2 isoenzymes by chromatography on DEAE-Sephadex A-50.

A DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) column (2.2 x 40 cm) was equilibrated with 0.15 M sodium chloride pH 6.0. Enzyme preparations (5 to 10 ml) were applied to the column and eluted with the starting buffer at a rate of 15 to 20 ml per hour. Fractions of 2.5 ml were collected and assayed for enzyme activity, using the method of Hobson (1964).
(b) Relative heat stability of PG1 and PG2 isoenzymes.

PG2 activity is lost by heating extracts at 65°C for five minutes, whilst less than 10% PG1 activity is lost after this treatment (Tucker et al., 1980). By measuring the difference in enzyme activity of heated and unheated extracts, the amount of PG1 present was estimated.

(c) Separation of polygalacturonase isoenzymes by polyacrylamide gel electrophoresis (PAGE).

Enzyme samples were examined by disc electrophoresis using glass tubes of 5 mm internal diameter x 10 cm length. The method of Reisfeld (1962) was followed using 7% (w/v) acrylamide in the running gel; 3% (w/v) acrylamide in the spacer gel and β-alanine acetic acid pH 4.5 as the buffer. Methyl green (BDH Chemicals) was used as tracking dye. Electrophoresis was carried out at 4°C. Samples were separated cathodally under a current of 4 mA per gel. The protein bands were stained with Coomassie brilliant blue G250 in 50% (v/v) methanol and 10% (v/v) acetic acid in water for 20 to 30 minutes and destained in 50% methanol, 10% acetic acid aqueous solution.

Alternatively polygalacturonase activity in the gels was detected by the method of Lisker and Retig (1974). Immediately after electrophoresis gels were washed in distilled water several times then placed in 0.02 M sodium acetate buffer pH 4.5 for ten minutes. Gels were then incubated in 1% polygalacturonic acid in potassium phthalate buffer, pH 4.25 at 37°C for ten to 20 minutes, rinsed with water and stained in 0.05% ruthenium red (BDH Chemicals) for 30 minutes. The dye solution was replaced by distilled water after the bands appeared.
Glycoproteins were detected by the method of Felgenhauer (1970). Gels were soaked in 20 ml 0.5 M potassium iodate, 2 ml glacial acetic acid, 0.5 ml concentrated sulphuric acid, 0.2 g TCA for 16 hours, then transferred into a solution of 5 ml glacial acetic acid, 0.5 g TCA in 45 ml water for 16 hours, and finally stained with Schiff's reagent.

The polypeptides of polygalacturonase isoenzymes and their purity were examined under denaturing conditions by SDS electrophoresis. The method described by Tucker and Grierson (1982) was employed. A ten to 15% (w/v) acrylamide in Tris/hydrochloric acid 2.0 M pH 9.18 buffer running gel was overlaid by a 5% (w/v) acrylamide in Tris/sulphuric acid 0.5 M pH 6.1 stacking gel. The lower reservoir contained 0.4 M Tris/hydrochloric acid pH 9.18 buffer and the upper reservoir Tris/borate 0.04 M pH 8.64 buffer. Protein samples were suspended in 40 mM Tris/hydrochloric acid pH 9.18 buffer containing 50% (w/v) sucrose, 10% (w/v) sodium dodecylsulphate and 5% (v/v) mercaptoethanol and boiled for five minutes. The gels were run anodally at 17 mA and 120 mV. Bromophenol blue was used as tracking dye.

Gels were stained in 0.1% Coomassie blue G250 in 50% (v/v) methanol and 10% (v/v) acetic acid in water and then destained in 50% methanol, 10% acetic acid aqueous solution. Ovalbumin, bovine serum albumin and catalase were used as standards.

2.2.4. Examination of the possible in vitro conversion of PG2 to PG1

Tucker et al. (1981) found a factor, possibly a carbohydrate, in green fruit extracts which was capable of converting PG2 to a form similar to that of PG1. For my work it was necessary to establish whether conversion of PG2 to PG1 occurred during enzyme extraction
and purification. Three experiments were conducted, the first two to confirm the results of Tucker et al. (1981) and the third to establish whether their results were due to enzyme-substrate interactions.

**Experiment 1.** Samples of 10 g of mature green tomato pericarp tissue, 5 g of red tissue and a mixture of 10 g green and 5 g red tissue were extracted using Hobson's (1964) method. The crude extracts were centrifuged at 5000 g for 15 minutes to clarify them, then divided into subsamples, half of which were heat treated for five minutes at 65°C to destroy PG2 activity. Enzyme activity was assayed using the picric acid method and the isoenzymes examined by PAGE using the β-alanine acetic acid system.

**Experiment 2.** Unpurified polygalacturonase extracts from red tomatoes were incubated with extracts from NrNr (non-ripening mutant gene isogenic in cv. Ailsa Craig) for one hour and the polygalacturonase isoenzyme content examined by the heat test and by electrophoresis as described in Experiment 1.

The treatments were as follows:—

(1) 0.5 ml red extract + 0.5 ml 0.5 M sodium chloride,
(2) 0.5 ml NrNr extract + 0.5 ml 0.5 M sodium chloride,
(3) 0.5 ml red extract + 0.5 ml NrNr extract,
(4) 0.5 ml red extract + 0.5 ml NrNr extract (latter boiled for 5 minutes).

**Experiment 3.** PG2 purified by gel filtration and affinity chromatography (see Section 2.2.5.) was incubated with polygalacturonic acid (Sigma, grade 3) (PGA) solutions made up to 0.12%, 0.25% and 0.5% in 0.1 M potassium phthalate buffer, the pH of these solutions being adjusted to 3.0, 4.25 or 6.0.
The treatments were as follows:

1. 0.5 ml PG2 + 0.5 ml potassium phthalate buffer,
2. 0.5 ml PG2 + 0.5 ml 0.12% PGA pH 3.0,
3. 0.5 ml PG2 + 0.5 ml 0.12% PGA pH 6.0,
4. 0.5 ml PG2 + 0.5 ml 0.25% PGA pH 4.25,
5. 0.5 ml PG2 + 0.5 ml 0.5% PGA pH 4.25.

After one hour incubation, samples from the above treatments were assayed for polygalacturonase isoenzyme activity by the heat treatment method and by PAGE.

2.2.5. Purification of polygalacturonase isoenzymes

The most commonly used methods for the purification of PG1 and PG2 isoenzymes are ion exchange chromatography, using DEAE-Sephadex A-50 (Pressey and Avants, 1973; Tucker et al., 1980), or carboxymethyl-Sepharose (Ali and Brady, 1982), followed by gel filtration on Sephadex G100 or Sephacryl S-200. Since polygalacturonase isoenzymes are basic proteins, the use of anion exchange chromatography was surprising since the enzyme has a strong positive charge at pH below 7.0, and would not therefore be expected to bind to cations on the DEAE column. Thus, although many researchers use Sephadex A-50 columns, I found they gave poor separations (see 2.3.5.). Once the basic and glycoprotein nature of polygalacturonase had been established by Ali and Brady (1982) better separation procedures were employed.

Since PG1 can be separated from PG2 on the basis of molecular size, gel filtration chromatography was used as an early step in enzyme purification. Initially a Sephadex G100 column was used, but later an Ultrogel ACA44 column was employed. The rigid structure of the Ultrogel
beads made this column easier to use and gave consistent results over a long period of time. The glycoprotein nature of polygalacturonase was utilised by Ali and Brady (1982) in their purification schedule. They used a Concanavalin A-Sepharose affinity chromatography column to separate polygalacturonase from other proteins with good effect, and this procedure was examined as a second step in enzyme purification.

Gel filtration chromatography

A Sephadex G100 (Pharmacia Fine Chemicals) (2.2 x 80 cm) column was equilibrated with 1 M sodium chloride pH 6.0. Enzyme samples of approximately 10 ml were applied to the column and eluted with 1 M sodium chloride at a rate of 20 to 25 ml per hour.

An Ultrogel ACA44 column (LKB Products Ltd) (2.2 x 80 cm) was equilibrated with 1 M sodium chloride or 0.125 M sodium acetate pH 6.0. Enzyme samples of ten to 15 ml were applied to the column and eluted in the starting buffer at a rate of 15 to 20 ml per hour. Fractions (8 ml) were monitored for protein content by their absorption at 280 nm and assayed for enzyme activity.

Affinity chromatography

A column of Concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals) (1.5 x 20 cm) was equilibrated with 0.05 M sodium acetate, 0.5 M sodium chloride, 1 mM calcium acetate, 1 mM manganous sulphate, pH 6.0. Enzyme samples were dialysed against this buffer before application to the column. The column was eluted with the equilibration buffer until no protein, as detected by absorption at 280 nm, was observed in the fractions. A gradient of 0.0 to 0.05 M α-methyl-D-mannoside was used to elute the polygalacturonase enzyme. The flow rate for elution was
10 to 12 ml per hour. Fractions of 4 ml were collected and examined for enzyme activity. The column was regenerated by exhaustive washing with a buffer containing 0.1 M boric acid, 0.1 M sodium chloride, 1 mM calcium acetate, 1 mM manganous sulphate, pH 6.5.

Fractions with polygalacturonase activity were combined and concentrated between purification steps using an Amicon ultrafiltration cell (Amicon Ultrafiltration, Cambridge, MA, USA) with a YM10 diaflow membrane, which excludes molecules with a molecular weight more than 10 000. The apparatus was used under nitrogen at a pressure of 50 lb per in².

Protein estimation

Protein concentration was measured by the method of Hartree (1972) using bovine serum albumin (Sigma, Type F) as a standard. In crude extracts, the protein was precipitated by 5% (w/v) trichloroacetic acid (BDH Chemicals) and redissolved in 0.1 M sodium hydroxide before analysis by the method of Hartree (1972). Alternatively, where protein concentration was expected to be high, the method of Itzhaki and Gill (1964) was used.

2.2.6. Production of antibodies to PG2 isoenzyme

PG2 protein, purified by gel and affinity chromatography, was reduced to a concentration of 1.5 mg per ml, mixed with Freund's complete adjuvant and injected intramuscularly into a rabbit. Injection was repeated after five weeks and the rabbit was bled from a marginal ear vein ten days later. Serum was treated with ammonium sulphate to 50% saturation, the precipitate washed with 1.75 M ammonium sulphate, taken up in 10 ml sodium dihydrogen phosphate pH 7.0 and dialysed.
against water. The lipoproteins precipitated by this treatment were removed by centrifuging and the supernatant stored at -20°C.

Double diffusion analysis

The method of Ouchterlony (1948) was used to examine the reaction between polygalacturonase extracts and antibodies raised against purified PG2. Antibodies were loaded into the centre wells and the polygalacturonase preparations into the outer wells. The diffusion plates were incubated for 24 hours at room temperature after which the non-precipitated proteins were removed by washing in 0.15 M sodium chloride, 0.1 M sodium phosphate, pH 7.0. The precipitation bands were stained with Coomassie blue G250 as described for gel electrophoresis.

2.3. RESULTS

2.3.1. Methods for extraction of polygalacturonase

Comparison of extraction methods (Table 2.1 a and b) show that that of Hobson (1964) extracted significantly more polygalacturonase than those of Tucker et al. (1980) and Ali and Brady (1982). However, Hobson's method also extracted more protein, hence the specific activities of polygalacturonase extracts produced by the three methods were similar. The results in Table 2.1 a were obtained from fruit grown in the autumn whilst those of Table 2.1 b from fruit grown in mid-summer; this might account for the high enzyme activities reported in Table 2.1 b. The efficiency of the Ali and Brady method was increased by employing three sequential extractions (Table 2.2).

Electrophoretic analysis of these extracts (Fig. 2.1) shows that two extractions were required to release PG1, but at least three extractions to release all the PG2 from pericarp tissue.
Table 2.1 a. Polygalacturonase (PG) activity extracted from pericarp tissue of cv. Ailsa Craig at the red stage of ripeness using the methods of (a) Hobson (1964); (b) Tucker et al. (1980) and (c) Ali and Brady (1982). Fruit harvested in late autumn. Means followed by different letters were significantly different at the P 0.05 level by Students t-test.

<table>
<thead>
<tr>
<th>Method of Extraction</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG Activity (μkat g⁻¹ f.wt.)</td>
<td>125.2ᵃ</td>
<td>85.7ᵇ</td>
<td>78.7ᵇ</td>
</tr>
<tr>
<td>Protein (mg g⁻¹ f.wt.)</td>
<td>4.70</td>
<td>3.73</td>
<td>2.60</td>
</tr>
<tr>
<td>Specific Activity (μkat mg protein⁻¹)</td>
<td>26.63</td>
<td>22.96</td>
<td>30.26</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.1 b. Polygalacturonase activity extracted from red pericarp tissue of cv. Ailsa Craig fruit using the methods of (a) Hobson (1964); (b) Ali and Brady (1982). Fruit harvested in early summer. Figures are means of two experiments.

<table>
<thead>
<tr>
<th>Method of Extraction</th>
<th>(a)</th>
<th>(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG Activity (μkat g⁻¹ f.wt.)</td>
<td>212.6</td>
<td>175.5</td>
</tr>
<tr>
<td>(Spread of Values)</td>
<td>(18.5)</td>
<td>(18.6)</td>
</tr>
<tr>
<td>Protein (mg g⁻¹ f.wt.)</td>
<td>3.85</td>
<td>1.53</td>
</tr>
<tr>
<td>Specific Activity (μkat mg protein⁻¹)</td>
<td>55.23</td>
<td>114.70</td>
</tr>
</tbody>
</table>
Table 2.2. The efficiency of extraction of polygalacturonase from orange-red tomato tissue.

The total and percentage activity of PG in each of three successive extractions of 30 g of orange-red cv. Ailsa Craig tomato tissue was measured. PG was extracted by the method of Ali and Brady (1982). Enzyme activity was measured in the crude extract, without further purification, using the picric acid method. The activity and protein levels are the means of two experiments. Figures in brackets indicate variation of readings around mean. Tissue was sequentially extracted with 20 ml of a mixture containing 1.7 M sodium chloride, 50 mM sodium citrate and 15 mM EDTA pH 5.5.

<table>
<thead>
<tr>
<th>Extraction No.</th>
<th>PG Activity $\mu$kat g$^{-1}$ f.wt.</th>
<th>PG of Total Activity %</th>
<th>Protein Content mg g$^{-1}$ f.wt.</th>
<th>Protein Expressed as % of Total Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56.1</td>
<td>72.2</td>
<td>1.06</td>
<td>56.5</td>
</tr>
<tr>
<td></td>
<td>(9.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>17.7</td>
<td>22.8</td>
<td>0.48</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>(1.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td>5.0</td>
<td>0.34</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>(1.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>77.7</td>
<td>100</td>
<td>1.88</td>
<td>100</td>
</tr>
</tbody>
</table>
Fig 2.1. Electrophoretic separation of PG isoenzymes obtained from three sequential extractions of red tomato pericarp tissue.

PG obtained from three successive extractions of red tomato pericarp tissue, using the method of Ali and Brady (1982), was analysed by discontinuous gel electrophoresis using a β-alanine acetic acid pH 4.5 buffer system. The crude extracts were dialysed against 0.3 M sodium chloride for two hours prior to being separated cathodally. After electrophoresis, gels were incubated in 1% polygalacturonic acid in 0.02 M sodium acetate buffer pH 4.25 for 20 minutes, then stained in 0.05% ruthenium red. Enzyme activity is represented by the shaded areas. Gels 1, 2 and 3 represent the isoenzyme content of PG from the first, second and third extractions respectively. The scale on the left of the figure indicates the distance bands moved relative to methyl green.
In his method, Eichler (1964) used ethanol to partially purify the crude enzyme extract, while the other authors used protein precipitate fractions to achieve a degree of purification. Prety (pers. comm.) suggested that ethanol precipitation might cause aggregation of PG activities to a form similar to that of PG1. Major differences in enzyme distribution in extracts prepared by the three methods were observed during this work. The results of an experiment reported in Table 2 confirm this. Maximum activity and ethanol precipitation of proteins-free samples of the same enzyme extracts produced the most total polygalacturonase activity, while precipitation resulted in slight depression of statistically significant proportion of PG2 enzymes precipitation in the first test.

Oxidation of galacturonic acid by enzyme extracts of plant tissues and suspension cultures suggests that a large amount of galacturonic acid is produced in the cytoplasm and is oxidized by the enzyme. The analysis of extracts against standard Eichler method indicated a 25% loss in PG activity, which accounts for a partial inactivation of the enzyme. However, no precipitation of PG1 was observed.

21.2. Assay of polygalacturonase activity

All three methods for assaying polygalacturonase activity investigated have some advantages for their use, but the visual and
In his method Hobson (1964) used ethanol to partially purify the crude enzyme extract, while the other methods used ammonium sulphate fractionate to achieve a degree of purification. Brady (pers. comm.) suggested that ethanol precipitation might cause aggregation of PG2 isoenzyme to a form similar to that of PG1. No major differences in isoenzyme distribution in extracts prepared by the three methods were observed during this work. The results of an experiment reported in Table 2.3 confirm this. Ammonium sulphate and ethanol precipitation of proteins from samples of the same crude extracts produced the same total polygalacturonase activity; ethanol precipitation resulted in slightly less but statistically insignificant proportion of PG1 isoenzyme, as detected by the heat test.

Dialysis of polygalacturonase extracts was sometimes necessary prior to enzyme assay or purification procedures. However, Brady (pers. comm.) suggested that dialysing extracts, especially against buffers of low ionic strength, might reduce polygalacturonase activity, especially that of PG1. In a series of experiments designed to test this, on average 40% enzyme activity was lost during dialysis of extracts against 0.15 M sodium chloride for 14 hours. When the dialysed extract was centrifuged, the precipitate formed was found to have PG1 activity which accounted for up to 20% of that lost during dialysis. However, dialysis of extracts against 0.3 M sodium chloride resulted in less enzyme inactivation and no precipitation of PG1.

2.3.2. Assay of polygalacturonase activity

All three methods for assaying polygalacturonase activity investigated had some disadvantages for this work, but the picric acid
Table 2.3. Effect of ammonium sulphate and ethanol precipitation of polygalacturonicase from crude extracts on total enzyme activity and isoenzyme distribution.

PG was extracted from red pericarp tissue using the method of Tucker et al. (1981). After homogenisation of pericarp tissue in cold water, the residue was extracted with 1 M sodium chloride solution pH 6.0 for three hours. The supernatant recovered after centrifuging the slurry at 2 400 g for ten minutes was divided into two equal portions. The protein in each portion was precipitated either by ammonium sulphate to 75% saturation or by ethanol (5 ml:1 ml crude extract). Both precipitation procedures were conducted overnight at 4°C. The solutions were centrifuged at 0°C for 20 minutes and the resulting precipitate resuspended in 5 ml 0.15 M sodium chloride.

PG activity was assayed using the picric acid method and the isoenzyme content determined by heating extracts to 65°C for five minutes prior to assay. Values are means of four experiments.

<table>
<thead>
<tr>
<th>Method of Precipitation</th>
<th>Ammonium Sulphate 75% Saturation</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in precipitate</td>
<td>51.0 ± 6.1</td>
<td>50.3 ± 6.2</td>
</tr>
<tr>
<td>(µkat g⁻¹ f.wt.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG activity</td>
<td>6.1 ± 1.52</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>after heat treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µkat g⁻¹ f.wt.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.47 ± 0.16</td>
<td>0.24 ± 0.09</td>
</tr>
<tr>
<td>(mg g⁻¹ f.wt.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>107.26</td>
<td>206.06</td>
</tr>
</tbody>
</table>
method described by Hobson (1964) proved the most useful, since it was capable of measuring reducing groups over a wide concentration range, 0 to 0.4 mM galacturonic acid. The method of Gross (1982) proved to be more sensitive than the picric acid method for detecting changes in the 0 to 250 nmole galacturonic acid range, however, it was easily saturated by contaminating carbohydrates present in the crude and partially purified extracts. Although these could be removed by dialysis or gel filtration, such procedures resulted in marked loss of enzyme activity.

Viscometry was investigated as a method for measuring activity of extracts with low polygalacturonase activity and high amounts of carbohydrates, such as are produced from mutant non-ripening fruit or normal fruit at the beginning of ripening. Although the method could be used to detect low levels of enzyme activity (Fig. 2.2 a), it was found that enzyme extracts had to be dialysed prior to viscometric assay to remove sodium ions which caused gelling of the substrate. The comparison of decrease in substrate viscosity and release of reducing groups with time (Fig. 2.2 b), was consistent with assays for endopolygalacturonase.

2.3.3. Identification and separation of polygalacturonase isoenzymes

At the start of this work only two isoenzymes, PG1 and 2, had been characterised, using ion exchange and gel filtration techniques (Pressey and Avants, 1973; Tucker et al., 1980); although a third isoenzyme had been detected but not characterised by Rexova-Benkova et al. (1977). Initially the methods of Pressey and Avants (1973) and Tucker et al. (1980) were followed to identify and separate the two
Fig. 2.2 (a) Rate of change of specific viscosity of a 2% polygalacturonic acid solution brought about by polygalacturonase, extracted from red tomato pericarp tissue, at different concentrations. Two ml of polygalacturonase was added to 18 ml 2% polygalacturonic acid in 0.5 M sodium acetate pH 4.25 buffer and the viscosity changes with time measured in an Oswald viscometer, at 30°C.

Specific viscosity of the polygalacturonic acid solution =

\[
\left(\frac{\text{flow time of pectin solution}}{\text{flow time of buffer solution}}\right)^{-1}
\]

Polygalacturonase extracts with a relative concentration of 1.0 had an activity of 58 nkat g\(^{-1}\) f.wt., when incubated with the above substrate.

The figures presented are the results of two experiments 1(o) and 2(e). The correlation between rate of change of specific viscosity and relative enzyme activity was \( r = 0.993 \).

(b) Time course of decrease in viscosity (o) and increase in reducing groups (□) during the reaction of a partially purified polygalacturonase extract from red tomato pericarp tissue with 2% polygalacturonic acid in 0.5 M sodium acetate pH 4.25 buffer, at 30°C.
main isoenzymes. These were based on separation by ion exchange chromatography and an assay using the differential heat sensitivity of the isoenzymes.

Although the DEAE-Sephadex A-50 column partially separated PG1 and 2 isoenzymes in extracts of ripening tomato fruit (Fig. 2.3 a) in ripe fruit PG1 usually appeared only as a shoulder on the side of the much larger PG2 peak (Fig. 2.3 b), and in some experiments separation of the two isoenzymes was not achieved. Altering sample sizes and flow rates did not improve resolution.

The loss of activity of PG2 isoenzyme at 65°C, but a high degree of tolerance to heat shown by PG1 (Pressey and Avants, 1973) provided a useful indication of the isoenzyme composition of polygalacturonase extracts. However, later experiments (see 2.3.4.) showed that PG2 sometimes formed a heat tolerant aggregate during extraction and purification procedures, and the presence of this aggregate caused estimates of PG1 activity by this heat test to be inaccurate.

Since the ion exchange method required relatively large amounts of enzyme and did not achieve good separations, and the heating method gave unreliable results, separation of the isoenzymes by electrophoresis was investigated. Eventually the β-alanine acetic acid pH 4.5, cationic system described by Reisfeld et al. (1962) and Smith (1968) was found to give good separations of all three isoenzymes (Plate 2.1). PG1 appeared as a band with a relative mobility of 0.3 to 0.4, PG2A and PG2B had relative mobilities of between 0.45 and 0.6, depending on enzyme activity and protein loading of the gels. Dialysing extracts against 0.3 M sodium chloride for one hour prior to electrophoresis improved isoenzyme resolution. Specific staining of the bands showing
Fig. 2.3. The fractionation of polygalacturonase from orange, (Fig. 2.3 a) and red (Fig. 2.3 b) tomato cv. Sonatine pericarp tissue on DEAE-Sephadex A-50 columns.

PG samples were extracted from pericarp tissue using the method of Tucker et al. (1980). The precipitate following ammonium sulphate precipitation in the 40-80% saturation range was dissolved in 0.15 M sodium chloride, dialysed against the same solution for two hours and applied to the column.

Column dimensions 2.2 x 40 cm; flow rate 18.0 ml per hour; sample volume for (a) 7.0 ml, (b) 8.0 ml; buffer 0.15 M sodium chloride; fraction volume 2.5 ml. PG activity (O-O) is plotted against fraction number. The isoenzyme content of the fractions was determined by the heat test.
Fraction Number

PG Activity (A540 nm)

Graph a shows two peaks labeled PG1 and PG2.

Graph b shows three peaks.

The x-axis represents Fraction Number ranging from 40 to 70.

The y-axis represents PG Activity (A540 nm) ranging from 0.2 to 0.8.
Plate 2.1. Electrophoretic separation of PG isoenzymes by
natured PAGE

(a) Separation of PG1 and PG2 isoenzymes in a crude extract
from orange cv. Ailsa Craig pericarp tissue.

(b) Separation of PG isoenzymes in a crude extract from
red cv. Ailsa Craig pericarp tissue. This extract
contained predominantly PG2.

(c) Separation of PG2A and PG2B isoenzymes in an extract
from red cv. Ailsa Craig pericarp tissue partially
purified by ammonium sulphate fractionation.

Enzyme extracts were separated by discontinuous gel electrophoresis,
using the cationic $\beta$-alanine-acetic acid system, pH 4.5.

Gels labelled (1) were incubated in 2% polygalacturonic acid,
0.05 M sodium acetate for 20 minutes and PG activity detected
by 0.05% ruthenium red. Gels labelled (2) were stained for
proteins using Coomassie brilliant blue G250.
Adverse polygalacturonase activity, which helps to protect against the oligosaccharide nature of the polysaccharides, was also considered. (Plate 8, 9).

When isomeric content of the enzyme was observed by TMS analysis, the results were found to be quite interesting (Fig. 10).

Conversion of PG2 to PG1

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>PG1</td>
<td>PG2</td>
</tr>
<tr>
<td>b</td>
<td>PG2</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>PG2B</td>
<td>PG2A</td>
</tr>
</tbody>
</table>

The method of Shattil and Lebedy (1960) was used for the extraction of polygalacturonase from the various tissues.
positive polygalacturonase activity with Schiff's reagent confirmed the
glycoprotein nature of the enzyme in all its three multimolecular
forms (Plate 2.2b).

When isoenzymic content of an extract was examined by all three
methods, the results were found to be in close agreement (Fig. 2.4).

2.3.4. Conversion of PG2 to PG1

Extracting red tomato tissue in the presence of green tissue,
which contains no polygalacturonase, did not affect total polygalacturonase
activity of the red tissue extract, but did cause an increase in the
amount of the enzyme that was insensitive to heating to 65°C (Table 2.4).
Likewise, incubating extracts from red tissue with those derived from
a non-ripening mutant, NnRr, resulted in levels of heat tolerant
isoenzyme which were greater than the sum of those in the two extracts
prior to incubation together (Table 2.5). Boiled NnRr extracts caused
a similar increase in the levels of heat tolerant polygalacturonase
in red tissue extracts. However, when these extracts were analysed by
electrophoresis, no major changes in isoenzyme content of red extracts
were observed, after either extraction with green tissue or incubation
with NnRr extracts (Figs. 2.5 and 2.6). Incubating purified PG2 with
its substrate polygalacturonic acid (PGA) at various concentrations
and pH levels, (at which binding might be expected to occur) did not
confer the isoenzyme with stability to temperatures of 65°C (Table 2.6).

2.3.5. Purification of polygalacturonase isoenzymes PG1 and PG2

The method of Ali and Brady (1982) was used for the extraction
of polygalacturonase prior to isoenzyme purification, since the initial
Fig. 2.4. PG isoenzyme content of extracts from orange-red Spanish Winter fruit as assessed by:

(a) Discontinuous gel electrophoresis, using the cationic β-alanine acetic acid system, pH 4.5;

(b) Heat sensitivity test. Enzyme activity remaining after extracts were held at 65°C for five minutes was determined by measuring the increase in reducing power of 0.25% polygalacturonic acid solution, pH 4.25 at 30°C;

(c) Separation of polygalacturonase on DEAE-Sephadex A-50 column. Column dimensions 2.5 x 40 cm; sample volume 10.0 ml; buffer 0.15 M sodium chloride; flow rate 15 ml per hour; fraction volume 2.5 ml.

Polygalacturonase was extracted from the slow-ripening genotype Spanish Winter by the method of Hobson (1964), and the precipitate formed after ammonium sulphate fractionation in the 40-80% saturation range was dissolved in 0.15 M sodium chloride and dialysed against the same for two hours prior to estimation of isoenzyme content.
(a) +

(b) PG Activity (μkat g⁻¹ f.wt.)

Total 13.1
PG1 10.1
PG2 3.0

PG1 = 77% of Total PG Activity

(b) Fractions (A540 nm)

Fraction Number

PG1

PG2
Table 2.4. Distribution of PG isoenzymes in extracts prepared from green, red and a mixture of green and red tomato pericarp tissue.

Tomato pericarp tissue from mature green and red cv. Ailsa Craig fruit was extracted by the method of Hobson (1964). The isoenzyme distribution of PG crude extracts from green, red and a mixture of green and red tissue was determined by measuring total and heat (65°C) resistant PG activity by the measurement of release of reducing groups by the picric acid method, and by PAGE as described 2.2.3.c.

The values represent the mean of four experiments. Figures in brackets indicate the weight of tissue extracted. Means within a column followed by different letters were significantly different at the P 0.05 level by Students t test.

<table>
<thead>
<tr>
<th>Fruit tissue used for extraction</th>
<th>Total PG activity (µkat g⁻¹ f.wt.)</th>
<th>Heat resistant PG activity (µkat g⁻¹ f.wt.)</th>
<th>Heat resistant PG activity as % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green pericarp tissue (10 g)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Red pericarp tissue (5 g)</td>
<td>152.7⁺ 38.0</td>
<td>49.9⁺ 15.4</td>
<td>32.6</td>
</tr>
<tr>
<td>Red (5 g) and green (10 g) pericarp tissue</td>
<td>171.9⁺ 39.1</td>
<td>90.2⁺ 13.1</td>
<td>52.4</td>
</tr>
</tbody>
</table>
Fig. 2.5. Electrophoretic separation of PG isoenzymes in extracts from (a) mature green, (b) red and (c) a mixture of mature green and red pericarp tissues extracted together, using the method of Hobson (1964). Extracts were separated cathodally using the β-alanine acetic acid pH 4.5 cationic disc gel electrophoresis system. Gels were incubated in 2% polygalacturonic acid 0.05 M sodium acetate pH 4.25 for 20 minutes and stained for enzyme activity using 0.05% ruthenium red.

Figures on the left indicate the relative mobility of the bands to methyl green.
Fig. 2.6. Electrophoretic separation of PG isoenzymes in extracts of red tomato tissue before and after incubation, for one hour at 20°C, with extracts from yellow NNR fruit, some of which were boiled for five minutes and cooled prior to incubation with red tissue extracts. Extracts were separated cathodally using the β-alanine acetic acid pH 4.25 disc gel system and enzyme activity detected (shaded areas) using 0.05% ruthenium red after incubation of the gels in 2% polygalacturonic acid, 0.05 M sodium acetate pH 4.25.

Figures on the left indicate relative mobility of the bands to methyl green.

(a) Extracts from NNR fruit; (b) red tissue; (c) red tissue + NNR;
(d) red tissue + boiled NNR extracts.
Table 2.5. The distribution of PG isoenzymes in extracts prepared from red pericarp tissue of cv. Ailsa Craig fruit after incubation with extracts from slow ripening fruit of NnNn genotype.

PG crude extracts prepared from red pericarp tissue of cv. Ailsa Craig fruit were incubated for one hour at 20°C with either equal quantities of 0.15 M sodium chloride or extracts from yellow NnNn fruit, some of which had been boiled for five minutes to remove enzyme activity. The total polygalacturonase and heat resistant (65°C, five minutes) activity in the extracts was assayed by measuring the release of reducing groups from a 0.25% solution of polygalacturonic acid, pH 4.25. Means within a column followed by different letters were significantly different at the P 0.05 level by Students t test.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total Activity (nkat)</th>
<th>Heat Resistant Activity (nkat)</th>
<th>Heat Resistant Activity as % Total</th>
<th>Heat Sensitive Activity (nkat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ailsa Craig red fruit extract + 0.15 M sodium chloride. 5 ml each</td>
<td>21.3 ± 3.7</td>
<td>4.3 ± 0.1</td>
<td>20.1</td>
<td>17.1</td>
</tr>
<tr>
<td>NnNn yellow fruit extract + 0.15 M sodium chloride. 5 ml each</td>
<td>7.2 ± 0.2</td>
<td>6.3 ± 0.6</td>
<td>88.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Ailsa Craig red fruit extract + NnNn yellow fruit extract. 5 ml each</td>
<td>32.4 ± 2.3 (2.27)</td>
<td>15.8 ± 0.2</td>
<td>48.6</td>
<td>16.7</td>
</tr>
<tr>
<td>Ailsa Craig red fruit extract + boiled NnNn fruit extract. 5 ml each</td>
<td>26.9 ± 2.3</td>
<td>16.5 ± 2.1</td>
<td>61.3</td>
<td>10.4</td>
</tr>
</tbody>
</table>
Table 2.6. Examination of the possible interaction between PG2, purified by Ultrigel ACA44 and Concanavalin A-Sepharose chromatography, and its substrate polygalacturonic acid.

The total and heat resistant (65°C; five minutes) activity of purified PG2 was examined before and after incubation for one hour at 20°C with its substrate polygalacturonic acid at various concentrations in 0.2 M potassium phthalate and 0.1 M sodium chloride buffer adjusted to pH 3.0, 4.25 or 6.0. Values average of two experiments and values in parenthesis represent the spread of values around the mean.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PG Activity (nkat per ml enzyme extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>0.5 ml of PG2 isoenzyme was incubated with 0.5 ml of:</td>
<td></td>
</tr>
<tr>
<td>a) buffer</td>
<td>79.5 ± 14.9</td>
</tr>
<tr>
<td>b) 0.12% polygalacturonic acid pH 3.0</td>
<td>69.6 ± 10.5</td>
</tr>
<tr>
<td>c) 0.12% polygalacturonic acid pH 6.0</td>
<td>80.1 ± 10.1</td>
</tr>
<tr>
<td>d) 0.25% polygalacturonic acid pH 4.25</td>
<td>92.2 ± 3.5</td>
</tr>
<tr>
<td>e) 0.50% polygalacturonic acid pH 4.25</td>
<td>98.6 ± 8.6</td>
</tr>
</tbody>
</table>
extraction in 17 mM Tris buffer removed soluble proteins but not wall bound polygalacturonase (Ali, 1983). The first purification step employed was ammonium sulphate fractionation, the enzyme precipitated in the 40 to 80% saturation range being collected. This step resulted in a five- to seven-fold increase in specific activity (Table 2.7 a and b); although some loss of enzyme activity occurred. The protein sample was then desalted by dialysis prior to further purification on ion exchange or gel filtration columns.

Ion exchange chromatography was tried as an initial purification step, as described by Pressey and Avants (1973). However, this did not give satisfactory isoenzyme separation (Section 2.3.3). Since PG1 (MW 80 000 to 110 000) and PG2 (MW 42 000 to 47 000) can be separated on the basis of molecular size, gel filtration was examined as a purification step. Initially a Sephadex G100 column was used following the procedures outlined by Tucker et al. (1980); however, later this was replaced by an Ultrogel ACA44 column. Ultrogel ACA44 has a fractionation range of 10 000 to 130 000, which is very suitable for the separation of polygalacturonase isoenzymes. This column gave reasonable separations of PG1 and PG2 isoenzymes. (Fig. 2.7 a, b, c.). All separations were conducted at 4°C. The results obtained using 0.125 M sodium acetate pH 6.0 as eluting buffer were similar to those obtained using 1 M sodium chloride, but the latter was used in the preparation of PG1 isoenzyme to prevent the loss, through precipitation, that occurred when this isoenzyme was suspended in solutions of low ionic strength (Section 2.3.1.).

The glycoprotein nature of polygalacturonase isoenzymes (Section 2.3.3.) was utilised in their purification. A Concanavalin A-Sepharose affinity chromatography column was employed by Ali and Brady (1982) to
Table 2.7a. Purification of PG1 isoenzyme from extracts of orange-red 
NurNur tomato fruit. Polygalacturonase extracted by the method of Ali and 
Brady (1982).

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>PG Activity (nkat g(^{-1}) f.wt.)</th>
<th>Yield (%)</th>
<th>Protein (mg g(^{-1}) f.wt.)</th>
<th>Specific Activity (nkat mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7M NaCl extract</td>
<td>20.4</td>
<td>100</td>
<td>1.18</td>
<td>17.3</td>
</tr>
<tr>
<td>40-80% (NH(_4))(_2)SO(_4) after dialysis</td>
<td>5.8</td>
<td>28</td>
<td>0.07</td>
<td>85.9</td>
</tr>
<tr>
<td>Ultrogel ACA44</td>
<td>5.2</td>
<td>25</td>
<td>0.05</td>
<td>111.7</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose</td>
<td>1.4</td>
<td>7</td>
<td>0.01</td>
<td>194.3</td>
</tr>
</tbody>
</table>

Table 2.7b. Purification of PG2 isoenzyme from extracts of red 
cv. Sonatine fruit prepared by the method of Ali and Brady (1982).

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>PG Activity (nkat g(^{-1}) f.wt.)</th>
<th>Yield (%)</th>
<th>Protein (mg g(^{-1}) f.wt.)</th>
<th>Specific Activity (nkat mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.7 M NaCl extract</td>
<td>52.7</td>
<td>100</td>
<td>1.25</td>
<td>42.1</td>
</tr>
<tr>
<td>40-80% (NH(_4))(_2)SO(_4)</td>
<td>40.6</td>
<td>77</td>
<td>0.15</td>
<td>274.3</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose</td>
<td>11.8</td>
<td>22</td>
<td>0.03</td>
<td>357.6</td>
</tr>
<tr>
<td>Sample 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.7 M NaCl extract</td>
<td>30.1</td>
<td>100</td>
<td>0.95</td>
<td>31.6</td>
</tr>
<tr>
<td>40-80% (NH(_4))(_2)SO(_4)</td>
<td>20.4</td>
<td>68</td>
<td>0.09</td>
<td>232.1</td>
</tr>
<tr>
<td>Dialysis against 0.3 M NaCl</td>
<td>11.2</td>
<td>37</td>
<td>0.07</td>
<td>152.9</td>
</tr>
<tr>
<td>Ultrogel ACA44</td>
<td>9.4</td>
<td>31</td>
<td>0.03</td>
<td>376.4</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose</td>
<td>2.6</td>
<td>9</td>
<td>0.01</td>
<td>434.2</td>
</tr>
</tbody>
</table>
Fig. 2.7. The fractionation of PG from (a) orange, (b) red cv. Sonatine fruit and (c) yellow Nrrr fruit on Ultrogel ACA44 columns.

Fig. 2.7 a. Column dimensions 2.2 x 80 cm; sample volume 10.0 ml; buffer 0.125 M sodium acetate pH 6.0; flow rate 12.0 ml per hour; fraction volume 8.0 ml.

Fig. 2.7 b. Column dimensions 2.2 x 80 cm; sample volume 12.0 ml; buffer 0.125 M sodium acetate pH 6.0; flow rate 15.0 ml per hour; fraction volume 8.0 ml.

Fig. 2.7 c. Column dimensions 2.2 x 80 cm; sample volume 12.0 ml; buffer 1.0 M sodium chloride, 0.05 M sodium acetate pH 6.0; flow rate 12 ml per hour; fraction volume 8.0 ml.

Samples used were extracted by the method of Ali and Brady (1982); partially purified by 40-80% saturation of ammonium sulphate. The resulting precipitate was taken up in either 0.125 M sodium acetate pH 6.0 (Fig. 2.7 a and b) or 1.0 M sodium chloride (Fig. 2.7 c) and dialysed for two hours against the column buffer. Absorbance at 280 nm (---) and PG activity (o-o) are plotted against fraction number. PG1 and PG2 were identified in fractions by the heat test.
separate polygalacturonase from other proteins, especially pectinesterase, with good effect. This column was used here as a purification step after the isoenzymes had been separated by gel filtration.

A gradient of 0 to 0.10 M \( \alpha \)-methyl-D-mannoside was used to see if an extract of partially purified polygalacturonase could be resolved into its isoenzymic components using this method, but the isoenzymes were eluted as one peak at low methyl-mannoside concentrations (Fig. 2.8). Shallower gradients were tried with no effect. However, considerable purification was achieved by this method (Table 2.7 a and b), although in many cases enzyme activity was inhibited by passage through the column; thus the specific activities reported do not reflect the usefulness of this column in removing unwanted proteins. A summary of the purification procedure is given in Fig. 2.10.

SDS electrophoresis of PG2 isoenzyme after purification by gel filtration and affinity chromatography show the relative purity of the samples (Plate 2.2). The PG2 isoenzyme was resolved into its two isoenzymes, PG2A and PG2B, even under denaturing conditions. The position of these isoenzymes relative to ovalbumin suggests they have molecular weights between 42 000 and 44 000. The small contaminating band below that of PG2 in Plate 2.2 a, was probably a breakdown product of the stored enzyme and was removed by gel filtration before the sample was used to raise antibodies to PG2 protein. Fig. 2.9 shows results of gel filtration and affinity chromatography on isoenzyme purity as assessed by natured gel electrophoresis. Although gel filtration separated PG2 from PG1 isoenzymes, the Concanavalin A step was required to remove the remaining contaminating proteins.
Fig. 2.8. Fractionation of PG from red tomato pericarp tissue on Concanavalin A-Sepharose 4B column after partial purification by ammonium sulphate fractionation in the 40-80\% saturation range. Samples were dissolved in and dialysed against the column buffer: 0.05 M sodium acetate, 0.5 M sodium chloride, 1 mM calcium acetate, 1 mM manganous sulphate pH 6.0. Column dimensions 1.5 x 20 cm; sample volume 8.0 ml; flow rate 10 ml per hour, fraction volume 4.0 ml.

A gradient of 0.0-0.1 M α-methyl mannoside was applied at A. Absorbance at 280 nm (---) and PG activity (o - o) are plotted against fraction number.
Plate 2.2. Analysis of denatured PG2 by SDS-polyacrylamide gel electrophoresis. PG2 purified from an extract of red cv. Ailsa Craig by gel and affinity chromatography as outlined in Fig. 2.10

(a) SDS-vertical slab electrophoresis. A 10-15% acrylamide running gel with a 5% acrylamide spacer gel was used to separate PG isoenzymes as described in 2.2.3.

1. Purified PG2.
2. Ovalbumin. MW 43 000.

(b) SDS-PAGE in tubes. Seven-and-a-half percent acrylamide gel with a 3% acrylamide spacer gel in Tris/HCl pH 9.18 buffer as described for vertical slab electrophoresis in 2.2.3.

2. Purified PG2. Gel stained to detect proteins with Coomassie brilliant blue G250.
3. Crude enzyme extract from red cv. Ailsa Craig fruit stained as for (2).
4. Ovalbumin. MW 43 000.
5. Purified PG2. Gel stained as for (1).

Gels 1 and 2 were not run at the same time as gels 3, 4 and 5. The position of PG relative to the cathode was slightly different in the two experiments.
Fig. 2.9. Electrophoretic separation of proteins in extracts of red tomato tissue after:

(1) Extraction from pericarp tissue with 1 M sodium chloride pH 6.0;

(2) Precipitation with ammonium sulphate in 40–80% saturation range;

(3) Fractionation on an Ultrogel ACA44 column (fractions containing PG2 activity were pooled prior to electrophoresis);

(4) Further fractionation of Ultrogel PG2 fractions on Concanavalin A-Sepharose 4B column.

Gels labelled (a) were stained for proteins using Coomassie brilliant blue G250; those labelled (b) were incubated in 2% polygalacturonic acid, 0.05 M sodium acetate for 20 minutes and PG activity detected by 0.05% ruthenium red. Blocked areas denote bands with intense staining; those hatched were less intensely stained.

The figures on the left show the relative mobility of the bands to methyl green.
**Fig. 2.10.** Scheme for the purification of tomato PG1 and PG2.

Tomato pericarp tissue, kept at -20°C

↓

Partially thawed and homogenised with 0.017 M Tris pH 10.5

↓

Residue extracted with 1.7 M sodium chloride, 50 mM sodium citrate, 15 mM disodium EDTA pH 5.5

↓

Protein precipitated by ammonium sulphate fractionation in the 40–80% saturation range and redissolved in 1.0 M sodium chloride

↓

Sample (10–12 ml) applied to Ultrogel ACA44 (2.2 x 80 cm) column. Eluted at 15–20 ml per hour with 1 M sodium chloride

Fractions containing PG1

↓

As for PG2

Fractions containing PG2, combined and reduced in volume by ultrafiltration

↓

Dialysed against Concanavalin A-Sepharose buffer for one hour

↓

Applied to Concanavalin A-Sepharose column (1.5 x 20 cm). Eluted with 10 mM α-D-methyl-D-mannoside in 0.05 M sodium acetate buffer pH 6.0. Fractions containing PG2 concentrated by ultrafiltration

↓

Sample reapplied to Ultrogel ACA44 column
2.3.6. Immunodiffusion assay

The antibodies raised against PG2 were used to examine the antigenic properties of polygalacturonases from various sources (e.g. wild *Lycopersicon* species, Chapter 5). Both PG1 and PG2 formed separate precipitin lines when incubated with PG2 antibodies (Plate 2.3). No precipitin line was formed against extracts from mature green tomatoes, nor when PG2 extracts were incubated with pre-injection serum.

2.4 DISCUSSION

To study the role of polygalacturonase and its isoenzymes in the softening and ripening processes of tomatoes it was necessary to obtain an efficient extraction of the enzyme, with minimum loss of total activity or alteration of isoenzyme identity. Polygalacturonase isoenzymes have high isoelectric points (Ali and Brady, 1982), suggesting that they interact electrostatically with their substrates in the acid environment of the cell wall (Babbitt *et al.*, 1973). Thus the extraction of polygalacturonase from cell walls requires solutions of either high pH or high ionic strength. Since polygalacturonase activity is inhibited above pH 7.0, it is generally extracted using solutions containing 1.0 to 1.5 M sodium chloride, pH 6.0 to 6.5 (Hobson, 1964; Pressey and Avants, 1973; Rexová-Benková *et al.*, 1977; Takehana *et al.*, 1977; Tucker *et al.*, 1980; Ali and Brady, 1982).

The methods of Hobson (1964) and Ali and Brady (1982) employed EDTA in the extraction media to aid the release of wall-bound polygalacturonase by sequestering calcium ions, thereby weakening wall structure as discussed in Chapter 1. Although Ali and Brady (1982)
Plate 2.3. Reaction of PG1 and PG2 with antiserum to PG2. Serum well (a) contained immune serum, and well (b) serum collected prior to immunization of the rabbit. Sample wells contained: (1) PG2; (2) PG1; (3) crude extract from red pericarp tissue from cv. Ailsa Craig, which contained mainly PG2; (4) crude extract from orange pericarp tissue from cv. Ailsa Craig, which contained about equal amounts of PG1 and PG2; (5) and (6) extracts from mature green pericarp tissue having no detectable PG activity.
found that EDTA inhibited PG1 activity, the concentration required to do so to any great extent was $5 \times 10^{-2}$ M; at $1 \times 10^{-2}$ M, only 4% inhibition of PG1 occurred. Since the concentration of EDTA used in Hobson's method was only $2.3 \times 10^{-4}$ M, it was unlikely to have any inhibitory effect on PG1. This method also has the advantage for quantitative extraction in that it extracts soluble proteins, which are discarded in the first steps of Tucker et al. and Ali and Brady's methods. In ripening tomato fruit up to 20% of polygalacturonase may be in soluble form (Hobson, 1963).

Wherever possible, polygalacturonase activity of the crude extract was assayed and examined for isoenzyme composition soon after extraction, to minimise loss of enzyme activity. However, for some extracts with very low polygalacturonase activity e.g. green-orange coloured fruit or those from slow or non-ripening genotypes, it was necessary to concentrate the extract and remove some of the contaminating carbohydrates before enzyme assay. None of the assay techniques tried were able to measure accurately low enzyme activity against a background of either high reducing sugars (picric or cyanoacetamide methods) or high salt concentrations (viscometry method), although the picric acid method was the least affected by contaminants. Dialysis of extracts against water or low ionic strength buffers removed salts and reducing sugars from extracts, enabling enzyme activity to be measured by the cyanoacetamide and viscometric methods. However, such treatment of extracts was found to not only reduce total polygalacturonase activity, but to cause the precipitation of PG1. Since polygalacturonase activity in fruit during the early stages of ripening and in slow ripening mutants was found to be largely due to PG1 (Chapters 3 and 4) prolonged dialysis of extracts from these fruit was avoided. Instead extracts were concentrated and purified by precipitation.
Although Hobson (1964) used ethanol to precipitate polygalacturonase from crude extracts, Brady (pers. comm.) found that this sometimes caused an apparent increase in PG1 content of the extracts, due to aggregation of PG2 molecules. The slight increase in PG1 content of extracts precipitated with ethanol compared to those precipitated with ammonium sulphate (Table 2.3), was not found to be significant in these experiments. However, in subsequent work most extracts were purified where necessary by ammonium sulphate precipitation. Although ammonium sulphate fractionation caused some loss in total enzyme activity (Table 2.7), it was generally not more than 25% and no alteration in the relative isoenzyme composition of the extracts after precipitation was observed (Fig. 2.9).

During polygalacturonase extraction and its subsequent assay in crude extracts, there was the possibility that enzyme activity might be impaired by the presence of inhibitors. However, that polygalacturonase was detected after purification or electrophoresis only when its activity was observed in the crude extracts suggests that inhibitors were not present in these extracts. Phenolic compounds, which inhibit many enzymes, were found not to affect tomato polygalacturonase activity (Hobson, 1964). Furthermore, Brady et al. (1982) found that polygalacturonase activity correlated well with the amount of polygalacturonase protein in fruit, again implying that enzyme inactivation during extraction is not a problem.

The close agreement of isoenzyme content of extracts assessed by heat treatment, ion exchange and electrophoretic methods (Fig. 2.4), also shows that the isoenzyme content of crude extracts is not altered by the presence of contaminants.
Polygalacturonase hydrolyses the $\alpha-1\rightarrow4$-galacturonosyl linkages of pectic or polygalacturonic acid, releasing galacturonic acid residues. The optimum in vitro conditions for the assay of tomato polygalacturonase have been well researched (Hobson, 1964; Pressey and Avants, 1973; Ali and Brady, 1982), thus were not investigated further in this work. The optimum pH for activity against polygalacturonic acid lies between 3.5 to 4.5, most researchers using a pH of 4.25 to 4.5 for enzyme assays (Hobson, 1964; Pressey and Avants, 1973; Takehana et al., 1977; Tucker et al., 1980; Ali and Brady, 1982).

Potassium and sodium ions stimulate polygalacturonase activity (Ali and Brady, 1982), the optimum sodium chloride for activity lying between 0.15 and 0.3 M (Pressey and Avants, 1973; Takehana et al., 1977; Marković and Slezárik, 1977; Ali and Brady, 1982). Rexova-Benková and Marković (1976) reported that the optimum pH for enzyme activity depended on the sodium chloride concentration present, the higher it was the lower the optimum pH; Pressey and Avants (1973) suggested that the salt disrupts aggregation of the polygalacturonic acid that occurs at low pH's due to hydrogen bonding. Thus for this work all assays using polygalacturonic acid as substrate were conducted at pH 4.25 with the addition of 0.12 M sodium chloride.

The suggestion that tomato polygalacturonase might exist in more than one form was first put forward by McColloch and Kertez (1948) and later by Fatel and Phaff (1960), who found that some enzyme activity was resistant to high temperatures but some was not. These ideas were later confirmed by Pressey and Avants (1973), who separated two isoenzymes using ion exchange chromatography and called them PG1 and PG2 respectively. Subsequently, others confirmed the existence of multimolecular forms of polygalacturonase (Takehana et al., 1977;
Tucker et al., 1980; Ali and Brady, 1982; Moshrefi and Luh, 1983). The estimates for the molecular weight of PG1 from these workers varied between 84,000 and 115,000, and for PG2 between 42,000 and 47,000. However, Moshrefi and Luh (1983) reported a very high molecular weight for PG1 of 199,500; whether they have found a third isoenzyme or whether this high value is due to protein aggregation requires further investigation.

Hexová-Benková et al. (1977) resolved PG2 isoenzyme into two components using a column of cross-linked pectic acid. This third isoenzyme can also be resolved by gel electrophoresis (Plate 2.1), as was confirmed by Ali and Brady (1982). They estimated its molecular weight to be 45,000 and called it PG2B. The predominant PG2 isoenzyme, termed PG2A had an estimated molecular weight of 43,000. The molecular weight of PG2A and B estimated in this work by SDS electrophoresis (Plate 2.2), agrees well with these estimates of Ali and Brady (1982).

Tucker et al. (1980) found that PG1 and PG2 isoenzymes gave similar polypeptides after proteolysis, and reacted to antibodies raised against PG2. They suggested PG1 may be a dimer of PG2, the difference in molecular weight between PG1 and a dimer of PG2 being due to additional carbohydrate. The same group later found that extracts from mature green fruit when added to extracts from red fruit, caused conversion of PG2 to a form similar in heat sensitivity and molecular weight to PG1 (Tucker et al., 1981). The substance causing conversion was found to be heat-stable and non-dialysable and appeared to increase during fruit ripening. These results were confirmed in this work (Table 2.4) and in addition extracts from NNR tissue were found to cause similar aggregation of PG2. However, analysis of these
extracts by gel electrophoresis indicated that no major change in isoenzyme composition of red tissue had occurred due to the green or NrNr tissue extracts.

Tucker et al. (1981) analysed their extracts by the heat test, gel filtration and density in caesium chloride. It is possible that a simple aggregation of PG2 molecules, which conferred heat stability to the isoenzyme, was not disrupted by gel filtration and density measurements, but was resolved into its components by electrophoresis. Brady (pers. comm.) has observed aggregation of PG2 isoenzymes under some extraction and purification procedures, while Biggs and Honda (1983) found that polygalacturonic acid combined with some PG2 isoenzyme during heating of the two to 65°C, to form a heat-stable aggregate. Although the results in Section 2.3.4. did not confirm this report, it seems likely that some substance, probably a carbohydrate (Tucker et al., 1981), which increases in fruit during ripening causes the aggregation of PG2 to a form similar, but not identical, to PG1 in terms of molecular weight and heat tolerance.

The conditions of extraction and incubation of the extracts may be important. Pressey and Avants (1973) found that in low ionic buffers polygalacturonase may form relatively stable complexes with acidic polysaccharides, however, they did not investigate whether these complexes were stable under electrophoretic conditions. The results of this chapter would suggest that they might not be.

Since PG1 remains a discrete isoenzyme under electrophoretic conditions, whilst the PG2 aggregate was resolved into its components, it is suggested that PG1 is an in vivo isoenzyme of polygalacturonase and not an artifact of the extraction procedure. Tucker et al. (1981)
reported that the conversion factor increased in concentration during ripening, yet the amount of PG1 extracted from fruit tissues after the red stage of ripening remains fairly constant (Tucker et al., 1981; Ali and Brady, 1982. Chapter 3.). This also suggests that the factor is not involved in regulating in vivo isoenzyme levels.

The fact that PG2 can be converted into a form stable to incubation at 65°C, renders the heat test method for isoenzyme identification much less reliable than first thought. Thus polyacrylamide disc gel electrophoresis was utilised as the main method for the identification of polygalacturonase isoenzymes in this work.

For investigations into the possible in vivo roles of PG1 and PG2 it was necessary to have purified preparations of the two main isoenzymes. No attempt was made in this work to resolve PG2 into its component A and B isoenzymes on a preparative scale. The main problems faced in purifying PG1 and PG2 were firstly, to separate the two isoenzymes from one another, and secondly to separate them from other pectic enzymes such as pectinesterase (Rexova-Benkova and Tibenský, 1972; Ali, 1983). Although many researchers have used either anion (Pressey and Avants, 1973; Tucker et al., 1980; Moshrefi and Luh, 1983) or cation (Takehana et al., 1977; Ali and Brady, 1982) exchange chromatography as a first step in polygalacturonase purification, neither method solves these main problems. Anion exchange chromatography partially resolved the two isoenzymes (Fig. 2.3; Tucker et al., 1980), but did not separate polygalacturonase from the bulk of the proteins (Ali, 1983). Cation exchange chromatography gave greater purification but did not resolve the isoenzymes (Ali, 1983).
Gel filtration using Ultrogel ACA44, not only resolved PG1 and PG2, but gave a 1.3- to 2.5-fold increase in specific activity (Table 2.7a and b). Although Ali (1983) reported that Ultrogel ACA54 gave poor recoveries of poylgalacturonase activity, no such inhibition of activity by Ultrogel ACA44 was observed (Table 2.7a and b).

Once the isoenzymes had been separated, further purification was achieved by utilising the glycoprotein nature of polygalacturonase, which had been established by Takehana et al. (1977) and confirmed by this work and that of Ali and Brady (1982) and Nooshiri and Luh (1983). A column of Concanavalin A-Sepharose was found to bind polygalacturonase but not most other proteins in the extract (Fig. 2.8). Ali (1983) confirmed that pectinesterase, which is one of the most difficult proteins to remove from polygalacturonase extracts by ion exchange or gel filtration chromatography methods (Rexova-Benkova and Tibenský, 1972; Ali and Brady, 1982), was not bound by Concanavalin A.

Concanavalin A-Sepharose chromatography unfortunately also caused loss of enzyme activity, as also observed by Ali (1983), thus the specific activity of the purified extracts were lower than might be expected from the removal of most contaminating protein. This loss of activity might be due to inhibition by manganese chloride and calcium chloride ions which are incorporated into the elution buffer to stabilise Concanavalin A activity (Ali and Brady, 1982).

A specific activity of 0.3 to 0.4 was achieved for PG2 and of 0.2 for PG1. These results are slightly higher than those of Patel and Phaff (1960) and Tucker et al. (1980) who obtained specific activities of 0.1 µkat mg⁻¹, but not as great as the 1.7 µkat mg⁻¹ achieved by Ali and Brady (1982). The loss of activity during affinity chromatography
is largely responsible for the lower specific activity of extracts in this work, but the efficiency of the Concanavalin A column in purifying polygalacturonase was considered to outweigh the loss of some enzyme activity.

Purified PG1 and PG2 both formed precipitin bands when incubated in dchtelony plates with antibodies raised against PG2, although they formed discrete lines, indicating a reaction of partial identity (Plate 2.3), Tucker et al. (1980) and Ali and Brady (1982) obtained the same results. SDS electrophoresis of PG2 (Plate 2.2) suggested that it has a molecular weight of about 43 000 average for the two isoenzymes A and B, which is in agreement with published estimates of PG2 molecular weight (Pressey and Avants, 1973; Tucker et al., 1980; Ali and Brady, 1982; Moshrefi and Luh, 1983); thus I am confident that the isoenzymes studied in this work are the same as those reported previously.

The study of methods for extraction, identification and purification of polygalacturonase and its isoenzymes concludes that for quantitative assays of enzyme activity the method of Hobson (1964) efficiently extracts polygalacturonase without causing major changes in isoenzyme content. The three isoenzymes of polygalacturonase PG1, PG2A and PG2B, can be routinely separated by polyacrylamide disc electrophoresis using the β-alanine acetic acid pH 4.5 system of Reisfeld et al. (1962). Whilst the differential heat sensitivity test gave a useful estimate of PG1 content of enzyme extracts it was not always reliable, due to the aggregation of PG2 under certain circumstances to a heat stable form.
Purification of PG1 and PG2 isoenzymes (Fig. 2.10) by ammonium sulphate fractionation in the 40 to 80% saturation range followed by gel filtration on Ultrogel ACA44 and then affinity chromatography using Concanavalin A-Sepharose produced extracts with a specific activity of 0.2 to 0.4 μkat mg⁻¹.
CHAPTER THREE  
ACCUMULATION OF POLYGALACTURANASE ISOENZYMES 
IN RIPENING TOMATO FRUIT FROM DIFFERENT CULTIVARS

3.1. INTRODUCTION

The many varieties of cultivated tomato now grown throughout the world display great diversity in the rate and degree of softening of their fruit during ripening (Shafshak and Winsor, 1964; Hobson, 1965; Sobotoka and Watada, 1970; Cornley and Egan, 1978). Although much of this diversity may be due to differences in fruit morphology e.g. thickness of fruit walls or number of locules (Shafshak and Winsor, 1964), it is well documented that the major changes in fruit firmness occurring during ripening are associated with changes in the pectin fraction of the fruit cell walls. Insoluble protoplastin declines with a concomitant increase in water soluble uronides (Poda, 1957; Knecht et al., 1975; Sawamura et al., 1978; Brady et al., 1982; Malis-Arad et al., 1983).

In tomato fruit, endo-polygalacturonase has been identified as the enzyme predominantly involved in the degradation of cell wall pectic substances (Poda, 1957; Hobson, 1964; Brady et al., 1982; Themmen et al., 1982). Hobson (1965) found a highly significant positive correlation between the firmness and polygalacturonase activity of fruit at the orange-red stage of ripeness; while other studies have shown that cultivars such as Ailsa Craig and Immuna, which soften relatively quickly, have higher polygalacturonase activity than slower ripening cultivars such as Potentate (Hobson, 1964; Tucker et al., 1980). However, Ramadan (1981) found polygalacturonase levels in a firm fruited cultivar to be higher than in a soft variety, and observed that the differences in firmness seemed to be due to
differences in cell wall content of the two varieties prior to ripening.

Although researchers have examined polygalacturonase changes in tomatoes at different stages of ripeness (Hobson, 1964; Sobotka and Watada, 1970; Tucker et al., 1980; Brady et al., 1982), or firmness changes during ripening (Shafshak and Winsor, 1964; Ramadan, 1981) only Hobson (1965) has examined changes in firmness and polygalacturonase activity in two varieties of tomato. No detailed studies of the accumulation of polygalacturonase in its different isoenzymic forms in ripening tomato fruits of different cultivars have been published. In addition, despite the fact that the isoenzymes of polygalacturonase have been well characterised in vitro (Pressey and Avants, 1973; Tucker et al., 1980; Ali and Brady, 1982; Moshrefi and Luh, 1983), little is known about their roles in vivo.

This study was concerned with elucidating the relationship between the activity of polygalacturonase isoenzymes and the rate of softening of tomato fruit by, firstly, examining the accumulation of isoenzymes in fruit of cultivars with different rates of ripening, and secondly, to examine the activity of isoenzymes in vitro against natural substrates e.g. cell walls and pericarp tissue discs.

3.2. MATERIALS AND METHODS

3.2.1. Tomato cultivation and assessment of fruit ripeness

Tomato plants of cultivars Ailsa Craig, Bellina, Moneymaker, Potemtate and Sonatine, were grown in a peat/sand (3:1) mixture under glasshouse conditions. In each experiment eight to ten plants per
cultivar were grown. Liquid fertiliser, containing 167 μg l⁻¹ nitrogen, 278 μg l⁻¹ potassium, 25 μg l⁻¹ magnesium, 20 μg l⁻¹ phosphorus, 0.5 μg l⁻¹ boron and 1.5 μg l⁻¹ iron, was fed daily. The day/night temperatures during fruit development were 25°C/15°C. Plants were grown to the third truss.

Fruit age was determined by tagging flowers at anthesis. Although it has been observed that fruit age is not a good criterion of maturity (Janes, 1941; Grierson and Tucker, 1983), fruit of the same cultivar growing on similar positions on the plant (i.e. with respect to truss number and position in truss) were found to mature at approximately the same time.

The colour of tomato fruit changes in a predictable fashion during ripening and is thus a popular criterion for assessing fruit ripeness (Hobson, 1963; Kader and Morris, 1976; Hobson et al., 1983a). For routine harvesting of samples at specific stages of ripeness, a subjective method of fruit colour assessment, based on published descriptions of fruit appearance at different stages of ripeness (Hobson, 1963), and on colour charts (USDA colour chart, Anon, 1975; Tomato colour chart, Anon, 1977) was used. Five colour grades were chosen: mature green, green-orange, orange-green, orange and red.

The colour of harvested fruit samples was also measured objectively using a Hunterlab Color Difference Meter (D25A-9, Hunter Associates Laboratory Inc., USA), as described by Hobson et al. (1983a). Light reflected by the tomato is received by an optical sensor and converted into colour specifications. These consist of three parameters: 'L' a measure of lightness on a scale 0(black) to 100(white), 'a' a measure of greenness when negative and red when positive, and 'b'
which denotes blueness when negative and yellowness when positive. These parameters, especially the 'a' values and a/b ratio have been found to be good indices of fruit ripeness (Hobson et al., 1983a).

Fruit firmness was measured on an instrument which records the longitudinal compression occurring when fruit are subjected to a load of 1 kg for five seconds. Shafshak and Winsor (1964) have shown that such direct compression measurements partially compensate for differences in fruit size, and that this parameter is to be preferred to measuring percentage compression. Firmness values were expressed as mm compression or converted to a firmness index value, which is 1/mm compression x 10. To facilitate comparison of softening rates of fruit of different cultivars, some data was transformed to express firmness as a percentage of the value at the mature green stage.

Whilst the firmness of whole tomatoes is a good criterion of their ripeness and quality, it could depend not only on the softness of pericarp tissue but also on the relative thickness and internal structure of the inner and outer pericarp, placental tissue, and the amount of locular jelly. Since polygalacturonase activity mostly occurs in the outer pericarp of the tomato (Hobson, 1964), this tissue was used for extraction of polygalacturonase. Thus it was felt that a better correlation between firmness and polygalacturonase activity might be obtained if the firmness of pericarp tissue alone was measured.

A method was devised to measure the compression of pericarp discs. Discs of tissue 1.5 cm diameter were cut from pericarp tissue, avoiding areas adjoining the radial walls. These were blotted dry and placed epidermis uppermost in a perspex cylinder of 1.5 cm diameter.
Four or five discs were placed on top of each other in the cylinder, which was then placed between the compression plates of the firmness meter. A solid perspex cylinder placed on top of the discs connected them to the upper compression plate. In this way the longitudinal compression occurring when the discs were subject to varying loads for five seconds was measured.

Many methods of cutting the discs and applying the load were tried, but in all experiments it was found that the pericarp discs did not give firmness readings consistent with stage of ripeness. Regression values of firmness index of fruit discs against colour, as assessed by a/b ratio, were $r = 0.044$. In contrast, there was a high degree of correlation between whole fruit (from which the discs were prepared) firmness index and colour $r = -0.901$. As a result firmness measurements of whole fruit were retained as the best method available for measuring changes in pericarp texture during ripening.

In some experiments the onset and development of fruit ripening was followed by measuring ethylene production. Replicate samples of two to four fruit per cultivar or treatment were individually placed in 440 ml gas-tight Kilner jars fitted with sampling ports. The fruit were held at 20°C in the jars for periods up to 90 minutes. One ml samples of air were then removed from the jars and injected into a Pye Unicam Series 204 gas chromatogram fitted with an alumina column and flame ionisation detector. A standard gas mixture of 1 ppm ethylene was used to calibrate the instrument. The carrier gas flow rate was 40 ml min$^{-1}$ and the column temperature 120°C.
3.2.2. Accumulation of polygalacturonase isoenzymes in ripening fruit

Samples (n > 5) of fruit from the different cultivars were harvested at various stages of ripeness according to colour (mature green, green-orange, orange-green, orange and red) and age. The colour, firmness, and in some experiments, ethylene production of the fruit were measured before the pericarp tissue from all fruit examined was removed. Longitudinal segments from opposite sides of each fruit were used to form subsamples of pericarp tissue for polygalacturonase extraction by the method of Hobson (1964), (Chapter 2).

Isoenzyme content of these extracts was assessed using the heat test and by polyacrylamide disc gel electrophoresis (Chapter 2).

In one experiment fruit ripening off-the-vine was investigated. Fruit samples at the mature green stage were harvested from cvs. Ailsa Craig, Bellina, and Potentate on the same day and stored at 20°C. Fruit were kept in trays enclosed in perforated polyethylene bags to reduce water loss during storage. Sub-samples of ten fruit per cultivar were measured every two days for colour and firmness, and three of these fruit were used to determine ethylene production. When fruit samples reached the green-orange, orange and red stages of ripeness, samples of four fruit were removed for polygalacturonase determinations.

3.2.3. Analysis of cell wall yield from tomato fruit of different cultivars at two stages of ripeness

Pericarp tissue from tomato fruit of the five cultivars at mature green and red stages of ripeness, previously stored at -20°C, was partly thawed and the skin removed. The skinned tissue
(approximately 50 g) was accurately weighed and macerated in 150 ml cold 30 mM potassium phosphate buffer pH 9.5 with two drops of octanol to prevent frothing. The mixture was left on ice for 30 minutes, centrifuged for ten minutes at 2500 g, and the upper layer removed. Any material floating on the upper layer was removed to the solid layer by deaeration. The residue was washed twice in 150 ml cold 20 mM potassium phosphate buffer pH 6.5, then twice with 50 ml cold acetone and once with 50 ml cold chloroform:methanol (1:1 v/v). After this the residue was freeze-dried to constant weight and the final dried weight noted. At least two determinations of yield were made for each cultivar and stage of ripeness.

3.2.4. Degradation of cell wall preparations from mature green fruit of different cultivars

Cell wall material (100 mg) was pre-incubated twice with 150 mM sodium chloride, 50 mM sodium acetate pH 4.0, then incubated in 9.75 ml of the same buffer at 30°C. The final pH of the mixture was 3.8. Polygalacturonase from red Ailsa Craig fruit was extracted, partially purified by ammonium sulphate precipitation in the 40-80% saturation range, and taken up in 0.3 M sodium chloride to give an activity of 650 μkat ml⁻¹. An aliquot (0.25 ml) of this enzyme preparation was added to the cell wall suspension. At 15 minute intervals, 3 ml of the reaction mixture was removed, filtered through Miracloth (Calbiochem Ltd.) and Whatman No.1 filter paper. Two 1 ml samples of the filtrate were used to assay enzyme activity using the picric acid method. Enzyme extract, boiled for five minutes, was used as control.
3.2.5. The activity of purified PG1 and PG2 against cell wall material and pericarp tissue discs

PG1 and PG2 isoenzymes were purified by gel filtration and affinity chromatography as described in Chapter 2. The method of Hobson et al. (1983b) was used to assay PG1 and PG2 activity against cell wall material from mature green tomato fruit. Six 125 mg samples of cell wall material were each incubated with 9.25 ml water, 2.5 ml 0.1 M potassium phthalate pH 4.25, and 0.25 ml saturated (at 20°C) sodium chloride at 30°C. The final pH of the incubating medium plus cell wall material was 3.8, which is optimum for polygalacturonase against cell wall material (Pressey and Avants, 1982a). To this, 0.5 ml of PG1 was added to two samples, 0.5 ml PG2 to another pair of samples and 0.5 ml of 0.3 M sodium chloride to the remaining two samples. After mixing, one tube from each pair of samples with PG1, PG2, or without enzyme, was immediately filtered through Miracloth (Calbiochem) and Whatman No. 541 filter paper with suction. The filtrates were held at 30°C for one hour. The second tube from each pair was incubated under similar conditions for the same length of time and then filtered. All filtrates were centrifuged at 0°C for 20 minutes at 20,000 g and the supernatant decanted. After dilution with an equal quantity of water, four 2 ml samples from each extract were used to determine protein content by the method of Itzhaki and Gill (1964); and two 1 ml samples used to determine the release of reducing groups, by the picric acid method.

The degradation of discs of pericarp tissue from mature green fruit by purified PG1 and PG2 was measured according to the method of Buescher and Hobson (1982). Cylinders (5 mm diameter) were cut
from the outer pericarp walls of green Ailsa Craig fruit using a cork borer, washed under running distilled water for ten minutes and kept in a beaker of water for a further hour at 20°C. The cylinders were then blotted dry. Six cylinders per replicate were weighed (approximately 500-600 mg) and placed in 10 ml tubes with 4.5 ml 20 mM sodium acetate, 50 mM sodium chloride and 0.01% benzoic acid, pH 4.2 and allowed to equilibrate for 30 minutes at 30°C. Either 0.5 ml of 0.3 M sodium chloride, FG1 or FG2, (which had been dialysed against 0.3 M sodium chloride), were added to the cylinders and the tubes incubated at 30°C for 26 hours. The enzyme activity applied to the discs was 40 μkat for FG1 and 28 μkat for FG2. After incubation the cylinders from each replicate were rinsed under water for 30 seconds, kept in water for another hour at 20°C, blotted dry and carefully weighed.

3.3. RESULTS

3.3.1. Ripening characteristics and polygalacturonase content of tomato fruit ripened on-the-plant

In the first season's experiments, the firmness and polygalacturonase content of fruit of several cultivars was examined at two or more stages of ripeness, as judged by subjective colour ratings, to determine whether there was any relationship between fruit firmness and polygalacturonase activity.

The cultivar Ailsa Craig produced fruit which were quite soft when red, whilst another cultivar, Bellina, produced fruit which softened more slowly than those of Ailsa Craig. At the mature green
stage no polygalacturonase activity was detectable in either cultivar (Fig. 3.1), but it was found in green-orange fruit of both cultivars, and increased slowly until fruit reached the orange-green stage of ripeness. Until this stage enzyme activity was due solely to PG1. The slow rate of softening of Bellina fruit at the onset of ripening was reflected in lower PG1 content at the green-orange stage compared to Ailsa Craig fruit at the same stage.

The accumulation of polygalacturonase increased markedly between the orange-green and orange stages of ripening. In Ailsa Craig this increase was due almost entirely to the appearance of PG2, but in Bellina fruit PG1 accumulation continued until the orange stage of ripeness. Red Bellina fruit contained about 40% less polygalacturonase activity than Ailsa Craig but had a higher PG1 content. On a percentage basis the content of PG1 in red Ailsa Craig fruit was about 7% and in red Bellina fruit about 37%. The greater softness of Ailsa Craig fruit compared to Bellina fruit at ripeness could be due to the fact that it accumulated higher amounts of PG2 during the later stages of ripening.

When the firmness and polygalacturonase activity of fruit of several cultivars was examined at two or three stages of ripeness, the correlation between polygalacturonase activity and fruit firmness was less clear. Fruit from different cultivars at the red stage of ripeness were often found to have similar firmness but very different polygalacturonase content. If the firmness of fruit samples was plotted against the polygalacturonase content, determined from pericarp tissue from the same samples (Fig. 3.2), a clearer picture of the possible relationship between polygalacturonase activity and fruit
Figure showing the stage of fruit ripeness for (a) Ailsa Craig and (b) Bellina.
Fig. 3.2. Total PG activity plotted against firmness of fruit samples from several cultivars examined at various stages of ripeness.

Samples of at least five fruit were collected from various cultivars at different stages of ripeness, as assessed subjectively by fruit colour: (MG) Mature green; (GC) green-orange; (OG) orange-green; (O) orange; (R) red. The average firmness of each sample was converted to a firmness index value. Pericarp tissue from all fruit in a sample was bulked and subsampled for analysis of PG activity.

(●) Ailsa Craig; (o) Bellina; (▲) Noneymaker; (▲) Sonatine; (□) Potentate; (■) Rutgers; (▲) Kingley Cross; (▼) Sonato.
Stage of Fruit Ripeness
firmness emerged. During the early stages of fruit ripening and softening (in general up to the orange stage) there was a good correlation \( r = -0.942 \) between firmness and polygalacturonase content of fruit samples. After the orange stage the correlation coefficient was \(-0.002\).

At the same time of year, during spring and early summer, a study was made of the time taken for fruit of different cultivars to ripen. Although many factors other than genotype influenced fruit ripening, such as nutrition (Besford and Maw, 1975), position of fruit on a truss and environmental factors, some differences in the average time taken for fruit of different cultivars to ripen was evident (Table 3.1). The cultivars Ailsa Craig and Sonato ripened more quickly than Moneymaker and Bellina, which in turn ripened faster than Potentate. The problem of determining whether fruit were at the mature green stage was partly overcome by choosing light green fruit which were close in truss position to fruit already ripening, and by checking random samples of such fruit to see if their locular contents showed signs of becoming jelly-like in consistency, a criterion of independent ripening.

Comparing these results with those in Fig. 3.2, shows that cultivars which produced high levels of polygalacturonase when ripe (e.g. Ailsa Craig) produced fruit that on average ripened at a relatively fast rate. Thus polygalacturonase production by tomatoes affects the rate of softening rather than the intrinsic firmness of fruit at a standard red colour.

To examine this idea further, the age, colour, firmness and polygalacturonase activity of fruit from five cultivars was examined.
Table 3.1. Time taken for tomato fruit of different cultivars to ripen

The mean time (in days) taken for tomato fruit of different cultivars to reach successive ripening stages, as judged by colour, from the mature green stage. Figures are means of twenty fruit. Observations were made on fruit on plants grown under glasshouse conditions during spring and early summer.

<table>
<thead>
<tr>
<th>Stage of Ripeness</th>
<th>Ailsa</th>
<th>Craig</th>
<th>Sonato</th>
<th>Bellina</th>
<th>Moneymaker</th>
<th>Potentate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green-orange</td>
<td>3.04  ± 1.50</td>
<td>3.46  ± 1.70</td>
<td>3.55  ± 1.30</td>
<td>3.69  ± 1.8</td>
<td>6.0  ± 0.78</td>
<td></td>
</tr>
<tr>
<td>Orange-green</td>
<td>4.87  ± 1.87</td>
<td>4.78  ± 1.03</td>
<td>5.93  ± 1.28</td>
<td>5.69  ± 1.4</td>
<td>8.5  ± 1.23</td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td>6.44  ± 1.57</td>
<td>5.87  ± 1.4</td>
<td>7.29  ± 1.48</td>
<td>7.00  ± 1.6</td>
<td>10.36  ± 1.22</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>8.66  ± 2.17</td>
<td>8.0  ± 2.1</td>
<td>9.76  ± 1.51</td>
<td>10.07  ± 2.3</td>
<td>12.50  ± 1.70</td>
<td></td>
</tr>
</tbody>
</table>
at five stages of ripeness, (mature green, green-orange, orange-green, orange and red) (Table 3.2).

Since this experiment was conducted in the autumn, the time taken for fruit to ripen differed slightly from the results in Table 3.1, which were obtained from spring-grown plants. However, the differences between cultivars remained the same. Ailsa Craig fruit reached the red stage of ripening, and produced a more intense red coloured fruit as judged by the a/b ratio in 11 days as compared to 13 to 14 days for Moneymaker, Sonatine and Bellina and 16 days for Potentate. Although Moneymaker appeared red by subjective evaluation, the a/b ratio for the fruit sample was much lower than that of the other varieties. However, the firmness of its ripe fruit was similar to that of Sonatine and Bellina. The cultivar Potentate appeared to take longer to produce red pigment, as judged by the a/b ratio than other varieties, but eventually at the red stage had a colouring similar to those of Sonatine and Bellina fruit.

Although the softer cultivar, Ailsa Craig, also produced fruit of a more crimson colour than the other cultivars, there was a poor correlation between fruit colour and firmness at the red stage \((r = 0.516)\) when fruit of different cultivars were compared. However, within each cultivar the development of colour measured by the a/b ratio correlated well with the loss of firmness, (Ailsa Craig \(r = 0.966\); Moneymaker \(r = 0.996\); Sonatine \(r = 0.975\); Bellina \(r = 0.923\) and Potentate \(r = 0.984\)) and with polygalacturonase activity \((r = 0.99)\) up until the orange stage of ripeness.

The five cultivars can be divided into three groups on the basis of rate of softening. Ailsa Craig fruit softened rapidly
Table 3.2. Firmness, colour and PG activity of tomato fruit samples of five cultivars at various stages of ripening on-the-plant.

Fruit samples (n = 10) were harvested from five cultivars at five stages of ripeness: (MG) Mature green; (GO) green-orange; (OG) orange-green; (O) orange; (R) red, and in one cultivar (R') red soft. Colour (a/b ratio) and firmness of individual fruit in the sample were determined, and pericarp tissue bulked and subsequently subsampled for determination of PG activity. Values means of three determinations.
<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Days from M.C.</th>
<th>Stage of Ripeness</th>
<th>Colour a/b Ratio</th>
<th>Firmness (mm compression)</th>
<th>PG Activity (μkat g⁻¹ f.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ailsa Craig</td>
<td>0</td>
<td>NG</td>
<td>-0.550</td>
<td>0.88 ± 0.21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>GO</td>
<td>-0.433</td>
<td>1.62 ± 0.44</td>
<td>0.8 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>OG</td>
<td>-0.152</td>
<td>1.64 ± 0.23</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>O</td>
<td>0.186</td>
<td>1.88 ± 0.12</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>R</td>
<td>1.186</td>
<td>2.33 ± 0.44</td>
<td>129.9 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>R¹</td>
<td>1.400</td>
<td>2.68 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Moneymaker</td>
<td>0</td>
<td>NG</td>
<td>-0.517</td>
<td>0.80 ± 0.16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>GO</td>
<td>-0.424</td>
<td>0.98 ± 0.17</td>
<td>0.9 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>OG</td>
<td>-0.293</td>
<td>1.29 ± 0.15</td>
<td>1.3 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>O</td>
<td>0.153</td>
<td>1.51 ± 0.24</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>R</td>
<td>0.587</td>
<td>1.89 ± 0.18</td>
<td>59.1 ± 0.6</td>
</tr>
<tr>
<td>Sonatine</td>
<td>0</td>
<td>NG</td>
<td>-0.528</td>
<td>0.88 ± 0.16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>GO</td>
<td>-0.329</td>
<td>1.05 ± 0.14</td>
<td>1.1 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>OG</td>
<td>-0.083</td>
<td>1.12 ± 0.15</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>O</td>
<td>0.176</td>
<td>1.58 ± 0.34</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>R</td>
<td>0.926</td>
<td>1.89 ± 0.51</td>
<td>52.0 ± 5.7</td>
</tr>
<tr>
<td>Bellina</td>
<td>0</td>
<td>NG</td>
<td>-0.573</td>
<td>0.65 ± 0.12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>GO</td>
<td>-0.185</td>
<td>0.91 ± 0.21</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>OG</td>
<td>0.154</td>
<td>1.02 ± 0.21</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>O</td>
<td>0.173</td>
<td>1.03 ± 0.16</td>
<td>11.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>R</td>
<td>0.767</td>
<td>1.70 ± 0.38</td>
<td>83.8 ± 14.6</td>
</tr>
<tr>
<td>Potentate</td>
<td>0</td>
<td>NG</td>
<td>-0.541</td>
<td>0.70 ± 0.11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>GO</td>
<td>-0.462</td>
<td>0.83 ± 0.21</td>
<td>0.9 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>OG</td>
<td>-0.318</td>
<td>0.89 ± 0.17</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>O</td>
<td>0.125</td>
<td>1.23 ± 0.43</td>
<td>12.8 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>R</td>
<td>0.891</td>
<td>1.44 ± 0.32</td>
<td>54.9 ± 13.1</td>
</tr>
</tbody>
</table>
during the early ripening phases. Fruit from cultivars Bellina, Sonatine and Honeymaker declined in firmness in a more regular fashion and at an intermediate rate throughout ripening, whilst Potentate fruit softened slowly until they reached the orange-green stage and then ripened at a rate similar to that of the other cultivars. Thus differences in rates of softening between the cultivars were established during the early stages of ripening (Fig. 3.3).

The rate and extent to which fruit softened during the transition from mature green to orange-green appeared to relate better to the firmness of ripe fruit than the rate at which they softened after the orange stage. Ailsa Craig fruit which softened rapidly during early ripening were softer at the red stage than Honeymaker and Bellina, which in turn were softer than Potentate. The exception was Sonatine fruit which softened little after the orange stage, and thus were as firm as those of Potentate.

Polygalacturonase production by fruit of the five cultivars reflected their rates of softening (Fig. 3.4). Ailsa Craig fruit produced significantly more polygalacturonase between the mature green and orange-green stages of ripening i.e. during the first five days of ripening, than the other cultivars, paralleling its early loss of firmness (Fig. 3.3). In Potentate fruit the increase in polygalacturonase production after day 11, at the orange-green stage, reflected the increase in softening at this time.

The correlation between polygalacturonase production and softening during the later stages of ripening was less obvious. All cultivars showed a marked increase in polygalacturonase production about the orange stage of ripeness (Fig. 3.4); however, in Ailsa Craig,
Fig. 3.3. Firmness of fruit samples from five tomato cultivars during ripening on-the-plant.

Ailsa Craig (●); Moneymaker (○); Sonatine (□); Bellina (■); Potentate (▲).

Firmness expressed as percent of the firmness value (compression mm) of fruit samples (n = 10) at the mature green stage.

The points on graph give firmness means at five stages of ripeness: Mature green; green-orange; orange-green; orange and red. In Ailsa Craig fruit an extra sample at red stage was included.
Days from Mature Green

Firmness (as % of Mature Green Fruit)
Fig. 3.4. Total PG activity in fruit samples from five tomato cultivars during ripening on-the-plant.

PG was extracted from pericarp tissue subsampled from the ten fruit used to estimate firmness (Fig. 3.3).

Ailsa Craig (●); Moneymaker (○); Sonatine (□); Bellina (■); Potentate (▲). Values are means of two determinations, the bars indicate sample variation.
Sonatine, Moneymaker and Potentate this was accompanied by a decrease in the rate of softening. Only in Bellina fruit did the rate of softening increase after the orange stage.

Studies of the enzyme extracts by electrophoresis (Fig. 3.5 and Plate 3.1) show that in Potentate, Bellina and Moneymaker only PG1 was present in extracts from fruit up to the orange-green stage. Sonatine, and to a lesser extent Ailsa Craig, fruit had a small amount of PG2 activity at the orange-green stage. It was not until fruit reached the orange stage that PG2A and 2B isoenzymes were evident in all cultivars. In extracts of red fruit, PG2 was predominant and PG1 was often difficult to detect in extracts; the high activity of PG2 in these extracts also made resolution of the PG2A and 2B isoenzymes difficult (Plate 3.1).

In all cultivars the sharp rise in polygalacturonase activity was associated with the production of PG2A and 2B isoenzymes. In some cultivars, Ailsa Craig, Moneymaker and Bellina, PG2A appeared before PG2B, in others they appeared together (Fig. 3.5).

The yield of cell wall material from mature green and red fruit samples of the five cultivars showed that wall content declined during ripening (Table 3.3). However, there was no correlation between firmness of mature green fruit and cell wall content, or between percent loss of firmness and percent loss of wall material. Furthermore, the correlation was poor between firmness at the red stage and wall content and between percent loss of wall content and polygalacturonase activity in red fruit.

One of the reasons for the lack of clear relationship between firmness and wall content between the varieties could be due to
Fig. 3.5. PG isoenzyme content of extracts from ripening fruit of five cultivars.

Polyacrylamide gel analysis of PG in extracts from ripening fruit of five cultivars. Extracts (0.1-0.2 ml) were loaded onto gels and developed cathodally in a β-alanine-acetic acid pH 4.5 system, as described in Chapter 2. Gels were stained for enzyme activity using ruthenium red, after incubation in 2% polygalacturonic acid. The figures to the left of the gels indicate the relative mobility of bands to methyl green. Enzyme activity is denoted by shading. Dotted lines indicate trace activity. The slower moving band is PG1 and the faster moving bands PG2A and B. Direction of travel is toward the cathode.

The days from Mature green and colour of fruit for each extract are given. (G) Green-orange; (O) orange-green; (O) orange; (R) red.
Plate 3.1. PG isoenzyme content of extracts from ripening fruit of four cultivars.

Extracts from pericarp tissue from ripening fruit of cvs. (a) Ailsa Craig, (b) Moneymaker, (c) Sonatine, (d) Bellina were separated by disc gel electrophoresis using the β-alanine-acetic acid pH 4.5 system. Gels were stained for enzyme activity (clear areas) using ruthenium red, after incubation in 2% PGA.

Gels labelled 1–4 are extracts from green-orange, orange-green, orange and red pericarp tissue respectively.
Table 3.3. Loss of firmness of fruit samples from five cultivars during ripening and their loss of cell wall material from pericarp tissue during the same period.

Firmness is expressed as firmness index = \( \frac{1}{\text{compression}} \times 10 \).

MG = Mature green; R = red stage of ripeness.

Cell wall content = yield in g per 100 g f.wt. pericarp tissue.

Variation around mean in parenthesis.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Firmness Index</th>
<th>% Loss</th>
<th>Cell Wall Content</th>
<th>% Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG</td>
<td>R</td>
<td>Firmness</td>
<td>MG</td>
</tr>
<tr>
<td>Ailsa Craig</td>
<td>11.36</td>
<td>4.29</td>
<td>62.20</td>
<td>1.76</td>
</tr>
<tr>
<td>Moneymaker</td>
<td>12.50</td>
<td>5.29</td>
<td>57.68</td>
<td>1.45</td>
</tr>
<tr>
<td>Sonatine</td>
<td>11.36</td>
<td>6.17</td>
<td>45.68</td>
<td>1.32</td>
</tr>
<tr>
<td>Bellina</td>
<td>15.38</td>
<td>5.88</td>
<td>61.77</td>
<td>1.29</td>
</tr>
<tr>
<td>Potentate</td>
<td>14.28</td>
<td>6.94</td>
<td>51.40</td>
<td>1.81</td>
</tr>
</tbody>
</table>

Correlation coefficient (r) between

Firmness of fruit at MG and cell wall content MG = -0.117
Firmness of fruit at R and cell wall content at R = 0.307
% loss firmness during ripening and % loss cell wall = -0.167
% loss cell wall and polygalacturonase activity of red fruit = 0.528
(see Table 3.2)
differences in fruit structure. Plate 3.2 illustrates cross-sections of mature green tomatoes from three cultivars studied. The cultivars Ailsa Craig, Bellina, Soratine and Moneymaker produced mainly bi- or tri-locular fruit whilst Potentate fruit were almost always multilocular.

The thickness of pericarp walls differed among the cultivars, those of Ailsa Craig fruit being thinner and Potentate much thicker than intermediate cultivars. Differences also existed between the thickness of the radial fruit walls, which must play an important role in maintaining fruit firmness.

The pericarp cell walls of various cultivars might also have different chemical or physical attributes which influence their susceptibility to enzyme degradation. However, when cell wall material prepared from mature green fruit from four of the cultivars was degraded by polygalacturonase extracted from red Ailsa Craig fruit, the slight differences in susceptibility to enzyme attack exhibited (Table 3.4), did not relate to the rate or extent to which fruit of those cultivars softened (Table 3.2).

3.3.2. Ripening characteristics and polygalacturonase content of tomato fruit ripened off the plant

The softening of fruit of three of the five cultivars was studied off the plant, so that environmental conditions could be controlled. Fruit from Bellina and Potentate ripened more rapidly off the plant than on, but Ailsa Craig fruit ripened at similar rates (Fig. 3.6 and Fig. 3.3). The differences in rate of softening between the cultivars were similar to those observed in Fig. 3.3, but the
Plate 3.2. Cross-section of mature green tomatoes of cvs. Potentate, Moneymaker and Ailsa Craig.
Table 3.4. Degradation of cell wall material, prepared from mature green pericarp tissue of fruit from four cultivars, by PG extracted from red Ailsa Craig fruit and partially purified by ammonium sulphate fractionation.

Degradation of cell wall material was measured as the release of reducing groups, expressed in terms of galacturonic acid, during incubation of wall material with 162 µkat enzyme over a period of ten and twenty minutes. The values given are the means of four experiments. Those sharing the same letter were not significantly different at the P<0.05 level. No reducing groups were released after incubation with boiled enzyme preparations.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Release of reducing groups nmole galacturonic acid sec⁻¹ per g cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bellina</td>
<td>127.49 ^a</td>
</tr>
<tr>
<td>Ailsa Craig</td>
<td>167.70 ^b</td>
</tr>
<tr>
<td>Potentate</td>
<td>164.41 ^bc</td>
</tr>
<tr>
<td>Sonatine</td>
<td>193.09 ^c</td>
</tr>
</tbody>
</table>
Fig. 3.6. Colour, ethylene production, loss of firmness and total PG activity of samples of fruit from three cultivars at various stages of ripening off-the-plant.

Firmness and colour values are the means of ten fruit samples; ethylene production and polygalacturonase activity the means of three determinations. Bars indicate standard errors.

Ailsa Craig (●); Bellina (■); Potentate (△).
Days of Ripening

Ethylene Production (µl 100 g⁻¹ f.wt. h⁻¹)

Colour a/b ratio

-0.6

2 4 6 8
rate of softening was more regular under the controlled conditions off-the-plant, especially in Ailsa Craig fruit. However, some loss of firmness in this experiment must be due to water loss, despite fruit being kept in polyethylene bags with small perforations to reduce water loss without altering the oxygen and carbon dioxide concentrations around the fruit. Up to 0.5 to 1.0% loss of weight occurred in the samples of fruit during the first ten days of storage.

In all three cultivars the production of polygalacturonase activity and development of yellow colour closely followed the evolution of ethylene by fruit. However, the rate of fruit softening did not follow polygalacturonase activity as closely as colour development. As observed in fruit ripened on-the-plant, much of the softening observed to occur during ripening did so before the sharp rise in polygalacturonase activity, and this increased rate of enzyme production did not result in an increase in rate of softening over the same period.

3.3.3. Activity of purified FG1 and FG2 against cell wall material and pericarp tissue discs

The activity of FG1 and FG2 isoenzymes against cell wall material and pericarp tissue discs was investigated to see if differences in the action of the two isoenzymes in vitro could elucidate their in vivo roles.

Purified FG1 and FG2 samples were assayed using polygalacturonic acid immediately prior to their application to cell wall material and tissue discs. The quantity of enzyme activity applied was similar to that found in red fruit, i.e. 1.6 µkat to 1 g tissue with a cell wall
content of 1.5%. This is similar to the content of polygalacturonase activity found in orange or red fruit.

Despite its activity against polygalacturonic acid, PG1 caused very little degradation of cell wall material compared to an equivalent applied activity of PG2 (Table 3.5), as measured by release of reducing sugars and proteins from the cell wall. The activity of PG2 in degrading cell walls was over ten times greater than that of PG1.

A difference was also observed when tissue discs were used as substrate, although it was not as great. Due to the problem of precipitation of PG1 in solutions of low ionic strength (Chapter 2) both isoenzymes were dissolved in solutions of 0.3 M sodium chloride. Since this was therefore used as the control solution in the tissue disc experiment, some weight loss due to osmotic effects was observed. However, the loss of tissue weight due to enzyme activity was clear (Table 3.6). Since the applied activity of PG1 and PG2 in extracts was different (40 µkat PG1, 28 µkat PG2), the weight loss was calculated on the basis of an applied activity of 50 µkat for both isoenzymes. This shows that PG2 caused about twice the weight loss of discs than PG1.

3.4. DISCUSSION

The firmness of tomato fruit is an important aspect of quality of interest to exporters, retailers and consumers alike. Whilst some softening of fruit during ripening is desirable, to produce an edible texture, fruit that become too soft during post-harvest handling and storage are not only unpalatable, but also very easily bruised and susceptible to attack by pathogens. Thus a number of
Table 3.5. Protein and reducing groups released from cell wall material from mature green pericarp tissue after incubation with purified PG1 and PG2 isoenzymes.

Cell wall material from cvs. Ailsa Craig, Soratime and Bellina was incubated for one hour with 100 µkat purified PG1 and PG2 isoenzymes. Protein and galacturonic acid content of the supernatant was assayed before and after incubation of the cell wall material with isoenzymes. Values are means of six experiments. ND = not detectable.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reducing groups released during incubation (nmole gal. acid per g cell wall)</th>
<th>Protein released during incubation (mg per g cell wall)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boiled PG2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PG1</td>
<td>22.28 + 5.08</td>
<td>2.06 + 1.42</td>
</tr>
<tr>
<td>PG2</td>
<td>330.32 + 74.36</td>
<td>37.25 + 6.05</td>
</tr>
</tbody>
</table>
Table 3.6. Loss of weight of mature green tomato pericarp tissue after incubation with PG1 and PG2 isoenzymes

Discs of mature green tomato pericarp tissue cv. Ailsa Craig were incubated in 20 mM sodium acetate buffer pH 4.2 with purified PG1 and PG2 in 0.3 M sodium chloride for 26 hours. Discs were rinsed with water, blotted dry and weighed before and after incubation. Two experiments were conducted using pericarp tissue from different tomatoes. The treatments in each experiment were replicated four times. Figures in parenthesis are standard errors of the means.

PG1 activity applied in 0.5 ml was 40.0 µkat, and PG2 28 µkat. Values of percent weight loss have been transformed to percent weight loss per 50 µkat applied enzyme to facilitate comparison of the activities of the two isoenzymes against pericarp tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent weight loss of discs</th>
<th>Percent weight loss due to 50 µkat applied enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>Control (0.3 M sodium chloride)</td>
<td>14.10</td>
<td>16.46</td>
</tr>
<tr>
<td></td>
<td>(1.59)</td>
<td>(2.74)</td>
</tr>
<tr>
<td>PG1</td>
<td>25.37</td>
<td>29.68</td>
</tr>
<tr>
<td></td>
<td>(4.72)</td>
<td>(4.27)</td>
</tr>
<tr>
<td>PG2</td>
<td>33.44</td>
<td>45.98</td>
</tr>
<tr>
<td></td>
<td>(4.25)</td>
<td>(4.56)</td>
</tr>
</tbody>
</table>
researchers have evaluated methods for measuring tomato firmness and observed differences in the firmness and rates of softening of different cultivars (Hamson, 1952; Foda, 1957; Shafshak and Winsor, 1964; Hobson, 1965; Kader and Morris, 1976; Gormley and Egan, 1978) similar to those observed here (Table 3.2, Figs. 3.3 and 3.6). The most widely used method of assessing tomato firmness is non-destructive measurement of fruit deformation under a fixed load, as described by Shafshak and Winsor (1964). An instrument similar to the one they describe was used in this study, and the rates and patterns of softening observed here were similar to those reported by them and Hobson (1959).

A method of measuring softening of pericarp tissue, instead of whole fruit, was investigated by adapting the firmness instrument available. Unfortunately the compression of pericarp tissue discs did not correlate with softening or ripening of whole fruit, and this method was abandoned. However, studies using penetrometer-type instruments or shear-presses to evaluate the softness and texture of pericarp tissue of ripening tomatoes (Kader and Morris, 1976) showed that rates of softening of this tissue were similar to those obtained by measuring the compression of whole fruit. The data on fruit softening produced by Foda (1957) using a penetrometer to measure firmness of pericarp tissue showed rates and patterns of softening very similar to those reported here.

Thus whilst physical characteristics of fruit (such as their skin thickness and elasticity, pericarp wall structure, and number of locules) may affect their firmness values before and after ripening, it seems that the rate and pattern of softening of whole fruit
during ripening follows closely that of the pericarp tissue. This is confirmed by Hamson (1952) who found that the firmness of pericarp tissue in tomatoes influenced fruit firmness more than fruit structure.

To study the role of polygalacturonase in tomato fruit softening, only pericarp tissue was therefore sampled for enzyme extraction. In addition, Hobson (1964) found no polygalacturonase activity in locular tissue. Although Sobotka and Watada (1970) found some activity in the locules of tomatoes in their study, it was a small proportion of the total amount extracted from whole tomatoes, and did not appear before the enzyme was detected in the pericarp tissue. They found polygalacturonase had little effect on the locular tissue texture, and their results have been recently confirmed by Huber (1984), who observed no enzymic modification of pectins in the locular tissue of ripening tomatoes. It was, therefore, felt that the polygalacturonase content of pericarp tissue, rather than whole fruit, would best reflect its role in fruit softening.

Polygalacturonase activity was not detected in mature green fruit of any variety in these experiments, and where ethylene evolution by ripening fruit was measured, it always preceded both polygalacturonase activity and colour development (Fig. 3.6). This has recently been confirmed by Brady et al. (1982) and Grierson and Tucker (1983) who have shown that ethylene production precedes both polygalacturonase protein and enzyme activity by at least 20 hours. Likewise, Sawamura et al. (1978) found fruit contained at least two to five μl l⁻¹ ethylene before polygalacturonase activity was detected.
Thus the hypothesis put forward by Tigchelaar et al. (1978a) that polygalacturonase plays a key role in initiating fruit ripening, based on work with mutant tomatoes, and supported by Poovaiah and Nukaya (1979) (who reported finding polygalacturonase activity in tomatoes six days prior to the climacteric) has not been substantiated by these more recent results. However, it is possible that polygalacturonase activity may affect the subsequent rate of ripening, as will be discussed in Chapter 7.

Fruit at the green-orange stage of ripeness had some polygalacturonase activity, and it accumulated slowly until fruit reached the orange colour. After this, enzyme activity increased at a remarkable rate. Accumulation during the early stages of ripening was due exclusively to PG1.

The timing of the appearance of PG2 varied slightly with cultivar; in most cultivars studied here PG2 first appeared in orange fruit. However, in Sonatine and occasionally Ailsa Craig fruit, it appeared in extracts from orange-green fruit. Tucker et al. (1980) observed that PG2 appeared in Ailsa Craig and Potentate fruit between the orange-green and orange stages of ripening, as measured using the heat test; and Ali (1983) found PG2 appeared at the orange-green stage in firm and soft fruited cultivars grown outdoors. Once PG2 isoenzyme appeared, its synthesis was rapid. Thus in red fruit over 80% of the activity was due to the smaller isoenzymes.

Since a fast ripening, soft fruited variety, Ailsa Craig, contained more PG2 than a slow ripening, firmer fruited variety, Potentate, Tucker et al. (1980) concluded that it was the amount of this isoenzyme that determined the loss of firmness in tomato fruit.
However, despite confirming their results in early experiments (Fig. 3.1), further studies (Figs. 3.2, 3.3, 3.4), suggested that a more complex relationship existed between fruit firmness and the accumulation of polygalacturonase isoenzymes.

The different cultivars studied not only softened at different rates, but their pattern of softening differed as well i.e. some fruit softened very rapidly during the early stages of ripening whilst others showed a more regular pattern of softening throughout ripening. Similarly, Poda (1957) found that some varieties lost up to 57% of their firmness in the first three days of ripening, whilst others showed a gradual loss of firmness. Both Hobson (1959) and Ramadan (1981) observed that maximum loss of firmness occurred early in ripening, as judged by fruit colour.

These changes in fruit firmness early in ripening were associated with small amounts of polygalacturonase activity, predominantly in the form of PG1. A good correlation was obtained between polygalacturonase activity during this time and loss of firmness. Hobson (1963) also found that small amounts of polygalacturonase activity were associated with changes in fruit firmness of two cultivars.

In contrast, rate of softening declined as the rate of PG2 activity increased. It appeared that the polygalacturonase content of red fruit of many cultivars, particularly those which soften quickly and have short shelf lives, such as Ailsa Craig, was in excess of that required to produce the loss of firmness observed; hence the poor correlation between polygalacturonase activity and fruit firmness at this stage.
Whether the changes in firmness during the early stages of ripening are due solely to polygalacturonase activity are debatable, and will be discussed more fully in Chapter 7. However, there is good evidence to suggest that firmness changes are associated with the degradation of pectin in the cell wall. Foda (1957), Sawamura et al. (1978), Brady et al. (1982) and Malis-Arad et al. (1983) observed increases in water-soluble uronides and decreases in insoluble pectins soon after the breaker stage. The results in Table 3.5 and those of Ramadan (1981) show significant losses occurred in the cell wall content of ripening fruit. Since polygalacturonase has been found to be the only enzyme capable of initiating cell wall degradation and of causing extensive wall degradation in vitro (Wallner and Walker, 1975), there is good reason to believe it is the major enzymic cause of softening in vivo. To confirm this, Gross and Wallner (1979) observed that the increase in water-soluble uronides during fruit ripening was closely associated with polygalacturonase activity.

However, other enzymes such as pectinesterase, cellulase and \( \beta \)-galactosidase or non-enzymic processes may play an important role, especially in the softening that occurs between the mature green and green-orange stages when polygalacturonase activity is very low indeed.

The role of the two isoenzymes of polygalacturonase in softening is not obvious from these results. Studies of the accumulation of the isoenzymes and associated rates of fruit softening might suggest that PG1 isoenzyme plays an important role early in softening, possibly by attacking large and heterogeneous pectin
molecules of the middle lamella layer causing reduction in intercellular cohesion and loss of tissue firmness. PG2 appeared to be less effective in causing fruit softening in vivo than PG1. However, in vitro studies using purified PG1 and PG2 indicate quite the opposite. PG2 was more effective by a factor of ten in degrading cell wall material isolated from a variety of tomato cultivars and by a factor of two in degrading pericarp tissue discs.

The reason for the difference in the results from the two substrates is unknown. Pressrey and Avants (1982a) examined the reaction of PG1 and PG2 against cell wall material and found PG2 to be only twice as active as PG1 in releasing reducing groups from the wall preparations. They prepared their cell walls by first boiling them in water to inhibit enzymic demethoxylation of the wall during extraction. This treatment could well have altered the structure of the pectin molecules in the wall, enabling PG1 to degrade them more rapidly than observed in the experiments reported here (Table 3.5).

Crookes and Grier son (1983) applied PG1 and PG2 extracts to tomato pericarp discs and examined the resulting degradation by electron microscopy and found no difference in the amount of degradation caused by the two enzymes, though rates of degradation were not estimated. One of the problems faced in using complex substrates in in vitro experiments such as these is that of ensuring that the substrate is accessible to the enzyme. The large molecular weight of PG1 relative to PG2 may be the reason for its apparent lower activity, since its size must preclude easy access to the inner sections of cell wall.

Studies of the action of the two isoenzymes against defined substrates of varying molecular weight showed that PG1 was less
sensitive to substrate size than PG2, but that the latter isoenzyme caused a more rapid loss of viscosity of a pectic acid solution (Pressey and Avanta, 1971, 1973). However, both Ali (1983) and Moshrefi and Luh (1983) observed no difference in viscosity changes in polygalacturonic acid substrate attacked by the two isoenzymes. It appears then that the conditions of the experiment and the substrate used influence isoenzyme action considerably, and that until more is understood of the chemical changes occurring in ripening cell walls the role of the two isoenzymes will remain undefined.

To establish the contribution made by polygalacturonase isoenzymes to tomato fruit firmness during ripening, it is necessary to examine all factors affecting fruit firmness. The variation in firmness observed to occur between fruit of different cultivars may be attributed to at least two sets of factors. A consideration of these may resolve the apparent anomalies between firmness and polygalacturonase that have been reported in the literature.

An important feature of fruit firmness is its structure, such as its skin and pericarp thickness, number of locules per fruit and structure of the radial walls. Variations in structure undoubtedly are responsible for the differences in fruit firmness observed at the mature green stage. For example, the thick-walled multilocular Potentate fruit had a higher firmness than the thin-walled, bilocular variety Ailsa Craig. If fruit having diverse structures have different firmness levels before ripening, then it is likely that such differences will exist during ripening, unless the fruit have very variable rates of softening.
This could explain why Ramadan (1981) and Ali (1983) observed no correlation between the firmness of a cultivar and its polygalacturonase content, since they were relating enzyme levels to absolute values of firmness rather than rates of softening. Close examination of Ramadan's results showed that in fact the two cultivars he studied had similar rates of softening during ripening, the difference between the two being their firmness at the mature green stage. Hence it is not surprising that he found a better correlation between firmness and total cell wall content than firmness and polygalacturonase activity.

In contrast, in these experiments the differences between the fruit of different cultivars at the mature green stage was not very great and did not correlate with cell wall content, although a better correlation was obtained at the red stage (Table 3.3). Instead, the other component of fruit firmness during ripening was more predominant i.e. the amount and rate of cell wall degradation leading to tissue softening. Fruit that softened rapidly during ripening had a higher polygalacturonase activity in the early stages of ripening than fruit softening more slowly (Fig 3.4).

Ali (1983) suggested that the differences in softness of ripe fruit from two cultivars she studied was not due to their polygalacturonase content but to the susceptibility of their cell walls to enzymic attack. Variations in the structure of cell walls, or their calcium content could affect their resistance to degradation (Wills and Tirmazi, 1979; Rigney and Wills, 1981). Wallner and Bloom (1977) observed that cell wall material prepared from fruit of several cultivars varied in its susceptibility to degradation by polygalacturonase. However, results obtained in these experiments
(Table 3.5) indicate that the hydrolysis of cell walls by polygalacturonase in vitro did not mirror the differences in softening observed to occur in the fruit of the cultivars from which the wall material was isolated. Many researchers have shown that wall degradation is the main cause of loss of fruit firmness (Pola, 1957; Brady et al., 1982). Malis-Arad et al. (1983) found that the difference in firmness in two cultivars was due not to variations in quantity or quality of pectins in the cell walls but to the rate at which pectins were solubilised during ripening. This confirms the importance of pectin degrading enzymes such as polygalacturonase in regulating the firmness of these cultivars.

Whilst polygalacturonase activity may no longer be considered an initiator of ripening events, its activity on the cell wall may indirectly affect the subsequent rate of other ripening events, such as colour development.

In all the cultivars considered changes in fruit colour, as judged by the a/b ratio, correlated with loss of firmness and with polygalacturonase activity, at least until the orange stage of ripeness. But comparisons of fruit colour and polygalacturonase activity between cultivars produced a poor correlation. Gormley and Egan (1978) similarly reported finding a good correlation between firmness and colour of fruit ripening within a cultivar, but found that differences existed between cultivars. In contrast Hobson et al. (1983a) reported a good correlation between colour and firmness of different cultivars. Possibly the stage of ripening at which the comparisons were made is important in determining the degree of correlation. The results of this work suggest that differences between firmness, polygalacturonase and colour of fruit of various
cultivars become less distinct after fruit reach the red stage of ripeness. In general, fruit having a high degree of red colour when ripe have a high polygalacturonase content, as also observed by McColloch et al. (1952).

However, it appears that some non-ripening mutant tomatoes which also contain genes that promote red colour development in ripe fruit, (such as high pigment, hp, and crimson characteristic, cc) also have long shelf lives and low polygalacturonase production (Foda, 1957; Sobotka and Watada, 1970). Whether these fruit contain genes with multiple effects on ripening such that firmness and colour were affected independently is not known. In addition, the synthesis of pigments by non-ripening mutant fruit, achieved by salt treating plants during fruit development, was not accompanied by an increase in polygalacturonase production (Chapter 6). Thus, the relationship between colour and polygalacturonase activity development is uncertain, but it is likely that they are both regulated by the same factor, possibly ethylene (Crookes and Grierson, 1983). Tomatoes stored under conditions which inhibit ethylene production showed no polygalacturonase activity or lycopene synthesis until removed to normal atmosphere when ethylene production commenced followed by polygalacturonase activity and lycopene synthesis (Goodenough et al., 1982). Later experiments confirmed this association of colour and pectic enzyme activity with ethylene (Jeffery et al., 1984). However, the nature of the association is as yet unknown, and there is no proof yet that polygalacturonase production is dependent on or regulated by fruit ethylene production.
In conclusion the results of this chapter show that the softening of tomato fruit during ripening was accompanied by loss of cell wall material from pericarp tissue and an increase in polygalacturonase activity, in the form of three isoenzymes. However, the marked increase in polygalacturonase production, due almost entirely to PG2 activity, observed to occur after fruit reached the orange stage of ripeness, was not accompanied by a similar increase in the rate of fruit softening. Instead most fruit softening occurred during the early stages of ripening, when polygalacturonase activity was accumulating at a slower rate, and in the form of FG1. The relative activities of the two isoenzymes in vivo were not reflected in their activities against cell wall preparations and tissue discs in vitro. This is possibly due to problems associated with size differences and conditions required by the isoenzymes for optimum activity. Although polygalacturonase appears to play an important role in regulating the rate of pericarp tissue softening during ripening, its effects on whole fruit firmness may be influenced by factors affecting the inherent firmness of fruit, such as their structure and the composition of their pericarp cell walls.
CHAPTER FOUR ACCUMULATION OF POLYGALACTURONASE ISOENZYMES IN NORMAL, NON-RIPENING MUTANT AND MUTANT HYBRIDS OF CV. AILSA CRAIG

4.1. INTRODUCTION

Three mutants having multiple effects on tomato fruit ripening have been characterised (Tigchelaar et al., 1978a) and used both in breeding programmes, designed to increase the storage life of tomatoes, and in biochemical studies, designed to elucidate the mechanisms of fruit ripening. The three mutants are: ripening inhibitor, rin, a recessive gene located on chromosome 5, non-ripening, nor, a recessive gene located on chromosome 10; and never ripe, Nr, an incomplete dominant gene located on chromosome 9 (Tigchelaar et al., 1978a). These genes have been incorporated into isogenic backgrounds by repeated backcrossing of the original mutants with commercial cultivars such as Ailsa Craig and Rutgers (Hobson, 1980; Brady et al., 1983), to facilitate physiological studies of their effect on fruit ripening.

Studies of these mutants in their homozygous forms have shown that, despite being non-allelic, rin and nor genes have similar effects on tomato ripening. Both virtually inhibit ripening changes with the exception of seed maturation, are non-climacteric and produce little or no ethylene (Denis, 1973; Herer and Sink, 1973; Ng and Tigchelaar, 1977), have severely reduced carotene and lycopene synthesis (Sink et al., 1974; Buescher and Doherty, 1978; Tigchelaar et al., 1978a), soften extremely slowly, and contain only trace amounts of polygalacturonase (Hobson, 1980; Tucker et al., 1980). The Nr gene,
in contrast, does not totally inhibit ripening, but delays the onset of ripening and reduces the magnitude of some ripening events, such as lycopene synthesis and the rate of softening and polygalacturonase production compared with normal fruit (Hobson, 1967; Denis, 1973).

The homozygous forms of these mutants have no practical use in the tomato industry because of their inability to ripen fully and their poor flavour characteristics (Tigchelaar et al., 1978a; Hobson, 1980). However, hybrids between these mutants and normal cultivars have ripening characteristics intermediate between their parents.

Buescher et al. (1976) found that nor x CV17 hybrids produced slightly firmer fruit than rin x CV17 hybrids, both of which were significantly firmer than the normal parents. This difference in firmness was reflected by levels of water-soluble pectins and polygalacturonase activities. However, Tigchelaar et al. (1978b) found that the levels of polygalacturonase in single and double hybrids of rin and nor were only roughly related to the rate of fruit softening; and Hobson (1980) observed that although a rin x Ailsa Craig hybrid had only 65% of the polygalacturonase activity of its normal parent there was no difference in the firmness of the ripe fruit. The Nr gene exerts a strong effect on fruit colour and flavour when hybridised with normal cultivars, but only a slight effect on fruit firmness when crossed with the slow-ripening cultivar Potentate (Hobson, 1967). However, despite the small difference in fruit firmness the polygalacturonase content of the hybrid was only 37% that of the normal parent.

The mutant genes, either in homozygous or heterozygous form, provide a useful tool to study the effects of polygalacturonase on fruit firmness when they are incorporated into isogenic backgrounds,
since differences in rates of softening due to variations in fruit structure can be almost eliminated. Although the studies above show that there might be some relationship between polygalacturonase content and softness of mutant tomatoes, most reported values are at only one or two stages of ripening and did not distinguish between isoenzymic forms of polygalacturonase.

To obtain a better understanding of the role of polygalacturonase in the ripening of fruit the accumulation of PG1 and PG2 in normal, mutant and F1-hybrid lines isogenic with cultivar Ailsa Craig during the later part of development (= ripening) was studied. Soon after the completion of this work Brady et al. (1983) published a report of a similar study conducted on mutant hybrids rin and nor crossed with a slow-ripening cultivar Rutgers. Their work complements this study conducted with mutant hybrids rin and Nr crossed with a fast-ripening cultivar Ailsa Craig, and their results will be discussed with respect to our own.

4.2. MATERIALS AND METHODS

4.2.1. Tomato cultivation and assessment of fruit ripeness

Seed homozygous for never ripe (Nr) and ripening inhibitor (rin) had been produced in a background nearly isogenic with cv. Ailsa Craig as a result of five successive backcrosses and selfing generations with progeny selection at each stage. A final cross with cv. Ailsa Craig produced the heterozygous lines. This work was done by Mr. P. Grimbly at the Glasshouse Crops Research Institute.
The plants and fruit referred to in this work as Ailsa Craig are the wild genotype, *nrnr RinRin*.

Plants of the cultivar Ailsa Craig and the *Rinrin*, *NnNr*, *Nnr* and *Nnr Rinrin* isogenic breeding lines were grown under glasshouse conditions as described in Chapter 3, during the summer months. Flowers were tagged at anthesis to determine fruit age. Fruit colour, firmness, ethylene production, polygalacturonase activity and its isoenzyme content were determined on samples of fruit harvested at different stages of ripeness as outlined in Chapters 2 and 3.

4.2.2. Characteristics of fruit ripening on-the-plant

Samples of at least ten fruit of the same age and truss position on the plant were collected from eight plants of each of the five breeding lines at intervals during ripening. Fruit firmness and colour were measured before pericarp samples were taken and these were frozen at -20°C for subsequent polygalacturonase analysis, as described in Chapters 2 and 3.

4.2.3. Characteristics of fruit ripening off-the-plant

Fifty fruit per genotype were harvested at the mature green stage and stored in trays covered with polyethylene film to reduce water loss. The film contained a few small perforations to prevent modification of the atmosphere around the fruit during storage. The fruit were kept in the dark at 20°C. A sample of ten numbered fruit from each genotype were used for measurement of colour and firmness and a sample of three of these, taken at random, were used for ethylene determinations. Measurements were made every two to three
days for three weeks. At intervals during ripening samples of four
fruit were removed and stored at -20°C prior to polygalacturonase
analysis. Pericarp samples from mature green and red fruit were also
taken for analysis of cell wall content as described in Chapter 3.

4.3. RESULTS

4.3.1. Characteristics of fruit ripening on-the-plant

The weather conditions during the late summer of 1983, when
the fruit used in this experiment were ripening, were very variable.
Since fruit of the different genotypes took varying lengths of time
to reach maturity and ripeness from anthesis (Table 4.1), it is
likely that they all ripened under slightly different environmental
conditions. This could explain why the hybrid mutant Nnr, ripened
faster than the normal parent, Ailsa Craig.

The Nr gene had a greater influence on fruit colour in hybrid
breeding lines than the rin gene. Rinrin fruit had a similar a/b
ratio to normal fruit when ripe, but Nnr and Nnr Rinrin fruit were
only orange coloured when at an equivalent softness to normal fruit,
while homozygous NnNr fruit remained yellow even when quite soft. The
development of colour, as measured by the a/b ratio did not correlate
with the loss of firmness during the ripening of fruit samples from
the five breeding lines. The best correlation was observed in Rinrin,
\( r = 0.851 \); all the other lines gave \( r \) values below 0.5. However,
reasonably good correlations were found between colour and
polygalacturonase accumulation during the ripening of each genotype.
The correlation coefficients were: Ailsa Craig 0.937; Rinrin 0.822,
Table 4.1. Ripening characteristics of normal and heterozygous mutant fruit of cv. Ailsa Craig ripened on-the-plant

Colour and firmness values are the mean of ten fruit samples. PG values are the mean of three determinations. The amount of PG1 isoenzyme was determined by the heat test; residual activity in extracts heated to 65°C for five minutes was assumed to be due to PG1 isoenzyme.

ND — not detectable
<table>
<thead>
<tr>
<th>Days from: Anthesis M.G.</th>
<th>Colour Stage</th>
<th>Colour a/b</th>
<th>Firmness (mm compression)</th>
<th>Total PG (μkat g⁻¹ f.wt.)</th>
<th>% PG1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ailsa Craig</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 0 MG</td>
<td>-0.574</td>
<td>1.27 ± 0.15</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34 2 GO</td>
<td>-0.339</td>
<td>1.71 ± 1.19</td>
<td>2.3 ± 0.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>37 5 OG</td>
<td>-0.202</td>
<td>1.94 ± 0.30</td>
<td>5.3 ± 0.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>42 10 O</td>
<td>0.237</td>
<td>2.46 ± 0.46</td>
<td>28.3 ± 4.2</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>44 12 R</td>
<td>1.006</td>
<td>2.99 ± 0.54</td>
<td>61.5 ± 4.8</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><strong>Rinrin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 0 MG</td>
<td>-0.567</td>
<td>1.01 ± 0.15</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 2 GO</td>
<td>-0.427</td>
<td>1.53 ± 0.13</td>
<td>0.8 ± 0.01</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>40 5 OG</td>
<td>0.177</td>
<td>1.76 ± 0.16</td>
<td>6.9 ± 0.8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>45 10 O</td>
<td>0.297</td>
<td>2.06 ± 0.32</td>
<td>21.5 ± 6.0</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>48 13 R</td>
<td>1.114</td>
<td>2.36 ± 0.28</td>
<td>77.9 ± 18.7</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td><strong>Nnr</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38 0 MG</td>
<td>-0.545</td>
<td>1.17 ± 0.14</td>
<td>ND</td>
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</tr>
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<td>39 1 GO</td>
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<td>1.69 ± 0.33</td>
<td>2.5 ± 0.5</td>
<td>100</td>
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</tr>
<tr>
<td>41 3 OG</td>
<td>-0.194</td>
<td>2.20 ± 0.24</td>
<td>4.9 ± 0.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>42 4 O</td>
<td>0.007</td>
<td>2.34 ± 0.27</td>
<td>45.6 ± 5.8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>47 9 OR</td>
<td>0.413</td>
<td>2.84 ± 0.21</td>
<td>75.9 ± 21.4</td>
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<td></td>
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<tr>
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<td>ND</td>
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<td>8.8 ± 0.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>50 11 O</td>
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<td>2.66 ± 0.51</td>
<td>17.5 ± 0.6</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>52 12 OR</td>
<td>0.321</td>
<td>3.28 ± 0.22</td>
<td>18.6 ± 1.0</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td><strong>Nlr</strong></td>
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<tr>
<td>38 0 MG</td>
<td>-0.449</td>
<td>1.57 ± 0.25</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
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<td>0.17 ± 0.1</td>
<td>100</td>
<td></td>
</tr>
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<td>2.41 ± 0.31</td>
<td>1.62 ± 0.6</td>
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<td></td>
</tr>
<tr>
<td>50 12 O</td>
<td>0.064</td>
<td>2.64 ± 0.20</td>
<td>23.0 ± 2.1</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>
Nrnr 0.924; Nrnr Rinrin 0.970 and NrKr 0.819. However, there was a very poor correlation between the colour and firmness or polygalacturonase activity when fruit of the different genotypes were compared at similar levels of softness. The colour differences observed to occur between genotypes did not appear to be closely related to the amount of polygalacturonase produced, despite the fact that mutant fruit with low polygalacturonase activity also had reduced carotenoid and lycopene levels.

Since fruit of the various genotypes had slight but not significantly different levels of firmness at the mature green stage (Table 4.1), firmness data was transformed to percent of firmness at the mature green stage to facilitate comparison of rates of softening occurring in the five genotypes during ripening (Fig. 4.1). Surprisingly, fruit of Nrnr hybrid softened more rapidly than fruit of normal or Rinrin genotypes. This might be accounted for by the variable environmental conditions under which the fruit ripened.

Differences in rates of softening between the genotypes were established early in ripening, during the first five days (Fig. 4.1). After this time the rates of softening were similar for fruit of all genotypes. Thus the softness of fruit from the five genotypes late in ripening was principally due to the rates of softening established early in ripening. Polygalacturonase activity in 'ripe' fruit generally reflected their degree of softness. Ailsa Craig, Rinrin and Nrnr fruit had high polygalacturonase activity compared to NrKr fruit and were much softer than the latter (Fig. 4.2). However, fruit of Nrnr Rinrin produced very little polygalacturonase activity during ripening, yet softened at a rate similar to that of the normal parent.
Fig. 4.1. Loss of fruit firmness during ripening of normal and heterozygous mutant fruit of cultivar Ailsa Craig. Fruit ripened on-the-plant.

(*) Ailsa Craig; (o) Rinrin; (■) NrNr; (□) NnRrinrin; (△) NrNr.
Fig. 4.2. Polygalacturonase activity of normal and heterozygous mutant fruit of cultivar Ailsa Craig. Fruit ripened on-the-plant.

(a) PG activity during the whole ripening period. Bars indicate standard errors.

(b) PG activity during the first six days of ripening.

(Scale of Fig. 4.2 a enlarged.)

(•) Ailsa Craig; (c) RInrin; (■) Nrnr; (□) NrnrRInrin;
(A) NrNr.
This accretion can be explained by a more detailed comparison of fruit softening and polygalacturonase action (Figures 4.1, 4.2, a and b). The high levels of polygalacturonase found in fruit of normal 'Allen Craig' (Fig. 4.1a) and of hybrid 'Hirini' fruit (Fig. 4.1b) were not accompanied by increased rates of softening during the same stage of ripening. However, when the rates of softening during the early stages of ripening were compared with the changes in polygalacturonase activity over the same period (Figs. 4.1a and 4.2b), then a better correlation was observed.

The fruit which showed an early and rapid softening had an early accumulation of polygalacturonase. However, the polygalacturonase activity was not reflected in the subsequent rate of softening, as it was in the hybrid 'Hirini' fruit. The polygalacturonase activity in 'Allen Craig' fruit was not as pronounced as in the hybrid 'Hirini' fruit, but the rate of softening was also not as rapid (Fig. 4.1a and b). This was reflected in the polygalacturonase production which was quite low after day 0 to 3 but rose rapidly after that. The hybrid fruit produced very low polygalacturonase activity initially, but the rate of softening was much greater than the rate of polygalacturonase production later in development. The papers also mention that the effect of gibberellic acid on polygalacturonase activity in 'Allen Craig' fruit was greater than in the hybrid 'Hirini' fruit. The results are presented in Figure 4.3.
This anomaly can be explained by a more detailed comparison of rates of fruit softening and polygalacturonase accumulation (Figs. 4.1, 4.2 a and b). The high levels of polygalacturonase found in fruit of normal, Rinrin and Nnr during the later stages of ripening, were not accompanied by increased rates of softening. However, when the rates of softening during the early stages of ripening were compared with the changes in polygalacturonase activity over the same period (Figs. 4.1 and 4.2 b), then a better relationship was observed.

Nnr fruit which showed an early and rapid loss of firmness had an early accumulation of polygalacturonase. Ailsa Craig and Rinrin fruit soften in a similar way, more slowly than Nnr, and this was reflected in their slower rate of polygalacturonase production. There was a slight delay in polygalacturonase production in Rinrin fruit compared with normal Ailsa Craig, but by day five their rates of softening and polygalacturonase production were similar. The double hybrid Nnr Rinrin softened slightly more slowly than Ailsa Craig during the first three days of ripening but thereafter softened more rapidly (Figs. 4.1 and 4.2 b); this was reflected in its polygalacturonase production which was quite low from day 0 to 3 but rose rapidly after that. Nnr fruit produced very little polygalacturonase during ripening, less than the amount of softening might suggest. However, it softened at a very slow rate compared to the other genotypes.

The isoenzyme content of polygalacturonase extracts was examined by heat test method (Table 4.1) and in some extracts by electrophoresis as well (Fig. 4.3). Fruit of Nnr, Nnr Rinrin and
Fig. 4.3. PG isoenzymes, separated by disc gel electrophoresis, from fruit of Ailsa Craig nmr and Nmr genotypes during ripening on-the-plant.

Table 4.3. Days of ripening on-the-plant and fruit weight in fruit ripened off-the-plant.

<table>
<thead>
<tr>
<th>Stage of Ripeness</th>
<th>OG</th>
<th>O</th>
<th>R</th>
<th>O</th>
<th>O-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ripening</td>
<td>2</td>
<td>10</td>
<td>12</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>
NrNr contained only PG1 during the first five days of ripening and thereafter contained at least 80% PG1, even in fruit that had become quite soft. Ripe Rinrin fruit contained 70% PG1 compared to a value of 30% in normal fruit at the same stage of ripeness, as judged by colour and degree of firmness. Thus the rate of softening of these genotypes did not appear to relate to the amount of PG2 produced.

4.3.2. Characteristics of fruit ripening off-the-plant

Under the controlled environmental conditions of this experiment the ripening characteristics of fruit samples from the five genotypes was studied in more detail (Table 4.2). The colour of Ailsa Craig and Rinrin fruits ripened on- and off-the-plant (Tables 4.1 and 4.2) were very similar, but NrNr, Nrnr and Nrnr Rinrin fruit ripened on-the-plant took only 12 days from the mature green stage to reach the same colour that similar fruit ripened off-the-plant took 21 days to attain after harvest.

The effect on softening of ripening fruit off-, rather than on-the-plant, varied among the five genotypes (Tables 4.1 and 4.2). Harvesting Ailsa Craig and Rinrin fruit slightly enhanced their rates of softening, but retarded softening in Nrnr, NrNr and Nrnr Rinrin fruit. These differences were reflected in polygalacturonase activity. Twelve days after mature green stage polygalacturonase levels in Ailsa Craig and Rinrin were 61.1 and 78.0 μkat g⁻¹ fresh weight respectively in fruit left on-the-plant, and 195.0 and 130.0 μkat g⁻¹ fresh weight in fruit ripened off-the-plant. As expected Nrnr, NrNr and Nrnr Rinrin fruit had less polygalacturonase in harvested fruit than comparable aged fruit on-the-plant.
Table 4.2. Ripening characteristics and accumulation of PG isoenzymes in normal and mutant heterozygous fruit of cultivar Ailsa Craig ripening off-the-plant.

Fruit samples harvested at the mature green stage were held at 20°C in the dark. Colour and firmness values are the means of ten fruit samples. PG activity values are the means of two determinations. The amount of PG1 was estimated by the heat test.

ND = not detectable
+ = one determination only
<table>
<thead>
<tr>
<th>Days of Ripening</th>
<th>Colour a/b Ratio</th>
<th>Firmness (mm compression)</th>
<th>% Weight Loss</th>
<th>Total PG Activity (μkat g⁻¹ f.wt.)</th>
<th>% PG1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ailsa Craig</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-0.587</td>
<td>1.19 ± 0.10</td>
<td>0.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-0.559</td>
<td>1.31 ± 0.18</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-0.527</td>
<td>1.44 ± 0.20</td>
<td>0.3</td>
<td>0.4 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>-0.154</td>
<td>2.28 ± 0.40</td>
<td>0.7</td>
<td>20.4 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>0.433</td>
<td>2.53 ± 0.47</td>
<td>0.9</td>
<td>169.5 ± 5.8</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>0.638</td>
<td>2.69 ± 0.43</td>
<td>1.2</td>
<td>196.7 ± 6.1</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>1.196</td>
<td>2.98 ± 0.34</td>
<td>1.5</td>
<td>273.9 ± 10</td>
<td>36</td>
</tr>
<tr>
<td>12</td>
<td>1.298</td>
<td>3.38 ± 0.28</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.440</td>
<td>3.63 ± 0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.591</td>
<td>3.82 ± 0.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>1.564</td>
<td>3.92 ± 0.45</td>
<td>2.29</td>
<td>412.2 ± 35.7</td>
<td>34</td>
</tr>
<tr>
<td><strong>Rinrin</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-0.595</td>
<td>1.21 ± 0.10</td>
<td>0.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
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<td>-0.558</td>
<td>1.18 ± 0.10</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-0.554</td>
<td>1.24 ± 0.10</td>
<td>0.2</td>
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<td>1.60 ± 0.30</td>
<td>0.4</td>
<td>10.1 ± 0.5</td>
<td>100</td>
</tr>
<tr>
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<td>1.78 ± 0.30</td>
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<tr>
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<td>1.87 ± 0.15</td>
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<td>27.5 ± 1.2</td>
<td>47</td>
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<td>0.9</td>
<td>129.2 ± 17.7</td>
<td>20</td>
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<tr>
<td>14</td>
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<td>2.77 ± 0.25</td>
<td>1.1</td>
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<td></td>
</tr>
<tr>
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<td>1.206</td>
<td>2.96 ± 0.30</td>
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<td>1.36</td>
<td>281.5 ± 51.4</td>
<td>20</td>
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<td><strong>Nnr</strong></td>
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<td></td>
</tr>
<tr>
<td>0</td>
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<td>1.19 ± 0.11</td>
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</tr>
<tr>
<td>1</td>
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<td>1.15 ± 0.13</td>
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<tr>
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<td>100</td>
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<tr>
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<td>1.93 ± 0.45</td>
<td>0.5</td>
<td>6.9 ± 2.2</td>
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continued ...
<table>
<thead>
<tr>
<th>Days of Ripening</th>
<th>Colour a/b Ratio</th>
<th>Firmness (mm compression)</th>
<th>% Weight Loss</th>
<th>Total PG Activity (µkat g⁻¹ f.wt.)</th>
<th>% PG1</th>
</tr>
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<td>Nrnr (cont.)</td>
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</tr>
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<td>18.6 ± 2.7</td>
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<td>0.8</td>
<td>21.6 ± 3.7</td>
<td>89</td>
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<td>3.04 ± 0.31</td>
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<td>100.3 ± 2.1</td>
<td>40</td>
</tr>
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<td>3.40 ± 0.31</td>
<td>1.6</td>
<td></td>
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</tr>
<tr>
<td>NrNr</td>
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</tr>
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<td>0</td>
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<td>1.34 ± 0.20</td>
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<td>1.28 ± 0.17</td>
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<td>0.4 ± 0.05</td>
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<td>0.8</td>
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<tr>
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<td>1.72 ± 0.50</td>
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<td>1.8 ± 0.1</td>
<td>100</td>
</tr>
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<td>1.0</td>
<td>3.2 ± 0.04</td>
<td>100</td>
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<td>2.55 ± 0.70</td>
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<td></td>
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</tr>
<tr>
<td>16</td>
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<td>2.76 ± 0.77</td>
<td></td>
<td>19.1 ± 4.8</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>-0.153</td>
<td>3.15 ± 0.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nrnr Rinrin</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-0.574</td>
<td>1.25 ± 0.20</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-0.571</td>
<td>1.23 ± 0.18</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-0.577</td>
<td>1.23 ± 0.21</td>
<td>0.2</td>
<td>ND</td>
<td></td>
</tr>
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<td>5</td>
<td>-0.583</td>
<td>1.44 ± 0.16</td>
<td>0.4</td>
<td>0.8 ± 0.1</td>
<td>100</td>
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<td></td>
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</tr>
<tr>
<td>9</td>
<td>-0.248</td>
<td>1.63 ± 0.28</td>
<td>0.6</td>
<td>1.0 ± 0.1</td>
<td>100</td>
</tr>
<tr>
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</tr>
<tr>
<td>14</td>
<td>-0.212</td>
<td>2.49 ± 0.39</td>
<td></td>
<td>1.6 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
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<td>2.71 ± 0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>-0.153</td>
<td>2.93 ± 0.37</td>
<td></td>
<td>12.1 ± 0.7</td>
<td>100</td>
</tr>
</tbody>
</table>
Differences in rates of fruit softening between genotypes were more pronounced in fruit ripened off, than those ripened on, the plant (Figs. 4.2 and 4.4). Ailsa Craig fruit softened rapidly after harvest and this was reflected by a high initial rate of polygalacturonase activity. Considerable softening of these fruit occurred by day five and analysis of extracts by electrophoresis and heat test (Table 4.2, Plate 4.1 and Fig. 4.5) showed that PG1 was the predominant form of the enzyme at this stage. Similarly rates of softening of the other genotypes during the first 12 days were reflected in fruit polygalacturonase activity (Fig. 4.4 b). Although the production of polygalacturonase by Nnr fruit was less than expected from its rates of softening.

After day 12 the rates of softening were similar for all cultivars and the extremely high levels of PG2 produced by Ailsa Craig and Rinnin fruit after day ten appeared to have little effect on their subsequent rate of softening. Polygalacturonase extracts from fruit of all genotypes up to day five of ripening were found to contain only PG1 (Table 4.2, Plate 4.1 and Fig. 4.5). Thus in all genotypes most fruit softening had occurred before the appearance of PG2 isoenzymes.

The amount of polygalacturonase produced by Ailsa Craig and Rinnin fruit in this experiment was considerably higher than that observed in most other experiments, although such high levels had been encountered in a previous experiment (Chapter 2).

Although some weight loss occurred from all the fruit samples, despite the use of polyethylene bags, it was less than 0.7% by day five. The amount of weight lost by samples of different genotypes did not relate to their relative losses of firmness (Table 4.2).
Fig. 4.4. a. Loss of fruit firmness during ripening of normal, mutant and heterozygous mutant fruit of cultivar Ailsa Craig. Fruit ripened off-the-plant, at 20°C in the dark.

Fig. 4.4. b. PG activity of normal, mutant and heterozygous mutant fruit of cultivar Ailsa Craig. Fruit ripened off-the-plant at 20°C in the dark. Bars indicate variation around the mean.

(*) Ailsa Craig; (o) Rinrin; (■) Nrnr; (□) NrnrRinrin; (△) NrNr.
Fig. 4.5. Polyacrylamide gel electrophoresis of PG in extracts from:

(a) normal Ailsa Craig fruit at day 2, 5, 9 and 14 of ripening
(b) Rinrin fruit at day 5, 7, 12 and 16 of ripening
(c) Nnr fruit at day 5, 12 and 19 of ripening
(d) Nrr fruit at day 5, 9 and 19 of ripening.

Extracts (0.1-0.2 ml) were loaded onto gels, developed cathodally in a β-alanine acetic acid pH 4.5 system, and stained for PG activity as described in Chapter 2.

The figures to the left of the gels indicate the relative mobility of the enzyme bands to methyl green.

The slower moving band is PG1, and the faster moving bands, PG2A and PG2B. Direction of travel was toward the cathode.
Plate 4.1. PG isoenzyme content of extracts from ripening fruit of four genotypes. (Fruit ripened off-the-plant.)

Extracts from pericarp tissue from ripening fruit of genotypes (a) Ailsa Craig (wild type); (b) Ailsa Craig (RnRn); (c) Ailsa Craig NnRr; (d) Ailsa Craig (NnNn). PG isoenzymes were separated by polyacrylamide gel electrophoresis using the β-alanine-acetic acid pH 4.5, cationic system. Gels were incubated in 2% PGA after electrophoresis and enzyme activity (shown by clear areas) detected using 0.05% ruthenium red.

Numbers above gels indicate the degree of ripeness, in days from mature green, of fruit from which the extracts were prepared.
Fruit samples of all the genotypes reached maximum ethylene production two days after harvest (Fig. 16). This complete production was reached by the time the fruit were three days old. Following harvest, many of the genotypes examined (e.g., Hindin and Hogg) had the highest ethylene production in the first 12 hours. The amount of ethylene evolved by the sample was calculated by area and compared to the amount that of Other Craig Fruit on day 1. They were the same in extent, but by only 30% in area. The area between the increase in ethylene production and the control reaction was calculated to determine the first time point where the curves were statistically different. Percentages of the area between the two curves were calculated to represent the amount of ethylene produced by each genotype over the same period of time.
Fruit samples of all the genotypes showed increased ethylene production two days after harvest (Fig. 4,6). Peak ethylene production was reached by day nine in fruit from Ailsa Craig, Rirrin, Nrnr Rirrin and Nrnr, but was delayed until day 14 in NrNr fruit. The amount of ethylene evolved at the peak was reduced by more than 50% that of Ailsa Craig fruit in Rirrin, Nrnr and the double mutant, but by only 20% in NrNr. The time between the increase in ethylene production and the onset of softening and polygalacturonase accumulation varied in fruit of the five genotypes. Softening and polygalacturonase activity were observed to occur within a day of ethylene production in normal fruit but the mutant hybrids took two days to respond to ethylene production by softening. There was no correlation between the amount of ethylene produced by fruit and their subsequent rate of softening, colouring or polygalacturonase production.

Cell wall content of pericarp tissue samples from fruit sampled at harvest and on days 16 or 19 was analysed for each genotype (Table 4.3). The firmness of fruit at the mature green stage was not determined by cell wall content, but a good correlation between fruit firmness and percent wall content of pericarp tissue was found in 'ripe' fruit, $r = 0.878$. There was also some relationship between the amount of softening during ripening and amount of cell wall material lost over the same period. However, fruit of Nrnr produced atypical results in that cell wall loss was less than might be expected from loss of firmness results. The correlation between percent wall loss and percent loss of firmness for all the five genotypes was 0.735; when the data for Nrnr were omitted from the correlation, $r = 0.974$. A good correlation was also obtained
Fig. 4.6. Ethylene production by normal, mutant and heterozygous mutant fruit of cultivar Ailsa Craig during ripening off-the-plant at 20°C in the dark.

Bars indicate standard errors.

(•) Ailsa Craig; (o) Rinrin; (■) NnR; (□) NnRinrin; (△) NnR.
Days from Nature Green
Table 4.3. Cell wall content and firmness of mature green and 'ripe' tomatoes from normal and mutant heterozygous fruit of cultivar Ailsa Craig

Firmness of fruit samples ripened off-the-plant (Table 4.2) were measured, and percent loss of firmness calculated. The cell wall yield of these samples was measured as detailed in Chapter 3.2.3. Values average of two determinations, range in parenthesis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Firmness</th>
<th>Index of:</th>
<th>% Loss of Firmness</th>
<th>Cell Wall Yield g 100 g⁻¹ f.wt.</th>
<th>% Loss of Cell Wall Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mature Green</td>
<td>Orange-Red</td>
<td>Nature Green</td>
<td>Orange-Red</td>
<td></td>
</tr>
<tr>
<td>Ailsa Craig</td>
<td>8.40</td>
<td>2.55</td>
<td>69.6</td>
<td>1.75 (0.01)</td>
<td>0.90 (0.04)</td>
</tr>
<tr>
<td>Rinrin</td>
<td>8.26</td>
<td>3.30</td>
<td>60.0</td>
<td>2.24 (0.57)</td>
<td>1.26 (0.01)</td>
</tr>
<tr>
<td>NnNn</td>
<td>8.40</td>
<td>3.08</td>
<td>63.3</td>
<td>1.91 (0.22)</td>
<td>1.24 (0.01)</td>
</tr>
<tr>
<td>NnNr</td>
<td>7.46</td>
<td>3.62</td>
<td>51.5</td>
<td>2.04 (0.08)</td>
<td>1.32 (0.10)</td>
</tr>
<tr>
<td>NnNnRinrin</td>
<td>8.00</td>
<td>3.42</td>
<td>57.3</td>
<td>1.93 (0.14)</td>
<td>1.18 (0.02)</td>
</tr>
</tbody>
</table>

Correlation coefficient between

- Firmness at MG and cell wall yield at MG: $r = -0.28$
- Firmness at 'ripe' and cell wall yield at 'ripe': $r = 0.878$
- Loss of firmness and loss cell wall yield: $r = 0.269$
- % Loss of firmness and % loss cell wall yield: $r = 0.735$
- % Loss cell wall and polygalacturonase activity of ripe fruit: $r = 0.804$
between percent loss of wall material and polygalacturonase activity in 'ripe' fruit at day 16 or 19.

4.4. DISCUSSION

Mutant non-ripening genes affected the rate of Ailsa Craig fruit softening either as homozygotes or heterozygotes, but the relative amount of softening of fruit of the five genotypes during ripening depended on the conditions under which ripening took place.

The Nrnr fruit ripened on-the-plant softened at a faster rate than normal fruit, but less fast when ripened under controlled conditions off-the-plant. Hobson (1967) also found that Nrnr in cv. Potentate fruit ripened off-the-plant had a firmness intermediate between that of normal and NrNr fruit. The mutant genotypes took a few days longer than Ailsa Craig fruit to mature (Table 4.1), ripening a little later when the ambient temperatures were exceptionally high. This could account for the accelerated rates of ripening observed in the mutant fruit on-the-plant, compared to those found off-the-plant, since Shafshak and Winsor (1964) observed that elevated temperatures during fruit ripening accelerated the rate of fruit softening.

The effect of the rin gene on fruit softening was also affected by conditions of ripening. Rinrin fruit ripened on-the-plant had softening rates similar to those of Ailsa Craig. A result also obtained by Hobson (1980), but in contrast, Buescher et al. (1976) observed a reduction in softening due to the rin gene in a hybrid fruit on-the-plant.
When ripened off-the-plant in these experiments, Rinnin fruit were firmer than Ailsa Craig fruit (Fig. 4.4 a), an observation also made by Tigchelaar et al. (1978b). However, Brady et al. (1983) found that Rinnin fruit crossed with Rutgers had an initial rate of softening similar to the fruit of the normal parent, but later in ripening softened less rapidly. Again conditions under which the fruit ripened could explain these different results. In addition, the cultivar into which the mutant genes were incorporated must also account for some of the observed differences in the effects of the heterozygous mutants on fruit softening.

In general the polygalacturonase activity of ripe fruit of the five genotypes reflected their rates of softening (Figs. 4.1, 4.2 and 4.4). Nrnr fruit ripened on-the-plant produced high levels of PG1, which reflected its rapid loss of firmness. The polygalacturonase levels and rate of softening of Nrnr fruit found by Hobson (1967) were much lower than those reported here. This is probably due to the fact that the Nr gene was incorporated into a slow-ripening cultivar, Potentate, in his study. His results were more in accordance with those produced from Nrnr fruit ripened off-the-plant (Fig. 4.4).

The similar rates of softening of normal and Rinnin fruit on-the-plant was also reflected in their similar accumulation of polygalacturonase; whilst the differences in softening of fruit of these two genotypes off-the-plant were also mirrored by differences in polygalacturonase activity, Rinnin having less activity than the fast ripening Ailsa Craig. The results of softening and polygalacturonase content for Rinnin ripened off-the-plant are similar to those obtained by Brady et al. (1983).
The polygalacturonase production in 'ripe' NnNn and Rrinrin fruit did not correlate so well with rates of softening observed. NnNn fruit contained very little enzyme yet softened appreciably during ripening, although at a much slower rate than the other genotypes. Rrinrin fruit on the plant softened at a rate slightly higher than normal fruit although it contained only 30% of the polygalacturonase activity of normal fruit at day 12. Off-the-plant, its rate of ripening was more consistent with its level of polygalacturonase activity (Fig. 4.4). Hobson (1980) noted that although Rrinrin and Ailsa Craig fruit softened at comparable rates the former had polygalacturonase levels 40% lower than the latter; whilst Brady et al. (1983) found that the lower enzyme activity in NnNn fruit compared to Nornor, Rrinrin and Rutgers fruit was not reflected in their rates of softening. Thus from the results of this chapter and other researchers it might appear that polygalacturonase activity does not correlate well with fruit softening in these mutant genotypes.

However, the differences in rates of fruit softening of the five genotypes were established during the first six days of ripening in fruit ripened both on- and off-the-plant. Brady et al. (1983) and Buescher et al. (1976) observed similar patterns of softening in Rrinrin and Nornor fruit. In fruit ripened on-the-plant much of the loss of firmness occurred as the fruit turned from mature green to green-orange stage. However, after day five of ripening off-the-plant, and about day ten on-the-plant, the rates of fruit softening were similar for all the genotypes (Figs. 4.1 and 4.4). Buescher et al. (1976) also found this to occur in Rrinrin and Nornor fruit, as did Brady et al. (1983) for Rutgers, NnNn and Nornor fruit.
The marked rise in polygalacturonase production occurring later in ripening coincided with a decrease in rate of fruit softening, whilst on close examination the rates of softening during early ripening were closely reflected by accumulation of polygalacturonase, in the form of PG1. Brady et al. (1983) also observed that the differences between the rates of softening of Nornor, Rinrin, Nornor Rinrin and normal fruit were established during the first six days of ripening, when fruit contained only PG1, and suggested that the accumulation of this isoenzyme may have a particular and specific role in fruit ripening.

Although the rates of PG1 accumulation and softening mirrored each other during early ripening, equivalent loss of firmness e.g. 50% in fruit of different genotypes was not associated with equal levels of PG1 activity. In fruit ripened on-the-plant PG1 levels at 50% firmness were: Ailsa Craig 7.7; Nnrn Rinrin 9.1; Rinrin 26.0; Nnrn 20.8; and a similar variation was found in fruit ripened off-the-plant. Thus, although within a genotype PG1 accumulation appeared to reflect softening rates, when comparing softening and polygalacturonase activity between genotypes other factors seemed to impinge on the relationship.

It is interesting to observe that fruit of Nnrn and Rinrin ripened off-the-plant contained more PG2 at the advanced stages of ripeness than fruit at a similar stage ripened on-the-plant (Tables 4.1 and 4.2). Although Brady et al. (1983) suggested that PG2 was accumulated only when polygalacturonase production reached a certain threshold; these results suggest that other factors may influence the appearance of PG2, since Nnrn fruit ripened off-the-plant accumulated PG2 when they contained lower levels of the enzyme than similar fruit on-the-plant.
In all genotypes loss of firmness was accompanied by loss of cell wall material during ripening. The correlation between percent loss of firmness and percent loss of cell wall material was better in this experiment than obtained in the comparison of cultivars (Chapter 3). This might reflect the importance of fruit structure in ameliorating the softening effects of wall degradation during ripening. The mutant genotypes were all incorporated into a nearly isogenic background in Ailsa Craig fruit, thus all had similar fruit structures and firmness before ripening, unlike individual cultivars. Thus the differences in softness between the mutant genotypes and normal fruit were due to differences in rates of wall degradation, softening and polygalacturonase activity. In cultivars the effect wall degradation had on tissue and whole fruit softening may be affected in part by fruit and tissue structure, hence the lower correlation between softening and wall degradation observed.

In both cultivars and mutant genotypes there was a better correlation between percent loss of cell wall material and polygalacturonase than between percent loss of cell wall material and firmness. Since polygalacturonase production late in ripening had little effect on softening rates it is suggested that beyond a certain point wall degradation by polygalacturonase (generally in the form of PG2) does not affect fruit firmness. Instead this wall degradation may contribute to textural changes in the pericarp cell walls. It is noticeable that whilst fruit of Ailsa Craig, Rinrin and Nrnr are quite edible when ripe, those of NrNr and the double mutant have an unpleasant, leathery texture.

The development of colour in ripening tomato fruit is due to the synthesis of carotenoids, which are responsible for the yellow
and orange pigments evident during the early stages of ripening, and of lycopene which is the red pigment predominant at the ripe stage of normal fruit.

The synthesis of these pigments, especially of lycopene, is severely retarded by the mutant \( \text{Nr} \) and \( \text{rin} \) genes in their homozygous form (Simpson et al., 1976; Buescher and Doherty, 1978; Tigchelaar et al., 1978a). In a heterozygous state the \( \text{Nr} \) gene appears to exert a greater influence on fruit colour than the \( \text{rin} \) gene (Tables 4.1 and 4.2). \( \text{Rinrin} \) fruit had a similar colour to normal cultivar Ailsa Craig; whilst fruit of genotypes \( \text{NrNr} \), \( \text{Nrnr} \) and \( \text{Nrnr Rinrin} \) all remained an orange colour even when quite soft. Buescher et al. (1976) found that both \( \text{rin} \) and \( \text{nor} \) hybrids with normal fruit produced fruit which developed a normal red colour. Although Herner and Sink (1977) observed that colour development was delayed in \( \text{rin} \) hybrid fruit, no such delay was observed here. Crookes and Grierson (1983) studied ripening \( \text{NrNr} \) tissue by electron microscopy and found that fruit at an orange-red colour had modified plastids which contained carotenoid and xanthophyll pigments, but lacked lycopene crystalloids characteristic of normal fruit.

Ripening fruit off-the-plant did not alter the rates of colour development of normal and \( \text{Rinrin} \) fruit but retarded the rate of pigment synthesis in \( \text{NrNr} \), \( \text{Nrnr} \) and \( \text{Nrnr Rinrin} \) fruit quite considerably. This suggests that metabolites from the plant may contribute to colour development in these genotypes. Kopeleovitch et al. (1980) found that a slow-ripening race of tomatoes called Alcobaca, ripen on- but not off-the-plant. It is possible that the influence of the plant in controlling ripening of this genotype also affects
the ripening of other mutant genotypes, but to a lesser extent. In addition, fruit off-the-plant ripened in the dark. This may have affected its rate of pigment synthesis since light has been found to stimulate carotene and lycopene production in some mutant genotypes (Buescher and Doherty, 1978). However, it is therefore surprising that normal and Rinrin fruit colour was unaffected by absence of light during ripening.

The rates of softening of fruit on- and off-the-plant follow the same pattern. Normal and Rinrin lines showed enhanced rates of softening off-the-plant, in contrast, genotypes containing the Nr gene exhibited slower rates of softening off- as compared to ripening on-the-plant. These differences were reflected by polygalacturonase production; normal and Rinrin fruit ripened off-the-plant contained much higher levels of the enzyme than fruit at similar colour stages on-the-plant. The reverse was true for other genotypes containing the Nr gene. The very high levels of polygalacturonase activity found in normal and Rinrin fruit ripened off-the-plant were unusual but may well have been due to conditions of growth of the fruit as well as of ripening. Hobson (1967) observed that fruit grown during the spring months had higher polygalacturonase activity than those grown during the late summer. Thus environmental factors seem to affect the total polygalacturonase production.

The differences in colour development and softening were not related to peak ethylene production (Figs. 4.4, 4.6 and Table 4.2). The high rates of ethylene production in NrNr fruit and low rates in Rinrin fruit did not reflect their rates of colour development or softening. These ethylene results are similar to those of Herner
and Sink (1973); Tighelaar et al. (1976) and Brady et al. (1983) for Rinrin and NrNr fruit. They also measured respiration in these genotypes and found that it was not reduced in Rinrin but was in NrNr fruit. Thus Ali (1983) suggested that colour development and softening were more closely related to respiration than ethylene production. However, Buescher and Doherty (1978) found that fruit containing rin and nor genes responded to ethylene treatment by increasing carotenoid synthesis. Thus while ethylene might be required to stimulate colour development in tomato fruit, the actual amount of ethylene produced does not seem to affect the intensity of colour or polygalacturonase development.

As found in the cultivars (Chapter 3), there was a good correlation between fruit colour development (a/b ratio) and polygalacturonase activity, but a lower correlation between colour and firmness. Those fruit developing a red colour when soft (e.g. normal and Rinrin fruit) contained FG2, whilst orange or yellow coloured fruit contained only FG1, despite being quite soft. Thus development of red colour due to lycopene might be associated with FG2 activity, since the two developed at about the same time. However, despite containing some FG2, Nrnr fruit ripened on-the-plant did not turn red (Table 4.2, Fig. 4.4).

In conclusion the results of this chapter support those of Chapter 3. The differences in the rate of fruit softening among five genotypes of cultivar Ailsa Craig were established during the first few days of ripening, when polygalacturonase accumulation was in the form of FG1 only. The rate of FG1 accumulation appeared to
reflect quite closely the rates of softening observed. The good correlation between loss of firmness and loss of cell wall content, and between loss of cell wall and polygalacturonase activity supports the hypothesis that fruit softening is regulated by polygalacturonase activity.

Although PGI may play an important role in regulating whole fruit softening, the high levels of PG2 isoenzyme produced by 'ripe' fruit may cause more extensive degradation of the primary cell wall and thus affect the texture of pericarp tissue.

The role of polygalacturonase in regulating other parameters of ripening is less clear. Whilst colour development and polygalacturonase activity appear to be closely related, the appearance of lycopene was not always associated with the appearance of PG2 activity during the later stages of fruit development.

Whilst ethylene production occurred prior to the onset of other ripening events, and thus might initiate them, its rate of production was not related to rates of fruit softening, polygalacturonase activity or colour development.
5.1. Introduction

The genus *Lycopersicon* contains several closely allied and intercrossable species, all of which produce a berry fruit with, except for some forms of cultivated tomato (*L. esculentum*), a bilocular structure (Luckwill, 1943). Fruit of the different species exhibit considerable variation in morphology and in ripening characteristics (Luckwill, 1943; Rick, 1979; Grumet et al., 1981). This variation in ripening physiology is useful in studies of the physiology and biochemistry of ripening, and a survey of the polygalacturonase content and isoenzyme distribution in tomato fruit of closely related species might provide an insight into its role and interaction with other ripening events.

Within the genus *Lycopersicon* the species have been subdivided into two groups: *Eulycopersicon*, the species which produce glabrous fruit which turn red or orange when ripe, and *Eriopersicon*, the fruit of which are pubescent and green or purple-green when ripe (Luckwill, 1943; Davies and Hobson, 1981). Grumet et al. (1981) studied the ripening physiology of several wild tomato species and subsequently divided the genus into three groups based on physiological differences.

The first group comprised the subgenus *Eulycopersicon*, i.e. fruit that change colour, soften when ripe accompanied by an increase in ethylene production. Included in this group were the
cultivated tomato, *L. esculentum*, the wild cherry tomato *L. esculentum var. cerasiforme*, which is thought to be the ancestor of the cultivated tomato (Luckwill, 1943) and *L. pinninellifolium*, which is also closely related to cultivated tomato. A member of the Eriopersicon, *L. cheesmanii*, which is a perennial plant found on the Galapagos islands, was also included in this group since it produces some pigments when ripe, and also produces ethylene during ripening and softening (Grumet et al., 1981).

The second group was composed of two unusual species, *L. chilense* and *L. peruvianum* which do not colour during ripening and will only ripen and soften after fruit abscission. These two species do not hybridise easily with the *L. esculentum* group (Rick, 1979).

The third group consisted of the rest of the species in Eriopersicon, the fruit of which remain green but soften on the plant e.g. *L. chmielewskii* and *L. parviflorum*. Also included in the species *Solanum pennellii* which is very closely allied to Lycopersicon species, not only because of its chromosomal similarity but also because of its morphology (Rick, 1979). *L. chmielewskii* and *L. parviflorum* produce ethylene and soften during ripening. In contrast, *S. pennellii* produces ethylene erratically during fruit growth and production is not related to fruit ripening (Grumet et al., 1981).

The wild tomato species studied in this work were:-

*L. esculentum var. cerasiforme* and *L. pinninellifolium*, since they are most closely related to the cultivated tomato; *L. cheesmanii* because of its high ethylene production yet low pigment synthesis;

*L. chmielewskii* and *S. pennellii* because they are genetically
compatible with *L. esculentum* yet have unusual ripening characteristics. *L. peruvianum* and *L. chilense* were not studied because they are less closely related to *L. esculentum* than these other species.

5.2. MATERIALS AND METHODS

Six plants of the following species were grown under glasshouse conditions, as described in Chapter 3, during the summer months: *L. esculentum* cv. Ailsa Craig; *L. esculentum* var cerasiforme; *L. pimpinellifolium*; *L. cheesmanii*; *L. chmielewskii* and *S. pennellii*. *L. cheesmanii* plants were shaded to expose them to only 12 hours daylight to promote flowering. The flowers of *L. chmielewskii* and *S. pennellii* required cross-pollination by hand. Flowers of all species were tagged at anthesis, and the fruit harvested as they showed signs of ripening, judged by subjective assessment of colour and/or softness.

Ethylene production of individual red *L. esculentum* cv. Ailsa Craig fruit and of ten fruit samples of green *L. chmielewskii* and *S. pennellii* fruit was measured as described in Chapter 3. The colour and firmness of the wild species fruit was not measured objectively due to the small size of the fruit (1 to 2 cm diameter) and the fragility of some species of fruit when ripe.

Polygalacturonase was extracted from previously frozen whole fruit, or in the case of *L. esculentum* var cerasiforme, from only pericarp tissue as well, using the method of Hobson (1964). Crude enzyme extracts were partially purified by ammonium sulphate precipitation in the 0-80% saturation range prior to enzyme assay, and analysed by natured and SDS gel electrophoresis, and by immunoassay studies, as described in Chapter 2.
5.3. RESULTS

The species *L. esculentum var cerasiforme* and *L. pimpinellifolium* were similar to the cultivated variety, Ailsa Craig, in the time they took to mature and ripen, and the degree of red colour developed by the fruit (Table 5.1). *L. pimpinellifolium* fruit took on average six days to ripen from breaker stage compared to eight days for *L. esculentum var cerasiforme* and 12 for *L. esculentum* cv. Ailsa Craig. The fruit of these species became quite soft during ripening and this was accompanied by polygalacturonase activity of a magnitude similar to that found in ripe cultivated tomatoes. Heat treatment of polygalacturonase extracts of these wild species reduced enzyme activity by 80 to 90%, suggesting a low level of PG1-like isoenzyme. However, analysis of these extracts by natured PAGE showed that in *L. esculentum var cerasiforme* PG1 appeared to account for at least half the polygalacturonase activity of ripe fruit, whilst the isoenzymes of polygalacturonase in *L. pimpinellifolium* were not separated (Fig. 5.1).

*L. cheesmanii* fruit took slightly longer to reach maturity and ripeness from anthesis than the *L. esculentum* species (Table 5.1). They did not turn red when ripe but remained a pale orange colour, suggesting these fruit lack the capacity to synthesise significant quantities of lycopene. Despite feeling soft to the touch when ripe, *L. cheesmanii* fruit produced very little polygalacturonase activity. The high standard error quoted is due to the fact that negligible polygalacturonase activity was recovered from two of the replicate extractions. The small amount of polygalacturonase activity found in the remaining two extracts was resistant to heating to 65°C,
Table 5.1. Ripening characteristics and polygalacturonase activity of normal and wild species of tomato

<table>
<thead>
<tr>
<th>Age of Ripe Fruit in Days</th>
<th>Colour of Fruit</th>
<th>Total PG (µkat g⁻¹ f.wt.)</th>
<th>FG¹ % of Total PG</th>
<th>Protein (mg g⁻¹)(i)</th>
<th>Ethylene Production in Ripe Fruit (nl 100 g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. esculentum cv. Ailsa Craig</td>
<td>40</td>
<td>Red</td>
<td>90.0</td>
<td>10</td>
<td>0.47(i)</td>
</tr>
<tr>
<td>L. esculentum</td>
<td>42</td>
<td>Red</td>
<td>0.78 ± 12.2</td>
<td>10-15</td>
<td>0.51 ± 0.05(i)</td>
</tr>
<tr>
<td>var cerasiforme</td>
<td></td>
<td></td>
<td>47.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>whole fruit pericarp tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. pimpinellifolium</td>
<td>40</td>
<td>Red</td>
<td>0.85 ± 16.1</td>
<td>20</td>
<td>1.74 ± 0.5 (i)</td>
</tr>
<tr>
<td>L. cheesmaniai</td>
<td>54</td>
<td>Orange</td>
<td>0.19 ± 0.02</td>
<td>100</td>
<td>0.41 ± 0.06(ii)</td>
</tr>
<tr>
<td>L. chmielewskii</td>
<td>50-60</td>
<td>Green</td>
<td>0.27 ± 5.2</td>
<td>60</td>
<td>0.74 ± 0.03(i)</td>
</tr>
<tr>
<td>S. pennellii</td>
<td>70+</td>
<td>Green</td>
<td>2.2 ± 1.0</td>
<td>100</td>
<td>0.86 ± 0.01(i)</td>
</tr>
</tbody>
</table>

a = mean of five determinations  
b = mean of four determinations  
c = data from Grumet et al. (1981)  
* = determined by heat test  
(i) = Protein  
(ii) = Specific Activity
Fig. 5.1. Polyacrylamide gel electrophoresis of PG in extracts from ripe fruit of:

(a) L. esculentum cv. Ailsa Craig;
(b) L. esculentum var. cerasiforme;
(c) L. pimpinellifolium;
(d) L. cheesmanii;
(e) L. chmielewskii;
(f) S. pennellii.

Extracts were loaded onto gels, developed cathodally in a β-alanine-acetic acid pH 4.5 system, and stained for PG activity as described in Chapter 2.

Figures to the left of gels indicate the relative mobility of the bands to methyl green. The slower moving band in (a), (b), (e) and (f) is PG1. The faster moving bands in (a), (b) and (e) are PG2A and B. The blocked bands represent PG activity. The hatched band was an unknown substance which have an orange-coloured band after ruthenium red staining.
indicative of a PG1-type of isoenzyme. Because enzyme activity was so low in extracts of these fruit no bands of isoenzyme activity were detected by electrophoresis (Fig. 5.1, Plate 5.1). However, a small band of enzyme activity which caused the ruthenium red dye to turn orange was noted on these gels, and also on those of *L. chmielewskii* and *S. pennellii*.

Fruit of *L. chmielewskii* and *S. pennellii* remained green throughout ripening, so ripe fruit were detected by their softness. For *L. chmielewskii* fruit this occurred between 50 to 60 days from anthesis, whilst *S. pennellii* fruit took another ten days to ripen. *L. chmielewskii* fruit produced ethylene prior to and during softening (Table 5.1). Once fruit started to soften they did so very quickly and easily split after a few days, especially under humid conditions. The polygalacturonase content of these fruit was about half that of cultivated tomatoes (Table 5.1), and was almost equally distributed between PG1 and PG2 isoenzymes (Fig. 5.1). *L. chmielewskii* fruit extracts contained both PG2A and B. *S. pennellii* fruit produced only trace amounts of ethylene when soft, and also produced very little polygalacturonase activity despite softening considerably. Polygalacturonase was present as PG1 (Fig. 5.1). A comparison of the relative mobilities of the polygalacturonase isoenzymes from wild and cultivated species under natured and SDS electrophoresis conditions suggests that they are similar in the different species.

Polygalacturonase extracts from four of the wild species and cv. Ailsa Craig were tested for their reaction against antibodies raised to PG2 (Plate 5.1 a). *L. pimplinellifolium*, *L. chmielewskii*, *S. pennellii* and cv. Ailsa Craig extracts all contained protein which
Plate 5.1. Examination of PG in extracts from ripe fruit of wild species of tomato by:

(a) Immunodiffusion. Reaction of extracts with antiserum to PG2.
    Serum well 1 contained immune serum. Sample wells contained extracts from:

(a) \textit{L. esculentum} cv. Ailsa Craig. Red fruit.
(b) \textit{L. cheesmanii}.
(c) \textit{L. pimpinellifolium}.
(d) \textit{L. chmielewskii}.
(e) \textit{S. pennellii}.
(f) Purified PG1 isoenzyme.

(b) SDS-polyacrylamide gel electrophoresis (10-15% gradient).
    Extracts (50-75 \mu l) denatured by SDS were separated as described in Chapter 2. Samples were:

(a) \textit{S. pennellii}.
(b) \textit{L. chmielewskii}.
(c) \textit{L. cheesmanii}.
(d) \textit{L. pimpinellifolium}.
(e) \textit{L. esculentum} var cerasiforme.
(f) PG1 purified by gel filtration and affinity chromatography.
(g) Ovalbumin. MW 43 000.
reacted with antibodies to L. esculentum fiber. Similar results were observed with L. esculentum extracts, but antibodies to L. esculentum extracts produced precipitin bands of antigenic properties to PO1. PO1 and PO2, produced by strains of L. esculentum of which one had identical to PO1 and the other the PO1 precipitin band due to PO1 production had by the .

Flattening of the precipitin lines were not observed. The presence of protein in the ume fruit juice and extracts (Plate 5a, b).

The manner of protein bands present in extracts from this fruit was seen than those produced by the other species studied.

In addition, during ripening, larger amounts of protein were still separating as indicated by the immunoblot analysis of the extracts. This suggests that the levels of protein increase as other studies have described in pectin.
reacted with antibodies to PG2. No precipitin lines were observed with *L. cheesmanii* extracts. *S. pennelli* and *L. chmielewskii* extracts produced precipitin bands with identical antigenetic properties to PG1. Ailsa Craig extracts, which were known to contain PG1 and PG2, produced a double precipitin line, one of which was identical to PG1 and the other formed a spur with the PG1 precipitin band due to PG2 protein. The precipitin band formed by the *L. pimpinellifolium* extract was broad, and separate precipitin lines were not observed. The lack of polygalacturonase protein in *L. cheesmanii* fruit was confirmed by SDS electrophoresis (Plate 5.1b). The number of protein bands produced by extracts from this fruit was lower than those produced by the other species studied.

5.4. DISCUSSION

Fruit of all the five wild species studied softened appreciably during ripening, but the amount of polygalacturonase produced by them varied considerably. The fruit of *L. esculentum var cerasiforme* and *L. pimpinellifolium* had rates of softening and polygalacturonase activity similar to those of the cultivated tomato, *L. esculentum*, to which they are closely related (Rick, 1979).

In contrast, soft fruit of *L. cheesmanii* produced negligible amounts of polygalacturonase activity, or even inactive protein separating at the appropriate point, as detected by electrophoresis or immunodiffusion assays (Fig. 5.1, Plate 5.1). This species produced fruit with very thin walls, and it is possible that its softening is less dependent on pectin degradation in the cell walls of pericarp tissue than other species. Changes in the consistency of the locale contents, which do not depend on changes in pectins
(Sobotka and Watada, 1970; Huber, 1984), may account for softening in this fruit.

Among the green- fruited tomato species a similar disparity between fruit softness and polygalacturonase content was observed. *L. chmielewskii* fruit softened rapidly, and its soft fruit had polygalacturonase levels only slightly less than those found in ripe *L. esculentum* fruit (Table 5.1). *S. pennellii* fruit softened as quickly as *L. chmielewskii* fruit yet produced very little polygalacturonase.

Thus high polygalacturonase activity was not always associated with fruit softening in these wild species, although it was high in those species which had relatively thick pericarp walls and softened rapidly during ripening. *L. cheesmanii* and *S. pennellii* fruit appear to have mechanisms of softening which do not involve extensive pectin degradation. Other wall degrading enzymes such as cellulases (Dickinson and McCollum, 1964; Sobotka and Watada, 1971; Sobotka and Stelzig, 1974), or glycosidases (Pharr et al., 1976; Pressey, 1983) may cause some softening in these fruit. Mechanisms which alter the viscosity of the locule contents might also be an important cause of softening in the thin-walled fruit of these species.

*L. esculentum* var. *cerasiforme*, and *L. chmielewskii*, species of tomato which accumulated high levels of polygalacturonase also contained three forms of this enzyme. Analysis of polygalacturonase extracts from these species by immunodiffusion and SDS electrophoresis showed that these isoenzymes were very similar to those found in cultivated tomatoes. However, both species contained more PG1 in ripe fruit than *L. esculentum* cv. Ailsa Craig.
Heat test analysis of *L. pimpinellifolium* fruit extracts suggested that two forms of polygalacturonase with different heat sensitivities were present, but they were not resolved by electrophoresis or immunodiffusion analysis. It is possible that these fruit contain substances that interfere with the separation of the isoenzymes, or more likely that the activity of one of the isoenzymes, probably PG2, was so great in comparison to the other that it interfered with the resolution of the bands.

Wild species that produced very little polygalacturonase produced only PG1, as was also observed to occur in non-ripening mutant fruit (Chapter 4; Tucker et al., 1980).

The existence of a substance which gave an orange-coloured band with ruthenium red stained PAGE gels could have implications for fruit softening. Pectin lyase enzymes have been reported to produce a similar reaction (Cruickshank and Wade, 1980). Although Besford and Hobson (1972) observed no activity of this enzyme in cultivated tomatoes, Ali (1983) found a reaction to ruthenium red in extracts of ripening cultivated tomatoes similar to the one observed in this work. Pectin lyase has only been observed to occur in fungi and one higher plant (Filnik and Voragen, 1970), but further investigations into cell wall degrading enzymes of fruit may reveal its wider existence in higher plants. If so, it could play a very important role in regulating softening during the early stages of tomato fruit ripening.

The relation of polygalacturonase accumulation to other ripening events is not clear. In the cultivated tomato ripening is generally thought to be initiated by ethylene (McGlasson, 1970;
Lieberman, 1979). The increase in its production is closely followed by polygalacturonase synthesis (Grierson and Tucker, 1983; Jeffery et al., 1984). Whilst fruit of *L. esculentum* var. cerasiforme and *L. pinnatifolium* had similar ripening patterns to cultivated *L. esculentum* fruit, the physiology of other wild species suggest that this pattern is not always followed.

*L. cheesmanii* fruit did not produce lycopene pigments or polygalacturonase (Table 5.1), yet Grumet et al. (1981) found that they produced two to three times more ethylene than *L. pinnatifolium* or *L. esculentum* species. In contrast, *S. pennelli* fruit which produced only trace amounts of ethylene, did accumulate polygalacturonase. Grumet et al. (1981) also observed that ethylene production by fruit of this species was low, erratic and not associated with softening.

Thus the idea that ethylene regulates the synthesis of enzymes related to fruit ripening and softening (Grierson and Tucker, 1983; Grierson and Slater, 1984; Tucker et al., 1984) may not hold true for this species. Alternatively, the sensitivity of this fruit tissue to ethylene may increase prior to ripening, rendering it susceptible to small fluctuations in fruit ethylene production. In which case ethylene may still regulate softening and ripening in this species. Cultivated tomato fruit become progressively more sensitive to ethylene as they mature (McGlasson and Adato, 1977), and it has been suggested that tissue sensitivity to ethylene may be an important regulatory factor in fruit ripening rather than the absolute levels of ethylene produced by pre-climacteric fruit (Trewavas, 1981).
As observed in Chapter 4, the amount and rate of ethylene production by fruit does not affect their colour development or polygalacturonase activity. *L. cheesmanii* fruit produced very high amounts of ethylene yet coloured only slightly and its polygalacturonase activity was negligible. Thus although ethylene may initiate synthesis of polygalacturonase and pigments, it does not appear to be involved in the regulation of their subsequent rate of production.

In *L. esculentum* species, colour development appears to be associated with polygalacturonase activity (Chapters 3 and 4; Crookes and Grierson, 1983), thus is possibly regulated by the action of this enzyme in releasing cell wall enzymes or regulatory molecules. However, *L. chmielewskii* fruit produced polygalacturonase but did not colour, whilst in contrast, *L. cheesmanii* fruit produced almost no polygalacturonase yet turned an orange-colour. The lack of colouring in *L. chmielewskii* fruit could be due to genetic inhibition of pigment synthesis. What the colouring of *L. cheesmanii* fruit suggests that either polygalacturonase is not associated with tomato colour development, or if it is, then very low amounts of the enzyme can release sufficient metabolites from the wall to regulate pigment synthesis.

In conclusion, the normal ripening sequence of ethylene production followed by polygalacturonase synthesis and then pigment development, observed in cultivated tomatoes is not always adhered to in the wild species. Softening is not always associated with high polygalacturonase activity. However, fruit which did not produce much polygalacturonase had less pericarp tissue in relation to locale tissue than cultivated tomatoes. Thus in species other than
L. esculentum, the softening of pericarp tissue by polygalacturonase, which usually determines the rate of firmness loss, does not appear to be of such importance, in regulating the extent to which the fruit soften.

While ethylene may initiate ripening events in cultivated tomatoes, the physiology of other wild species, especially that of S. pennelli, brings into question its role in stimulating pigment and polygalacturonase synthesis. Either ethylene does not initiate ripening in some tomato species, or factors which increase fruit sensitivity to low levels of ethylene are the primary regulators of ripening. Further investigations are necessary to establish which of these possibilities is correct.
CHAPTER SIX THE EFFECT OF SALT AND ETHREL TREATMENTS ON THE RIPENING AND POLYGALACTURONASE ACTIVITY OF SLOW AND NON-RIPENING TOMATO GENOTYPES

6.1. INTRODUCTION

Polygalacturonase isoenzymes appear to play an important role in regulating the rate of tomato softening and extent of pericarp tissue cell wall degradation (Chapters 3 and 4). However, the importance of these isoenzymes in regulating other fruit ripening events, either directly or indirectly has to be ascertained.

Many of the ideas of the role of polygalacturonase in tomato ripening have been generated from studies using mutant non-ripening tomato genotypes, such as rin, nor and hr (see Chapter 4). These genes in homozygous form, cause the fruit to change colour and soften at an exceptionally slow rate and little or no polygalacturonase synthesis takes place (Tigchelaar et al., 1978a). However, seed development and viability occurs at the expected time, at the end of fruit growth.

Other genotypes have recently been discovered which have unusual ripening characteristics. Spanish Winter and Longkeeper ripen very slowly, whilst Alcobaca genotypes behave similarly to nor nor mutants and are virtually non-ripening (Kopeliovitch et al., 1980). Like other slow ripening mutants these genotypes also are characterised by low rates of softening and polygalacturonase production, and poor colour development (Kopeliovitch et al., 1980; Pressy and Avants, 1982b).
Certain treatments have been found to overcome the genetic inhibition of ripening in some of these mutants. Exposure of rinrin and Alcobaca fruit to ethylene, whilst still on-the-plant, stimulated ripening (Mizrahi et al., 1975; Kopeliovitch et al., 1980), but had no effect on nornor fruit (Mizrahi et al., 1975). In contrast, treating nornor and rinrin plants with 0.3% sodium chloride solutions during fruit development caused nornor but not rinrin fruit to ripen (Mizrahi et al., 1982). Ripening in these studies was judged by fruit colour development and softening. However, although pectolytic activity was measured, polygalacturonase activity was not specifically assayed.

The aim of this study was to examine the effects of treating four non- or slow-ripening tomato genotypes, viz. Alcobaca, Spanish Winter, Longkeeper and nornor in cv. Ailsa Craig, with sodium chloride and ethylene on their polygalacturonase production and ripening characteristics.

6.2. MATERIALS AND METHODS

Plants of homozygous nornor mutant, incorporated into near isogenic lines of Ailsa Craig, and the partially isogenic mutant genotypes Alcobaca, Spanish Winter and Longkeeper were grown under glasshouse conditions during the 1983 and 1984 seasons. Sodium chloride, or in one experiment, sodium sulphate, solutions of either 0.5% or 1.0% were applied to plants, at a rate of 500 ml a day, from ten to 15 days after anthesis until fruit were harvested. The effect of ethylene on fruit ripening was examined by applying the ethylene releasing chemical 'ethrel' [(2-chloroethyl)phosphonic acid] at a
concentration of 0.1% to the surface of mature green fruit at two-day intervals until harvest. Fruit samples were harvested at one or two stages during development. Fruit age, colour, firmness and in some experiments, ethylene production, were measured as outlined in Chapter 3. Pericarp tissue samples were prepared for assay of total polygalacturonase activity and isoenzyme content as described in Chapter 2.

6.2.1. In a preliminary experiment the time taken for fruit of the four genotypes to ripen without salt treatment was followed. Samples of ten fruit were harvested during ripening, as judged by colour changes, and their firmness and colour measured. At the same time, three plants of each genotype were treated with either water, 0.5% or 1.0% sodium chloride solutions during fruit development. After 80 to 85 days from anthesis, samples of ten fruit of each genotype were analysed for firmness, colour and polygalacturonase activity.

6.2.2. A second experiment investigated the relative effects of sodium chloride and sodium sulphate, applied at 0.5% concentration, on the ripening of Spanish Winter, Alcobaca and Longkeeper fruit. Some plants were treated with sodium chloride solutions and, when fruit were mature, further treated with ethrel solutions. Samples of at least five fruit per genotype were harvested between 55 and 94 days from anthesis, depending on the genotype, when the salt treated fruit were judged to be ripe on the basis of colour. The firmness, colour and in some samples, ethylene production and polygalacturonase levels in fruit were measured.
6.2.3. In 1984 a more detailed experiment was conducted to compare the effects of sodium chloride and ethylene treatments on the ripening of nornor, Alcobaca and Spanish Winter fruit. Eight plants of each genotype were treated with either water or 0.5% sodium chloride during fruit development. When fruit were mature, half the water and salt treated plants were also treated with ethylene, by painting fruit with 0.1% ethrel solutions. Twice during fruit ripening samples of ten fruit were harvested and analysed for firmness, colour, ethylene production, and in one set of samples, polygalacturonase activity.

6.3. RESULTS

6.3.1. Ripening characteristics of nornor, Alcobaca, Spanish Winter and Longkeeper fruit with and without salt treatment

Spanish Winter and Longkeeper fruit had just started to ripen, as judged by colour, 55 days after anthesis (Fig. 6.1). However, although fruit of these genotypes continued to develop some colour, loss of firmness was very gradual even up to 90 days after anthesis.

Fruit of nornor genotype plants were only a green-yellow colour 85 days after anthesis, and very little softening occurred up to this time.

Alcobaca fruit ripening was retarded to an even greater extent. Seventy-five days after anthesis, fruit were green, and after 100 days were still only green-yellow. After an initial marked drop in firmness, these fruit exhibited the extremely slow rate of softening observed in the other genotypes.
Fig. 6.1. (a) Firmness and (b) colour of nornor (○); Alcobaca (△); Spanish Winter (○) and Longkeeper (▲) fruit during ripening.
Polyphenoloxidase activity of different potato varieties was high in variety with high polyphenoloxidase activity (Chapter 3), especially in variety and disease tests (Tables 5.1, 6.2 and 6.3). Despite higher activity seen in potatoes, fruit of different variety and genotype has higher polyphenoloxidase activity than petals and disease fruits.

Saline chloride treatment had a significant effect on the fitness of normal plants as seen during potato tuber growth. Treating plants with saline water increased the rate of solar development in the potato tubers. Chlorophyll content 0.5% and chloride content 0.01% for a significant increases in chlorophyll content in potato tubers. Due to increased growth, potato tubers also increased chlorophyll content.

(a) Fitness Index

(b) Carbon C/N Ratio

Days from Anthesis
Polygalacturonase production of ripening fruit of all four genotypes was very low compared to levels found in normal ripe fruit (Chapter 3), especially in normor and Alcobaca fruit (Tables 6.1, 6.2 and 6.3). Despite having similar rates of softening, fruit of Spanish Winter and Longkeeper had higher polygalacturonase activity than normor and Alcobaca fruit.

Treatment of all four genotypes with 0.5% and 1.0% sodium chloride solutions noticeably enhanced fruit colour (Table 6.1, Plate 6.1). Eighty days after anthesis the control fruit from all genotypes were green to yellow-green in colour, with a/b ratios less than 0.2, while fruit treated with sodium chloride were orange (normor and Alcobaca) or red (Spanish Winter and Longkeeper) with a/b ratios of 0.2 or above.

Sodium chloride treatments had a significant effect on the firmness of normor, Alcobaca and Spanish Winter fruit but not on Longkeeper fruit. Treating plants with 1.0% sodium chloride did not increase the rate of colour development or softening over that achieved with 0.5% salt solutions, (Table 6.1), except for a slight increase in softness of normor fruit. Thus in subsequent experiments 0.5% salt solutions were employed.

Salt treatment marginally increased polygalacturonase activity in Spanish Winter and Alcobaca fruit, but activity in 0.5% and 1.0% sodium chloride-treated Longkeeper and normor fruit was not consistently higher than water-treated fruit.
Table 6.1. Response of slow and non-ripening tomato mutants to applications of 0.5% and 1.0% sodium chloride (NaCl), or water (control) during fruit development

<table>
<thead>
<tr>
<th>Genotype and Treatment</th>
<th>Days from Anthesis</th>
<th>Colour a/b</th>
<th>Firmness (mm compression)</th>
<th>PG Activity (nkat g⁻¹ f.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPANISH WINTER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>80</td>
<td>0.207</td>
<td>2.62 ± 0.30</td>
<td>484 a</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>1.212</td>
<td>3.15 ± 0.22</td>
<td>0.22</td>
<td>511 a</td>
</tr>
<tr>
<td>1.0% NaCl</td>
<td>1.372</td>
<td>3.33 ± 0.28</td>
<td>0.28</td>
<td>498 a</td>
</tr>
<tr>
<td><strong>LONGKEEPER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>86</td>
<td>0.493</td>
<td>2.61 ± 0.31</td>
<td>2626 a</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>1.020</td>
<td>2.96 ± 0.54</td>
<td>0.54</td>
<td>3773 a</td>
</tr>
<tr>
<td>1.0% NaCl</td>
<td>0.920</td>
<td>2.69 ± 0.20</td>
<td>0.20</td>
<td>1408 a</td>
</tr>
<tr>
<td><strong>ALCOBACA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>86</td>
<td>0.129</td>
<td>0.99 ± 0.20</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>0.280</td>
<td>1.38 ± 0.20</td>
<td>0.20</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>1.0% NaCl</td>
<td>0.393</td>
<td>1.45 ± 0.20</td>
<td>0.20</td>
<td>49 ± 10</td>
</tr>
<tr>
<td><strong>nornor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>84</td>
<td>-0.415</td>
<td>1.44 ± 0.03</td>
<td>106 ± 36</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>0.406</td>
<td>1.91 ± 0.11</td>
<td>0.11</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>1.0% NaCl</td>
<td>0.397</td>
<td>2.34 ± 0.10</td>
<td>0.10</td>
<td>158 ± 12</td>
</tr>
</tbody>
</table>

a = average of two determinations
Plate 6.1. The appearance of (a) nornor, (b) Spanish Winter and (c) Longkeeper fruit from plants treated daily with 500 ml of 0.5% or 1.0% sodium chloride during fruit development. Fruit harvested approximately 80 days after anthesis.
CONTROL 0.5% NaCl 1.0% NaCl

CONTROL 0.5% NaCl 1% NaCl (500 ml per day)

SPANISH WINTER

CONTROL 0.5% NaCl 1% NaCl (500 ml per day)

LONGKEEPER
6.3.2. The relative effects of sodium chloride and sodium sulphate and of ethrel on the ripening of fruit

The effect of sodium chloride or sodium sulphate treatment on fruit ripening depended on the genotype.

The application of sodium chloride and sodium sulphate solutions to non-ripening Alcobaca fruit resulted in a decrease in fruit firmness compared to water-treated controls early in ripening (Table 6.2). However, very little softening of these salt-treated fruit occurred after 70 days from anthesis, yet control fruit softened slightly between 70 and 94 days from anthesis. Thus by 94 days there was no significant difference in the firmness of water- and salt-treated Alcobaca fruit. Exposure of fruit to ethrel did not increase fruit softening. The very low rate of softening of Alcobaca fruit from all treatments was reflected in the negligible polygalacturonase activity extracted from them (Table 6.2), which was due to PG1 (Fig. 6.2). Salt treatment only marginally increased polygalacturonase activity in these fruit.

In the slow-ripening genotypes Spanish Winter and Longkeeper, neither sodium salt treatment promoted softening compared to water-treated controls (Table 6.2). Applying ethrel to sodium chloride-treated fruit also did not affect fruit firmness of either genotype. Salt treatments did not consistently increase the polygalacturonase activity of Longkeeper fruit, but caused a slight increase in salt and salt-plus-ethrel-treated Spanish Winter fruit, despite the fact that little softening occurred in these fruit between sampling times.

The low levels of polygalacturonase activity recorded by enzyme assay were not due to the presence of inhibitors. When equivalent
Table 6.2. Response by some slow-ripening tomato mutants to application of 0.5% sodium chloride (NaCl) and sodium sulphate (Na$_2$SO$_4$), and sodium chloride plus ethrel (NaCl + E)

<table>
<thead>
<tr>
<th>Genotype and Treatment</th>
<th>Days from Anthesis</th>
<th>Colour a/b</th>
<th>Firmness (mm compression)</th>
<th>Ethylene (µl 100 g$^{-1}$ h$^{-1}$)</th>
<th>PG Activity (nkat g$^{-1}$ f.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPANISH WINTER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55</td>
<td>0.077</td>
<td>2.60 ± 0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>0.635</td>
<td>2.69 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl + E</td>
<td></td>
<td>1.138</td>
<td>2.44 ± 0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>64</td>
<td>0.266</td>
<td>2.78 ± 0.71</td>
<td>257 ± 15</td>
<td>1487 ± 20</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td></td>
<td>0.628</td>
<td>2.49 ± 0.36</td>
<td>408 ± 28</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>1.330</td>
<td>2.62 ± 0.71</td>
<td>512 ± 30</td>
<td>2792 ± 16</td>
</tr>
<tr>
<td>NaCl + E</td>
<td></td>
<td>1.140</td>
<td>2.94 ± 0.36</td>
<td>405 ± 62</td>
<td>2401 ± 30</td>
</tr>
<tr>
<td><strong>ALCOBACA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>70</td>
<td>-0.416</td>
<td>1.10 ± 0.05</td>
<td>104 ± 18</td>
<td>629 ± 194</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td></td>
<td>-0.179</td>
<td>1.39 ± 0.22</td>
<td>127 ± 7</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>0.359</td>
<td>1.59 ± 0.13</td>
<td>227 ± 20</td>
<td>786 ± 73</td>
</tr>
<tr>
<td>NaCl + E</td>
<td></td>
<td>0.386</td>
<td>1.70 ± 0.24</td>
<td>989 ± 147</td>
<td>666 ± 105</td>
</tr>
<tr>
<td>Control</td>
<td>94</td>
<td>-0.228</td>
<td>1.51 ± 0.22</td>
<td>22 ± 12</td>
<td></td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td></td>
<td>0.189</td>
<td>1.48 ± 0.25</td>
<td>58 ± 29</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>0.436</td>
<td>1.63 ± 0.31</td>
<td>113 ± 26</td>
<td></td>
</tr>
<tr>
<td>NaCl + E</td>
<td></td>
<td>0.670</td>
<td>1.68 ± 0.22</td>
<td>76 ± 19</td>
<td></td>
</tr>
<tr>
<td><strong>LONGKEEPER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>64</td>
<td>0.273</td>
<td>2.19 ± 0.27</td>
<td></td>
<td>6910 ± 872</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td></td>
<td>0.335</td>
<td>1.89 ± 0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td>0.400</td>
<td>2.32 ± 0.61</td>
<td></td>
<td>8648 ± 547</td>
</tr>
<tr>
<td>NaCl + E</td>
<td></td>
<td>0.821</td>
<td>2.39 ± 0.34</td>
<td></td>
<td>4963 ± 939</td>
</tr>
</tbody>
</table>
Fig. 6.2. Electrophoretic separation of PG extracts from (a) control, (b) 0.5% sodium chloride and (c) 0.5% sodium chloride plus 0.1% ethrel treated fruit of Spanish Winter, Alcobaca and Longkeeper slow-ripening tomato genotypes.

Extracts were loaded onto polyacrylamide gels, in quantities depending on enzyme activity, and run cathodally in a β-alanine acetic acid pH 4.5 system. Gels were stained for PG activity.

The figures to the left of the gels indicate the relative mobility of the bands to methyl green. Blocked bands indicate presence of PG activity. Hatched bands indicate presence of an orange colour in response to the ruthenium red stain.
The diagram shows the protein content of different types of fruit. It indicates that the protein content of Spanish Winter and Alcobaca fruits is higher than that of Longkeeper. The diagram also shows the effect of different treatments on protein content, with the highest protein content observed in Longkeeper treated with FG1 and FG2. This suggests that the treatment with FG1 and FG2 may have a positive effect on protein content in Longkeeper.
amounts of protein (within each genotype) were examined by electrophoresis, the polygalacturonase activity in extracts of control and salt-treated Longkeeper and Alcobaca fruit were similar, and only in Spanish Winter was an increase in activity in salt-treated fruit extracts observed. This increase was mostly due to the appearance of PG2 (Fig. 6.2, Plate 6.2).

In some extracts of salt-treated Spanish Winter and Longkeeper fruit, an orange-coloured band was observed after ruthenium red staining (as shown in Spanish Winter gel c, Fig. 6.2).

Both sodium salts stimulated the development of fruit colour in all three genotypes. The effect of sodium sulphate was slightly less that that of sodium chloride (Table 6.2, Plate 6.3). Treating the fruit of salt-stressed plants with ethrel further enhanced colour of Alcobaca and Longkeeper fruit, but although ethrel appeared to hasten the development of colour in Spanish Winter fruit, it did not increase the a/b ratio of fruit 64 days after anthesis compared to salt treatment alone.

Salt treatments increased ethylene production in Spanish Winter and Alcobaca fruit sampled at 64 and 70 days after anthesis respectively, but only in Alcobaca fruit did ethrel treatment further increase ethylene production of salt-treated fruit. After 94 days from anthesis ethylene production by Alcobaca fruit under all treatments had declined, but the differences between treatments still remained.

One of the most noticeable effects of sodium chloride treatment on fruit of all genotypes was to reduce fruit size, and this was particularly evident in Spanish Winter fruit (Plate 6.3 a).
Plate 6.2. Electrophoretic separation of PG extracts from fruit of Spanish Winter, Longkeeper and Alcobaca plants treated with 500 ml 0.5% sodium chloride and of salt-treated fruit given 0.1% ethrel during ripening.

(a) Control.
(b) NaCl.
(c) NaCl + Ethrel.
Plate 6.3. The appearance of (a) Spanish winter and (b) Alcobaca fruit from plants treated daily with 500 ml of 0.5% sodium sulphate or sodium chloride and of salt-treated fruit treated with 0.1% ethrel. Fruit harvested approximately 70 to 80 days after anthesis.
In this study, the activity was as noted (note 6.4).

After treatment only, fruit was exposed to ethrel.

Notice significantly sampling decay fruit took place (0.3). When the

SPANISH WINTER

CONTROL

0.5% SO₄

0.5% CI

0.5% CI + ETHREL

ALCOBACA

CONTROL

0.5% SO₄

0.5% CI

0.5% CI + ETHREL
6.3.3. The effects of sodium chloride treatment and the interactions between ethrel and salt treatment

Ethrel treatment caused an increase in ethylene production in fruit of the three genotypes studied in this experiment, viz. normor, Alcobaca and Spanish Winter. However, the treatment affected the ripening of each genotype differently.

Fruit of ethrel treated normor were significantly softer than water-treated fruit during early ripening, but this difference was insignificant after 86 days from anthesis (Table 6.3). Ethrel-treated normor fruit did not soften much during aging, and this is reflected in their negligible polygalacturonase content (Table 6.3). In this genotype ethrel affected the colour, especially the b and a/b values at both sampling dates (Table 6.3).

In contrast, neither fruit colour, firmness nor polygalacturonase activity was affected by ethrel treatment of Alcobaca fruit (Table 6.3, Plate 6.4).

Slow-ripening Spanish Winter fruit responded to ethrel treatment only late in ripening, and then only the red colour of fruit was increased, as measured by a and a/b values (Table 6.3). Fruit firmness and polygalacturonase activity remained unaffected by ethrel.

Sodium chloride treatment of plants produced fruit that were significantly softer than controls in all three genotypes at all sampling dates. However, very little softening of any salt-treated fruit took place between the first and second sampling dates (Table 6.3). When the firmness of salt-treated Spanish Winter fruit samples,
Table 6.3. Effect of 0.5% sodium chloride and ethrel treatment on the ripening of tomato mutant genotypes

<table>
<thead>
<tr>
<th>Days from Anthe</th>
<th>Hunterlab Colour Values</th>
<th>Firmness (mm comp.)</th>
<th>Ethylene (nl 100 g⁻¹ f.wt. h⁻¹) n = 2</th>
<th>PC Activity (nkat g⁻¹ f.wt.) n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>a</td>
<td>b</td>
<td>a/b</td>
<td></td>
</tr>
<tr>
<td>SPANISH WINTER</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-8.27</td>
<td>20.55</td>
<td>-0.404</td>
<td>0.99</td>
</tr>
<tr>
<td>Ethrel</td>
<td>-6.57</td>
<td>20.97</td>
<td>-0.347</td>
<td>1.12</td>
</tr>
<tr>
<td>NaCl</td>
<td>19.91</td>
<td>14.21</td>
<td>1.403</td>
<td>2.68</td>
</tr>
<tr>
<td>NaCl + E</td>
<td>19.24</td>
<td>15.35</td>
<td>1.253</td>
<td>2.50</td>
</tr>
<tr>
<td>73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-4.62</td>
<td>22.81</td>
<td>-0.213</td>
<td>1.67</td>
</tr>
<tr>
<td>Ethrel</td>
<td>0.29</td>
<td>23.69</td>
<td>0.011</td>
<td>2.00</td>
</tr>
<tr>
<td>NaCl</td>
<td>22.38</td>
<td>15.07</td>
<td>1.507</td>
<td>2.81</td>
</tr>
<tr>
<td>NaCl + E</td>
<td>19.91</td>
<td>14.34</td>
<td>1.403</td>
<td>2.58</td>
</tr>
<tr>
<td>SED</td>
<td>0.89</td>
<td>0.51</td>
<td>0.064</td>
<td>0.107</td>
</tr>
<tr>
<td>LSD 0.1%</td>
<td>3.06</td>
<td>1.75</td>
<td>0.219</td>
<td>0.38</td>
</tr>
<tr>
<td>LSD 1.0%</td>
<td>2.36</td>
<td>1.35</td>
<td>0.169</td>
<td>0.29</td>
</tr>
<tr>
<td>normer 79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-10.30</td>
<td>17.99</td>
<td>-0.573</td>
<td>0.83</td>
</tr>
<tr>
<td>Ethrel</td>
<td>-8.22</td>
<td>20.02</td>
<td>-0.416</td>
<td>1.14</td>
</tr>
<tr>
<td>NaCl</td>
<td>-2.85</td>
<td>24.60</td>
<td>-0.118</td>
<td>1.34</td>
</tr>
<tr>
<td>NaCl + E</td>
<td>11.23</td>
<td>20.35</td>
<td>0.564</td>
<td>1.63</td>
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<tr>
<td>SED</td>
<td>0.802</td>
<td>0.492</td>
<td>0.042</td>
<td>0.076</td>
</tr>
<tr>
<td>LSD 0.1%</td>
<td>2.75</td>
<td>1.69</td>
<td>0.143</td>
<td>0.27</td>
</tr>
<tr>
<td>LSD 1.0%</td>
<td>2.12</td>
<td>1.30</td>
<td>0.110</td>
<td>0.21</td>
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</tbody>
</table>

E = Ethrel
continued...
Table 6.3. continued

<table>
<thead>
<tr>
<th>Days from Anthesis</th>
<th>Hunterlab Colour Values</th>
<th>Firmness (mm comp.)</th>
<th>Ethylene (nl 100 g⁻¹ f.wt. h⁻¹) n = 2</th>
<th>PG Activity (nkat g⁻¹ f.wt.) n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>a</td>
<td>b</td>
<td>a/b</td>
<td></td>
</tr>
<tr>
<td>nornor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-9.09</td>
<td>18.99</td>
<td>-0.478</td>
<td>1.15</td>
</tr>
<tr>
<td>Ethrel</td>
<td>-6.30</td>
<td>19.66</td>
<td>-0.322</td>
<td>1.33</td>
</tr>
<tr>
<td>NaCl</td>
<td>7.18</td>
<td>20.64</td>
<td>0.353</td>
<td>1.76</td>
</tr>
<tr>
<td>NaCl + E</td>
<td>13.00</td>
<td>17.88</td>
<td>0.726</td>
<td>1.76</td>
</tr>
</tbody>
</table>

**ALCCBACA**

| 82                |            |            |           |                                    |                                |
| Control           | -9.77      | 17.75      | -0.549    | 0.67                              | 46                             | Trace                        |
| Ethrel            | -7.75      | 18.99      | -0.412    | 0.85                              | 195                            | Trace                        |
| NaCl              | 7.47       | 22.03      | 0.345     | 1.78                              | 80                             | 430                          |
| NaCl + E          | 11.61      | 20.07      | 0.589     | 1.46                              | 178                            | 370                          |
| SED               | 1.02       | 0.64       | 0.053     | 0.13                              |                                |                              |
| LSD 0.1%          | 3.51       | 2.21       | 0.183     | 0.47                              |                                |                              |
| LSD 1.0%          | 2.70       | 1.70       | 0.170     | 0.36                              |                                |                              |

| 92                |            |            |           |                                    |                                |
| Control           | -8.53      | 20.19      | -0.423    | 0.85                              | 95                             |
| Ethrel            | -9.36      | 18.57      | -0.504    | 1.13                              | 166                            |
| NaCl              | 12.08      | 19.32      | 0.640     | 2.04                              | 82                             |
| NaCl + E          | 12.61      | 18.64      | 0.675     | 1.76                              | 1502                           |

E = Ethrel
Plate 6.4. The appearance of Alcobaca fruit from plants treated with 500 ml water or 0.5% sodium chloride during fruit development and of water- and salt-treated fruit given 0.1% ethrel during ripening. Fruit harvested 90 days after anthesis.
CONTROL

ETHREL

0.5% CI + ETHREL

In all cases, the CI treatment increased the a/c ratio compared to the CONTROL.

Again, the CI treatment did not affect the development of fruit. However, the 0.5% CI treatment increased the a/c ratio of the fruit.
(n = 10) were examined at different stages of ripening, green-yellow fruit had a compression of 1.99 mm which increased to 2.26 mm as fruit turned orange-green, and thereafter softened to only 2.28 mm in orange and 2.49 mm in red fruit (the differences between the last three values being insignificant).

A significant difference in polygalacturonase activity was found between water- and salt-treated Spanish Winter fruit (Table 6.3). However, only a small amount of activity was extracted from salt-treated Alcobaca fruit and trace amounts from normor fruit.

In all three genotypes salt-treatment significantly increased a and a/b colour values of fruit at both sampling times (Table 6.3).

Again, one of the most noticeable effects of salt treatment on fruit of all three genotypes was on fruit size and weight (Plate 6.2). Salt-treated Spanish winter, Alcobaca and normor fruit weighed on average 54%, 42% and 51% respectively less than their control fruit.

The interaction between salt and ethrel treatments also varied with genotype. The addition of ethrel to salt-treated Spanish Winter fruit did not significantly affect their colour, firmness or polygalacturonase activity. In Alcobaca fruit it stimulated colour development but did not affect firmness or polygalacturonase content of the fruit. However, it increased colour, softness and polygalacturonase activity of normor fruit early in ripening, but the difference between salt and ethrel-plus-salt-treated fruit was negligible 86 days after anthesis (Table 6.3).
Although ethrel treatment of Spanish Winter fruit increased ethylene production, the effect of salt treatment was much greater and was not further enhanced by ethrel treatment. In contrast, ethrel treatment of nor nor and Alcobaca fruit increased ethylene production more than salt treatment alone. However, in nor nor fruit this enhancement of ethylene production was only temporary and was insignificant by 86 days from anthesis.

6.4. DISCUSSION

The four mutant slow or non-ripening genotypes used in this study fell into two groups based on the extent to which ripening, as assessed by colour development, was retarded. The genotypes nor nor and Alcobaca showed few signs of normal ripening, even 90 days after anthesis fruit were still a yellow-green colour and quite firm (Fig. 6.1). Fruit homozygous for nor are non-climacteric, produce little ethylene, and although they contain all the pigments present in normal fruit, their colour development is severely retarded, as is their rate of softening which is reflected in their negligible polygalacturonase production (Ng and Tigchelaar, 1977; Tigchelaar et al., 1978a).

Studies on homozygous Alcobaca fruit also show that they are non-climacteric and soften very slowly (Mutschler, 1981). Many studies on Alcobaca fruit appear to have been done on heterozygous fruit, and these have ripening characteristics similar to heterozygous nor fruit, i.e. fruit ripening is retarded but not inhibited (Kopeliovitch et al., 1980), or fruit show reduced rates of softening but normal colour development (Lobo et al., 1981). Since the Alcobaca
fruit studied in this experiment produced very little ethylene, and hardly softened or coloured it is assumed that they were homozygous for the Alcobaca gene.

In contrast to nornor and Alcobaca fruit, those of Spanish Winter and Longkeeper genotypes eventually turned an orange-red or red colour, although at a much slower rate than occurs in fruit of normal ripening genotypes (Fig. 6.1 and Chapter 3). However, these fruit did not soften appreciably between 50 and 90 days from anthesis. Pressey and Avants (1982b) and Tigchelaar et al. (1983) also reported that these fruit softened extremely slowly and had prolonged shelf-lives as a result.

Spanish Winter and Longkeeper fruit at the orange-red colour had higher polygalacturonase levels than nornor and Alcobaca fruit, despite having apparently similar rates of softening (Fig. 6.1). This perhaps reflects events occurring early in fruit ripening causing these fruit to be softer than nornor and Alcobaca fruit of similar ages.

Pressey and Avants (1982b) reported finding the Longkeeper fruit contained an isoenzyme of polygalacturonase which resembled PG1 of normal fruit but had slightly different molecular weight and enzymic activity to the PG1 of normal fruit. However, analysis of Longkeeper polygalacturonase by electrophoresis did not reveal any difference between it and normal PG1 (Fig. 6.2). The purification techniques employed by Pressey and Avants (1982b) did not seem adequate to remove PG2 from their PG1 sample, since they employed only one purification step using DEAE Sephadex A-50. Thus it is possible that their findings were due to PG2 contamination.
The results of this study showed that PG1 was the major form of polygalacturonase in both Longkeeper and Spanish Winter fruit. However, some PG2 appeared in very old, ripe fruit of both genotypes and in salt-treated Spanish Winter fruit. Press ey and Avants (1982b) confirm the appearance of PG2 in ripe Longkeeper fruit.

The only isoenzyme of polygalacturonase present in normor and Alcobaca fruit was PG1. The appearance of orange staining bands in some fruit (Fig. 6.2) might be due to the presence of a pectin lyase (as discussed in Chapter 5). However, since it appeared only occasionally in extracts from all treatments it is not thought to be responsible for any firmness differences between water- and salt-treated fruit. The four mutant genotypes studied here are capable of synthesising polygalacturonase isoenzymes and developing some colour, but the extent and rate of both ripening events are retarded under normal conditions of growth.

The physiological similarities between normor and Alcobaca fruit, and some similarities of these with Spanish Winter and Longkeeper, led Tigchelaar et al. (1983) to suggest that they were allelic. However, the differences in the reaction of Spanish Winter fruit to ethylene compared to normor and Alcobaca fruit (Table 6.3) suggests this genotype has a fundamentally different physiology and genetic composition to the nor type fruit.

It is possible that two alleles and/or modifier genes might exist at the nor locus which alter the expression of nor in Alcobaca, Spanish Winter and Longkeeper fruit (Tigchelaar et al., 1983). This would account for observed differences in ripening characteristics and reaction to salt and ethrel treatments between these genotypes.
However, whilst Lobo et al. (1981) felt that nor and Alcobaca were allelic, tests by Kopeliovitch et al. (1980) and Mutschler (1981) suggest that they were not. The results presented here suggest that whilst nornor and Alcobaca are physiologically similar, thus possibly allelic, Spanish Winter and Longkeeper fruit are very different to the nornor type fruit and therefore are unlikely to be allelic to nor.

Fruit of normal cultivars can be made to mature and ripen more quickly by treating the plants with sodium chloride solutions during fruit growth (Mizrahi, 1982). Such treatment not only increased the content of Cl- and Na+ ions in fruit but also increased fruit colour, acids, reducing sugars, flavour, ethylene production and activity of pectolytic enzymes. As observed in these studies, one of the major effects of salt treatment was in reducing fruit weight. However, Mizrahi (1982) found that the influence of salt on fruit ripening parameters was not primarily due to its effect on fruit size.

Later Mizrahi et al. (1982) found that similar treatment of non-ripening nornor fruit also stimulated fruit ripening in terms of colour development, taste, ethylene and carbon dioxide evolution. However, although pectolytic activity of these salt-treated fruit was found to increase slightly, polygalacturonase activity alone was not measured (Mizrahi et al., 1982). Ethylene treatment of rinrin fruit caused them to ripen, but salt treatments did not. This indicates that ripening in these two non-ripening mutants is blocked by different mechanisms. A greater understanding of the reasons for these differences might aid understanding of ripening events in normal fruit.
The treatment of the four genotypes used in this study with solutions of either sodium chloride or sodium sulphate during fruit development enhanced some parameters of ripening. Since the effect of sodium chloride on normor fruit ripening has been shown to be due to its effect in inducing water stress (Kalir Arad and Mizrahi, 1983), it is not surprising that sodium sulphate produced a similar effect on fruit ripening to sodium chloride, since both alter the osmotic potential of the soil water.

Salt treatment increased the colour development of fruit from all four genotypes. This was most noticeable in normor and Alcobaca fruit (Table 6.4). Mizrahi et al. (1982) found that sodium chloride increased lycopene levels in normor fruit to 44% of those in normal fruit. However, since Spanish Winter and Longkeeper control fruit did slowly become red in colour during ripening under normal conditions, the difference in colour between water- and salt-treated fruit of these genotypes diminished with fruit age.

Although in all genotypes studied salt treatment produced fruit that were softer than water-treated ones 50 to 60 days from anthesis, there was less difference in firmness of treated and untreated fruit beyond 80 days from anthesis (Table 6.3). Closer examination of the rate of softening, where more than one sampling time was included in the experiments (Tables 6.2 and 6.3) showed that very little softening of salt-treated fruit took place after about 60 days from anthesis. Untreated fruit softened slowly as they coloured and thus eventually approached the level of softness of treated fruit.

The reasons for the differences in firmness between salt-treated and untreated fruit remain to be elucidated. It is possible that they
are due to variations in fruit wall structure, brought about by growth under stress conditions which reduced fruit size (Plate 6.3 and 6.4), rather than differences in rates of cell wall degradation.

Alternatively, softening during the very early phases of ripening, brought about by small increases in polygalacturonase activity in salt-stressed fruit, may affect fruit firmness during the later stages of ripening. Such a mechanism of loss of firmness may account for the fact that Spanish Winter and Longkeeper fruit were softer and had higher polygalacturonase activity than Alcobaca and normor fruit.

Fruit of all the genotypes considered responded to salt stress by increasing ethylene production (Tables 6.1, 6.2 and 6.3). It is well documented that plants grown under stress conditions react in this way (Yang and Hoffman, 1984). Ethylene has been shown to play an important role in fruit ripening and treating normal tomato fruit with this gas hastens the onset of ripening (McGlasson et al., 1975). Since salt treatment enhanced fruit ethylene production, it is possible that the salt effects on ripening might be mediated through ethylene. It is known that treating fruit with the ethylene releasing chemical ethrel increases their internal ethylene content for 24 hours after application. After this time fruit ethylene production by normally-ripening fruit is due to endogenous production (Dennis et al., 1970). Thus if ripening in the non-ripening mutants is due to lack of ethylene production then ethrel treatment might overcome the block to ripening.

Treatment of the slow and non-ripening genotypes in this experiment with ethrel stimulated endogenous ethylene production in fruit of all four genotypes. However, although they all produced
some carotenoid pigments in response to ethrel, the effect of this
chemical on fruit colour was slight compared to the effect of salt
treatment (Table 6.3). Ethrel-treated nornor and Alcobaca fruit
produced more ethylene than salt-treated fruit. Thus high levels
of ethylene alone did not contribute to fruit colour development
(Table 6.4).

The firmness of ethrel treated nornor fruit was slightly
lower than control fruit but ethrel had no effect on firmness of
Alcobaca and Spanish Winter fruit. The results reported here for
nornor fruit are not in agreement with those of Mizrahi et al.
(1975), who observed no effect of ethylene on the ripening of
nornor fruit. However, the effect of ethrel alone on fruit colour,
reported here, was very slight and might easily be overlooked by
subjective evaluations. Possibly the cultivar into which the nor
gene was introduced affected the results. Mizrahi et al. (1975)
used nornor in cv. Rutgers, a slow-ripening cultivar compared to
Ailsa Craig, the cultivar into which nor gene was incorporated in
this study.

The block to ripening in these slow and non-ripening genotypes
cannot therefore be attributed to lack of ethylene production. Nor
can the effects of salt treatment on fruit colour and firmness
reported here (Tables 6.1, 6.2 and 6.3) be due solely to the effects
of salt stress on fruit ethylene production. Sisler (1982) found
no difference in the number of ethylene binding sites between normal-
ripening and nornor fruit. Thus it seems that, in this genotype at
least, the block to normal ripening occurs either (a) at the level
of ethylene action or (b) quite independently of ethylene metabolism
Table 6.4. Relative increase in ethylene production, fruit colour, fruit softness and PG content in nornor, Alcobaca, Spanish Winter and Longkeeper fruit due to treatment with ethrel, sodium chloride and salt—plus—ethrel over levels observed in water-treated fruit of the same genotypes. (Summary of data from Tables 6.1 to 6.3.)

<table>
<thead>
<tr>
<th>Treatment and Parameter of Ripening</th>
<th>nornor/Alcobaca</th>
<th>Spanish Winter/Longkeeper</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ETHREL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylene</td>
<td>XX</td>
<td>X</td>
</tr>
<tr>
<td>Colour</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Softness</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>PG Content</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>SALT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylene</td>
<td>X</td>
<td>XX</td>
</tr>
<tr>
<td>Colour</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>Softness</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>PG Content</td>
<td>X</td>
<td>X/O</td>
</tr>
<tr>
<td><strong>SALT + ETHREL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylene</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>Colour</td>
<td>XXX</td>
<td>XX</td>
</tr>
<tr>
<td>Softness</td>
<td>XX</td>
<td>XX</td>
</tr>
<tr>
<td>PG Content</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

**Symbols**

- **0** = No increase over levels in water—treated fruit of same genotype.
- **X** = Slight increase over levels in water—treated fruit of same genotype.
- **XX** = Significant increase over levels in water—treated fruit of same genotype.
- **XXX** = Major increase over levels in water—treated fruit of same genotype.
- **O/X** = Variable results.
or (c) that ethylene requires the addition of another metabolite to promote its action, and synthesis of this factor is required before non-ripening genotypes can be induced to ripen.

Salt treatment stimulates the production of factor(s) which promote ripening (Tables 6.1 to 6.4). Since polygalacturonase activity is very slightly enhanced by this treatment it is possible that this enzyme is one of the factors involved. Interaction of low levels of polygalacturonase activity and of ethylene could promote ripening of non-ripening mutants, and increase the rate of ripening of slow-ripening genotypes. Mizrahi and Malis Arad (1984) found a good correlation between lycopene levels and ethylene production in salt-treated nornor fruit in different isogenic backgrounds, suggesting that salt and ethylene effects on fruit metabolism interact to promote ripening in this genotype.

Since treating the slow and non-ripening genotypes with salt overcame the inhibition to most ripening parameters, it was thought that such salt-ripened fruit might respond to ethylene treatment in the same way as would be expected of normal fruit (McGlasson, 1970).

Adding ethrel to salt-treated nornor and Alcobaca fruit increased their colour development (more so in nornor than in Alcobaca fruit) and ethylene production but not firmness (Table 6.3). Ethrel plus salt-treated nornor and Alcobaca fruit produced about the same amount of ethylene as ethrel-treated fruit, yet coloured to a greater extent. This suggests that the colour development observed in these experiments was not solely due to the high levels of ethylene produced, but to an interaction between salt and ethrel treatments.
As expected from its lack of reaction to ethrel alone, salt-treated Spanish Winter fruit did not react further to ethrel treatments (Table 6.3). Since fruit of this genotype ripen slowly and therefore produce some ethylene during aging, adding extra ethylene in the form of ethrel probably had no further affect on fruit metabolism. It appears that low levels of ethylene produced during the early stages of ripening are more important to the initiation of fruit colour, and other ripening parameters, than the high amounts produced during the climacteric.

The main effect of salt treatment of mutant slow or non-ripening mutant fruit was on fruit colour and weight. Mizrahi et al. (1982) have also shown that salt-treated fruit have a good flavour compared to untreated mutants, with soluble solids and titratable acidity levels similar to those of normal fruit. Thus many parameters of fruit ripening are enhanced by water stress of plants during fruit development. However, salt and salt-plus-ethrel-treated plants still had slow rates of softening during ripening and very low levels of polygalacturonase activity in ripe fruit compared to levels found in similarly coloured normal-ripening cultivars. Mizrahi et al. (1982) measured some polymethylgalacturonase activity in salt-treated normor fruit. However, this was only 0.7% of that found in normal fruit and three times the trace amounts extracted from water-treated mutant fruit. These results are only slightly higher than the results for polygalacturonase activity in Alcobaca fruit reported here (Tables 6.2 and 6.3). Thus it appears that the genetic block to polygalacturonase synthesis that is present in these mutants is not entirely overcome by salt or ethylene treatment.
The fact that pigment synthesis, flavour development, increase in sugars and ethylene production are stimulated in salt-treated fruit, yet fruit polygalacturonase levels remain very low suggests one of two things. Either these events of ripening are not dependent on polygalacturonase activity but may occur independently of wall degradation. Or, salt-stressing the non-ripening genotypes may induce minimal polygalacturonase activity and cell wall degradation. This, combined with some ethylene production by fruit (also stimulated by salt) could lead to enhanced pigment synthesis and flavour development. This idea is supported by Mizrahi (1982) who suggested that salinity may affect the regulatory site of fruit ripening, which is normally blocked in these mutant fruit. However, if ripening of these fruit is dependent on removal of only one metabolic block, then the reasons why some ripening parameters, such as colour are affected more than others, such as softening, requires explanation. Further experimentation should throw light on the precise role of polygalacturonase in this interesting phenomenon.

In conclusion, salt-stressing slow and non-ripening plants stimulates colour development, ethylene production and minimal polygalacturonase activity. However, the effect of salt is not solely due to its effect on fruit ethylene production, since ethrel treatment of fruit did not produce the same marked enhancement of ripening as observed in salt-treated fruit. Salt-stress may promote the activity of other factors, possibly polygalacturonase or growth regulators, which interact with ethylene to stimulate ripening in these slow and non-ripening mutants.
CHAPTER SEVEN  

FINAL DISCUSSION: THE ROLE OF POLYGALACTURONASE ISOENZYMES IN TOMATO FRUIT SOFTENING AND RIPENING

7.1. The role of polygalacturonase in tomato fruit softening during ripening

The production and activity of endo-polygalacturonase has long been associated with tomato softening. As discussed in Chapter 3, the major changes in fruit firmness occurring during ripening are associated with a loss of insoluble pectic material and concomitant increase in water-soluble pectins (Foda, 1957; Sawamura et al., 1978; Brady et al., 1982). This change in pectin composition has been attributed to the action of an endo-polygalacturonase (Foda, 1957; Hobson, 1964; Brady et al., 1982; Themmen et al., 1982), but the precise relationship between the activity of this enzyme and its effects on fruit firmness have not been resolved.

The results of this thesis suggest that a very close relationship exists between polygalacturonase activity and fruit softening during the early stages of ripening. In contrast, the rapid accumulation of the enzyme that occurs as fruit turn orange colour is not associated with a similar increase in rate of fruit softening. There appears to be an over-abundance of the enzyme during the later stages of ripening (Chapters 3 and 4; Brady et al., 1982).

The discovery of more than one form of polygalacturonase (Pressey and Avants, 1973; Rexová-Benková et al., 1977; Ali and Brady, 1982) raises the possibility that its role changes during ripening. Polygalacturonase exists in at least three isoenzymic forms (Chapters
2 and 3; Ali and Brady, 1982). The largest isoenzyme PG1, always appears first, soon after the onset of ripening (as judged by an increase in fruit ethylene production) (Chapters 3 and 4; Tucker et al., 1980; Ali and Brady, 1982). It accumulates slowly during the early stages of ripening of both normal and mutant hybrid tomato fruit, compared with the high exponential rate of PG2 accumulation later in ripening (Chapters 3 and 4; Brady et al., 1982). PG1 is the only form of the enzyme present in fruit up to the green-orange stage of ripening (Chapters 3 and 4; Tucker et al., 1980; Ali, 1983).

Most fruit softening occurs during the early stages of tomato ripening, as observed by many researchers (Foda, 1957; Hobson, 1959; Ramadan, 1981). Thus if polygalacturonase does cause much of the marked loss of firmness that occurs during tomato fruit softening, then low activities of the enzyme must be able to bring about significant changes in the pectin fraction of the cell wall very early in ripening. Whether PG1 is able to bring about these changes alone or whether other processes both enzymic or non-enzymic are involved remains to be resolved.

7.1. Involvement of PG1 in early loss of firmness and the middle lamella pectins

The loss of tomato fruit firmness is due to the degradation of the middle lamella (Pilnik and Voragen, 1970). This is composed of pectic polysaccharides predominantly of the type which are simply substituted, methyl-esterified rhamnogalacturonan molecules (Albersheim et al., 1960; Albersheim and Killias, 1963; Knee et al., 1975).
Ultrastructural studies of ripening fruit have shown that wall degradation is first visible in the middle lamella of tomatoes (Crookes and Grierson, 1983), pears (Ben-Arie et al., 1979) and avocados (Platt-Aloia et al., 1980).

In tomatoes extensive dissolution of the middle lamella was observed three to four days after the increase in ethylene production, about the same time as PG1 activity was first detected (Crookes and Grierson, 1983). Most of the middle lamella was degraded whilst only PG1 was present. As fruit ripening progressed, more extensive degradation of the cell wall was noted together with an increase in the activity of PG2 isoenzymes.

Purified PG1 and PG2 preparations caused similar degradation of the middle lamella and outer cell wall to those observed in ripening fruit (Crookes and Grierson, 1983). Themmen et al. (1982) and Hobson et al. (1983b) also showed that polygalacturonase is capable of degrading cell wall material in vitro, but detailed comparisons of the various isoenzymic forms were not carried out. Although the results of Chapter 3 suggest PG1 is less active in degrading cell walls than PG2, this might well be due to the limitations of the in vitro experiment. The size of PG1 compared to PG2 must hinder its access to wall substrates, and it is possible that the optimum conditions of PG1 activity against natural substrates are slightly different to those of PG2, and that they were not met in these experiments. In particular the low rate of PG1 solubilisation of cell wall material could be due to alterations in middle lamella structure or even content during extraction and purification. If PG1 is primarily active against the middle lamella and its access to the cell wall limited because of
its size, then alterations to the middle lamella layer would affect its activity more than that of PG2. The latter isoenzyme is probably equally capable of degrading the middle lamella and cell walls.

Thus there is good evidence that polygalacturonase, in the form of PG1, can cause extensive wall degradation during ripening. What remains unresolved is whether the accumulation of small amounts of PG1 early in ripening can account for the marked changes in fruit firmness that occur over this period. One problem to be faced is whether the enzyme reaches the middle lamella in time to cause sufficient depolymerisation of the middle lamella pectins to weaken the intercellular structure.

Polygalacturonase is not detectable in mature green tomatoes, either as an active enzyme or as an inactive precursor (Tucker and Grierson, 1982). However, polygalacturonase synthesis begins about 20 hours after the increase in fruit ethylene production that is one of the more obvious signs of ripening (Grierson and Tucker, 1983).

It is likely that polygalacturonase glycoprotein is assembled in Golgi bodies located in the thin layer of cytoplasm that lines the enlarged fruit cell wall (Chrispeels, 1976). It may then be secreted to the wall, either after fusion of the vesicles with the plasmalemma, or by passage of intact vesicles via desmotubules of the plasmodesmata. Once within the plasmodesmata, polygalacturonase may be liberated by rupture of the vesicles in the immediate vicinity of the middle lamella (Bartley and Knee, 1982). Ben-Arie et al. (1979) observed that vesicles frequently occurred near plasmodesmata during the dissolution of the middle lamella, and that the plasmodesmata remained intact throughout ripening.
The synthesis and secretion of wall glycoproteins has been shown to occur within 15 minutes of each other (Chrispeels, 1976). Thus it is possible that PG1 could be at its site of action in the middle lamella in less than a day after the first detectable increase in fruit ethylene production. Whether the large PG1 molecule is secreted intact, or whether the component polypeptides and carbohydrates are assembled in the wall remain to be elucidated. However, a molecule of the size of PG1 could not pass through the available pore spaces in the cell wall (Knee et al., 1975; Carpita et al., 1979). Thus it is likely that PG1 is assembled or at least glycosylated in the middle lamella region.

Ultrastructural studies did not reveal middle lamella degradation until three days after the increase in fruit ethylene production (Crookes and Grierson, 1983). However, a rise in water-soluble pectins has been found to occur very early in ripening (Sawamura et al., 1978). Polygalacturonase is the only enzyme found so far that is capable of releasing water-soluble pectins from the cell wall (Wallner and Walker, 1975; Wallner and Bloom, 1977). Brady et al. (1982) found a good correlation between the rise in polygalacturonase activity and water-soluble pectins released from tomato pericarp tissue during early ripening. The results of this thesis support these results, and suggest that PG1 accumulation is closely related to fruit softening.

7.1.2. Processes that may contribute to tomato fruit softening

Observations by Besford and Hobson (1972) indicate that some softening occurs in tomato fruit prior to detectable polygalacturonase activity. These observations could be explained by several mechanisms.
Firstly, fruit cells continue to increase in size until just after the onset of fruit ripening (Reeve, 1959). The pericarp of mature green tomato fruit is composed of large, isodiametric, parenchymatous cells about 300 μm diameter (Crookes and Grierson, 1983). During the later stages of fruit growth partial separation of cell walls occurs at the middle lamella. Contact between adjacent cells is partially lost and large intercellular spaces form. If such cell expansion continues during the later stages of fruit ripening, then some weakening of intercellular cohesion must result as cells are forced apart. Cell turgidity is also an important factor in fruit firmness, and its reduction may contribute to some softening during ripening (Spurr, 1970). However, Vickery and Bruinsma (1973) showed that the plasmalemma and tonoplast persist 'intact' late into ripening so they probably become 'leaky' only with overripeness.

Secondly, throughout fruit growth there is a continuous turnover of cell wall material (Jermy and Isherwood, 1956; Knee, 1973; Wallner and Bloom, 1977; Van Buren, 1979; Lackey et al., 1980; Labavitch, 1981). During tomato ripening there is a noticeable loss of galactose as well as galacturonic acid residues from the cell wall (Lackey et al., 1980; Gross, 1983).

Enzymes involved with cell wall turnover e.g. β-galactosidase, β-glucosidase, α-mannosidase increase or change isoenzymic form during ripening, as determined by in vitro assays with artificial substrates (Wallner and Walker, 1975; Pharr et al., 1976; Yamaki and Matsuda, 1977; Hinton and Pressey, 1980; Pressey, 1983). In particular, the activity of one isoenzymic form of β-galactosidase increases three-fold during ripening. This isoenzyme was able to degrade β-1,4-linked galactose residues, which might explain the loss of
galactose from cell walls of ripening tomatoes. However, none of these enzymes has been found capable of initiating cell wall degradation (Wallner and Walker, 1975). Furthermore, Gross and Wallner (1979) concluded that β-galactosidase was not responsible for in vivo hydrolysis of cell wall galactans.

The loss of neutral sugar residue from cell walls of ripening tomatoes could be due to reduced rate of galactose polymer synthesis or metabolism of galactose residues in the ripening fruit cell (Lackey et al., 1980; Gross, 1983). Bartley and Knee (1982) suggested that wall degradation during the early stages of fruit ripening should be regarded as an extension of wall development, with the balance as ripening progresses being shifted increasingly in favour of degradation rather than synthesis. Such an hypothesis would explain the loss of neutral sugars from fruit cell walls prior to extensive pectin hydrolysis (Knee, 1973), and the slight increases in pectin content of some fruit during ripening (Fressey et al., 1971).

The fact that non-ripening mutant tomatoes lose galactose from cell walls during aging, yet do not soften appreciably strengthens the argument that galactosidase enzymes do not contribute greatly to tomato softening (Gross and Wallner, 1979).

However, these enzymes may prepare the wall for polygalacturonase action by changing the extent of covalent interconnections between polymer chains of the wall matrix and hemicellulose polymers (Karr and Albersheim, 1970; Van Buren, 1979; Hobson, 1980). They may also cause some textural changes in conjunction with polygalacturonase activity during fruit ripening. Although Wallner and Bloom (1977)
did not detect the release of neutral sugars when they incubated green
tomato cell walls with polygalacturonase; similar experiments by
Pressey and Avants (1983a) have proved that both PG1 and PG2 are
capable of releasing neutral sugars as well as pectic residues from
tomato cell walls. Since polygalacturonase has been found capable of
degrading cell wall material and pericarp tissue discs from mature
green tomato tissue (Chapter 3, Wallner and Bloom, 1977; Themmen et al.,
1982; Hobson et al., 1983b), the need for wall modifications prior
to polygalacturonase activity is questioned.

However, since structural changes may have occurred during
the preparation of cell walls, some caution should be exercised in
drawing conclusions about the in vivo situation. Other enzymes may
play a role in softening, including some that may have been lost in
the water-soluble fraction during preparation of wall-bound proteins.
Despite these reservations, the results of many studies on cell wall
metabolism of ripening fruit have concluded that polygalacturonase
plays a key role in wall degradation (Wallner and Bloom, 1977; Ahmed
and Labavitch, 1980; Themmen et al., 1982).

Another potentially important wall degrading enzyme involved
in the release of neutral sugars is cellulase. Cellulase activity
increases with ripening of many fruits (Hobson, 1968; Sobotka and
Watada, 1971; Yamaki and Matsuda, 1977), and could degrade both
cellulose and the β-1,4-glucan backbone of xyloglucan.

Sobotka and Stelzig (1974) found tomato fruit had exo- and two
endo-cellulases which could degrade artificial substrates, but
activity against wall cellulose was not demonstrated. Sobotka and
Watada (1971) found that firm-fruited cultivars had lower cellulase
activity than soft lines. The rapid softening rate that occurred
during the first six days of ripening was closely associated with
the change in cellulase activity. However, they found that the sharp
decrease in firmness that occurred in the very early stages of
ripening did so when cellulase levels were insignificant.

In contrast, Hobson (1968) found that cellulase activity in
tomato fruit was not correlated with fruit softening, and concluded
that it was not a major factor controlling softening of tomato fruit
during ripening. Likewise Ahmed and Labavitch (1980) could not assign
a role to cellulases in pear softening.

The non—ripening tomato mutant rin has been observed to contain
some $\beta$-1,4-glucanase and cellulase activity (Buescher and Tichelaar,
1975; Hobson, 1981) but these fruits soften slowly. Since
endo-polygalacturonase is absent from rin tomato fruit, the very
limited amount of softening that does occur over a long period of
time may be due to $\beta$-1,4-glucanase action. Thus cellulases may
contribute to limited softening of fruit but cannot be associated
with major changes in fruit firmness.

There are many problems associated with studies of enzymes on
cell walls (Ahmed and Labavitch, 1980). It is often unclear how
much of a given extracted activity was located in tissue free space
i.e. in a position to act on the wall pectic material. Cell walls
have the ability to trap cytoplasmic proteins due to their ion
exchange effects, thus some enzymes associated with the wall may be
cytoplasmic contaminants. In addition, some aspects of cellular
control may be eliminated during enzyme extraction and assay.
Changes in free space pH, such as occur during auxin stimulation of
cell wall change and elongation, could accompany ripening and might regulate *in situ* enzyme activity. Thus the precise contribution made by wall-degrading enzymes other than polygalacturonase to fruit softening is still unclear.

Thirdly, softening of the placental tissue could contribute toward limited fruit softening early in ripening, and may be responsible for much of the softening observed to occur in some wild species (Chapter 5), where the placental tissue formed a very high proportion of total fruit weight. Wallner and Walker (1975) found that placental tissue showed very low polygalacturonase activity but increasing \( \beta \)-galactosidase and \( \beta-1,4 \)-glucanase activities during ripening. Sobotka and Watada (1971) found that cellulase activity was four- to five-fold higher in the placental than pericarp tissue. They suggested that it caused softening of this tissue rather than pericarp cell walls.

Fourthly, calcium is important in maintaining fruit firmness. Addition of calcium to whole fruit or tissue slices delays or even inhibits softening (Wills and Tirmazi, 1979; Knee, 1982b), probably by stabilising the junction zones between calcium and pectin in the middle lamella (Grant *et al*., 1973; Bartley and Knee, 1982). Conversely, loss of calcium from the cell wall occurs during fruit ripening (Suwan and Poovaiah, 1978; Rigney and Wills, 1981). In addition, treating fruit with chelating agents such as EDTA or citrate has been shown to enhance ripening (Buescher and Hobson, 1982). Removal of calcium from pectin may be a prerequisite for tissue softening. Its removal could be brought about by endogenous regulators e.g. citrate (Buescher and Hobson, 1982).
Calcium may also regulate the activity of polygalacturonase and pectinesterase enzymes directly. Calcium has been found to stimulate pectinesterase but inhibit polygalacturonase at concentrations of $10^{-7}$ M (Wills and Rigney, 1979). However, Ali and Brady (1982) found much higher concentrations of calcium were required to inhibit PG2, whereas PG1 was stimulated by this ion.

Alterations of pH in the cell wall may also contribute to softening. Knee (1982a), after observing that apple softening could be inhibited by applying buffers of pH 8.0 to preclimacteric fruit, suggested that the onset of ripening and softening could involve an active ion pump which regulates the hydrogen ion status of the cell wall. This is analogous to the process that occurs in IAA-stimulated wall growth (Soll and Bottger, 1982). Since calcium binding to pectin is stabilised at high pH and weakened at low pH, acidification of the cell wall could initiate softening by causing the removal of calcium ions. Also it might activate polygalacturonase and glycosidase enzymes which have pH optima in the 3-5 range (Pressey and Avants, 1973; Wallner and Walker, 1975; Bartley and Knee, 1982). However, the in vivo pH changes required to verify this hypothesis have not yet been measured.

Fifthly, one process affecting cohesion of the middle lamella and solubility of pectin is the esterification of pectic acids (Worth, 1967; Knee, 1978). This controls not only the availability of carboxyl groups required for calcium binding, but also the action of polygalacturonase, since this enzyme cannot attack very highly methylated substrates (Jansen and McDonnell, 1945; Pressey and Avants, 1973). In general the degree of pectin esterification in
fruit cell walls declines throughout ripening (Jermyn and Isherwood, 1956; Roe and Bruemmer, 1981; Knee, 1982b).

The enzyme controlling pectin esterification, pectinesterase, has been found in all fruit studied so far (Hobson, 1963; Knee, 1978; Ahmed and Labavitch, 1980; Faull and Chen, 1983). In tomatoes, it exists in up to eight isoenzymic forms (Delinceba, 1976; Tucker et al., 1982). Although total pectinesterase content of ripening tomatoes increases, mainly due to isoenzyme 2 activity (Tucker et al., 1982); Hobson (1963) found no correlation of its total activity with softening. Tucker et al. (1982) found that NrKr fruit contained the same amount of pectinesterase as normal fruit yet softened at a much slower rate, thus its activity was not directly associated with softening.

Pressey and Avants (1983a) observed that for optimum activity of polygalacturonase the degree of esterification of pectin was about 40%. Pectinesterase assisted the degradation of pectin by polygalacturonase, especially PG1, at low pH's in vitro. However, considerable wall degradation occurred with polygalacturonase alone. They concluded that pectin solubilisation during tomato ripening is due to the action of polygalacturonase on highly esterified substrates at relatively low pH.

Since pectinesterase is present in tissue in non-limiting quantities in much of the period of development of many fruits, it must be the inaccessibility of the methoxyl groups on the side chains of pectin that prevents the enzyme from rapidly and completely de-esterifying the substrate. One factor regulating the activity of pectinesterase may be calcium. Nakagawa et al. (1971) found that
the concentration of divalent cations controlled the pectinesterase binding and activity in tomato cell walls. In addition, pectinesterase binds strongly to pectins by electrostatic forces. Thus, when it has de-esterified a chain segment it will remain attached there, freed only by polygalacturonase action (Jarvis, 1984). This must help prevent the indiscriminate de-esterification that would undoubtedly occur if all the pectinesterase present in the cell wall attacked the pectin (Hobson, 1980).

Pectin lyase enzymes could also degrade pectin molecules by a transeliminative cleavage of the α-1,4-glycosidic bonds to produce C4-C5 unsaturated galacturonic acid derivatives. Besford and Hobson (1972) examined ripening tomato fruit for activities of pectin transeliminase and pectic acid transeliminase and found no evidence for their existence. Only one such enzyme has been found in higher plants (Albersheim and Killias, 1963). However, in most cases higher plant pectin depolymerases have not been tested by methods that would distinguish between hydrolytic and β-elimination cleavage. This raises the possibility that pectin lyases may be more prevalent than would appear at present (Van Buren, 1979).

In this study and that of Ali (1983) it was observed that gels through which tomato extracts were fractionated contained a diffuse yellow-orange band, as well as the clear polygalacturonase bands. According to Cruickshank and Wade (1980) a yellow-orange reaction to ruthenium red may be due to pectin lyase degradation of pectin or polygalacturonic acid to an intermediate which gives a yellow-orange colour with ruthenium red. If pectin lyase does exist in tomato fruit it could have an important role in pectin degradation (Rexová-Benková and Marković, 1976).
Polymethylgalacturonase is another enzyme that could be involved in early loss of firmness. However, there are only a few reports of its existence in tomatoes (Mizrahi et al., 1982). Since these assays were done on unpurified extracts of fruit pericarp tissue it is likely that they contained pectinesterase. Thus it is difficult to ascertain whether the pectin degradation measured was due to polymethylgalacturonase, or a combination of pectinesterase and polygalacturonase.

A wealth of evidence shows that tomato softening is accompanied by an increase in soluble pectic compounds (Foda 1957; Sawamura et al., 1978; Gross and Wallner, 1979; Brady et al., 1982). Also that the degree of softening observed in various tomato cultivars is correlated with the amount of water-soluble pectins released during ripening (Malis-Arad et al., 1983), and this in turn is associated with polygalacturonase activity (Brady et al., 1982). This suggests that the activity of pectin degrading enzymes are paramount in the softening processes.

Observations so far show that only polygalacturonase is capable of bringing about such wall solubilisation, which lends support for the idea that small amounts of PG1, which this work has shown to be present in fruit at this time, are capable of causing wall degradation. In addition, not all the bond-splitting activity of this enzyme will be evident as water-soluble pectins. Because of steric hindrance, many large polymers may be held in the cell wall and middle lamella until degraded into small units. However, the fact that the polymer chain has been shortened, weakens the interpolymere bonding in the middle lamella and this affects fruit firmness immediately.
There are two ways in which polygalacturonases could break down a pectin gel in vivo (Jarvis, 1984). Firstly, pectic segments participating in junction zones could be attacked. Fragments would be released into solution when their non-esterified portions became shorter than the approximate 14 units required for optimum calcium binding. Short galacturonase oligomers have been observed to be released in ripe tomatoes. Slightly longer oligomers would not become soluble, but their formation would still reduce the cohesion of the gel.

Alternatively, the enzyme could attack unbranched areas of the interjunction segments. It is not certain whether apoplastic calcium levels are low enough to leave any non-esterified segments outside the junction zone; otherwise it would be necessary for polygalacturonase to act following demethylation by pectinesterases.

In vitro studies using purified polygalacturonase isoenzymes (Chapter 3, Themmen et al., 1982; Crookes and Grierson, 1983; Pressey and Avants, 1983a) have shown that PG1 and PG2 isoenzymes are capable of hydrolysing cell walls and pericarp tissue discs prepared from mature green tomato tissue. Thus processes other than polygalacturonase action are not necessary to explain the softening of fruit during ripening. The evidence from this thesis and of Brady et al. (1982) suggests that it is the rate of accumulation of PG1 that largely determines the rate of fruit softening during early ripening.

The effect polygalacturonase activity has on tomato fruit of different cultivars and species is also influenced by fruit and cell wall structure as discussed in Chapter 3. In this context, the calcium content of the cell wall appears to play an important role
in regulating the amount of fruit softening (Wills and Rigney, 1979; Brady pers. comm.).

As fruit ripening progresses so the middle lamella becomes completely degraded (Crookes and Grierson, 1983). According to Carpita et al. (1979), the large molecular size of PG1 precludes its entry into the cell wall. This would explain the in vitro differences between the effectiveness of PG1 and PG2 against cell wall material and pericarp tissue discs observed in Chapter 3. The PG2 molecules are small enough to pass freely through the pores of cell walls thus able to readily gain access to their pectic substrates within the cell wall, whilst the action of PG1 is confined to the outer regions of the wall and middle lamella.

7.1.3. The role of PG2 isoenzymes in wall degradation and fruit softening

As tomato cells ripen the cell walls become extensively degraded and the fibrils of cellulose show signs of becoming less organised (Crookes and Grierson, 1983). Hobson et al. (1983b) found that while applied polygalacturonase (mainly PG2) could release protein from mature green cell walls, during ripening proteins are not released until late in the ripening process. Most proteins are located in the wall, either as structural extensin or an enzymic proteins such as peroxidases. Thus the enzyme releasing these proteins must be acting on the cell wall itself. This could point to a specific role for PG2.

Texture, as discussed by Williams and Knee (1980), includes the ease with which cells can be split open and the ease with which
they can be separated. The former contributes mostly to fruit
juiciness and consumer appeal, the latter to fruit softening. Loss of
fruit firmness did not correlate well with loss of cell wall material
during ripening (Chapter 3), and the results suggest that some wall
degradation did not contribute to softening. However, loss of wall
material was associated with polygalacturonase activity. Thus, it
may be assumed that some of the effects of polygalacturonase on
degradation of the cell wall pectins is not associated with a significant
loss of fruit firmness.

PG1, by its action on the middle lamella, may cause cell
separation and thus marked fruit softening, whilst PG2 acting mainly
on the cell wall contributes to cell splitting and fruit texture
changes rather than further significant losses in fruit firmness.

It is noteworthy that fruit which contain only PG1 (e.g. non-
and slow-ripening mutants), though they soften, do not have the
juicy melting texture that normally ripening fruit have. This could
be explained by the fact that these fruit, having only PG1, undergo
less extensive cell wall degradation during ripening than fruit
containing PG2. In peaches, low levels of polygalacturonase and
pectinesterase are associated with mealiness and dry texture
(Ben-Arie and Sonego, 1980).

In conclusion, the role of polygalacturonase isoenzymes in
fruit softening and wall degradation is suggested as follows. The
early accumulation of PG1 is associated with weakening of the network
of pectic polymers in the middle lamella layer, leading to loss of
intercellular cohesion and thus slippage of cells against one another
rendering the whole tissue less firm. Other processes both enzymic
(e.g., β-galactosidase and cellulase) and non-enzymic (e.g., pH and calcium changes) may accompany this action of PG1. Because of its large molecular size, PG1 activity is limited to the middle lamella and outer cell wall regions. Thus PG1 accumulation ensures that some fruit softening occurs and may be important to fruit ripening by causing the release of calcium ions and/or other metabolites from the middle lamella layer. These may in turn affect other ripening processes. However, the limited ability of PG1 in attacking cell walls prevents loss of cell wall integrity from occurring too early in ripening. Such an event could alter membrane permeability and ionic fluxes across the plasmamembrane thus disrupting the highly regulated processes which take place in the cytoplasm during ripening.

When many ripening changes, such as starch hydrolysis, pigment synthesis and volatile production are well underway, fruit can be given a melting texture by more extensive wall degradation, so that they become more appealing as food.

It is hypothesised that the smaller PG2 isoenzymes rapidly attack not only the remains of the middle lamella, but also the pectin matrix of the cell wall. This changes the pericarp tissue texture from one that is mealy, as found in mutant hybrids, to one that is juicy and melting. It may also lead to loss of membrane integrity that is observed to occur late in fruit ripening (Vickery and Bruinsma, 1973). Since cell separation is complete by the time PG2 isoenzymes appear, their action on the cell wall does not influence tissue firmness as much as the action of PG1 on the middle lamella did early in ripening. If the fruit is not consumed by animals as it becomes ripe, the action of PG2 on the cell wall eventually destroys the wall
integrity, causing the whole fruit structure to collapse. At this stage the fruit become vulnerable to pathogenic attack and seed dispersal is ensured by the rotting of fruit tissue.

Thus tomato fruit may produce isoenzymes of polygalacturonase to regulate cell wall degradation during ripening. What factors determine the appearance of PG2 isoenzymes are unknown. Generally they appear at or soon after the orange-green stage of fruit ripeness (Chapter 3, Tucker et al., 1980; Ali, 1983). It is known that all three isoenzymes of polygalacturonase are the products of the same gene, since their protein subunits are very similar (Grierson and Tucker, 1983). However, the appearance of PG2 is not due to the splitting of PG1 (Grierson et al., 1981) since purified samples of the two isoenzymes were found to have similar specific activities. No evidence for the breakdown of PG1 to PG2 has been observed in any experiments so far. Thus controls must be exerted at the level of enzyme synthesis and assembly, in which case growth regulators such as ethylene and auxins may affect polygalacturonase activity, not so much by determining the total production of the enzyme, but by regulating PG1 accumulation and initiating PG2 production.

7.2. The role of polygalacturonase in tomato fruit ripening

The sequence of metabolic events in normal ripening tomato cultivars follows a highly co-ordinated path. Ripening is associated with a rise in fruit respiration and ethylene production (McGlennon, 1970) accompanied by the production of polygalacturonase and lycopene and changes in flavour, as discussed in Chapter 1. These events are largely brought about by alterations in enzyme activities due to either
synthesis, activation or transfer of protein across membranes (Sacher, 1973; Mattoo and Vickery, 1977). Both ethylene and polygalacturonase could contribute to the integrity and permeability of membranes (Solomos and La ties, 1973; Poovaiah and Mukaya, 1979).

Although ethylene has long been regarded as a growth regulator intimately involved in the initiation of fruit ripening (Lieberman, 1979), a suggestion was made that polygalacturonase might also be involved with these events. This idea was based on studies of mutant non-ripening tomatoes. Tigchelaar et al. (1978a) concluded that the failure of these fruit to produce more than trace levels of ethylene is a secondary effect of the mutant genes, and is due to inhibition of an event which precedes ethylene and respiratory rises. Since the main effect of the mutant genes is to block the synthesis of polygalacturonase this enzyme was seen to play a vital role in predisposing the fruit to ripen.

This idea was substantiated by the detection of polygalacturonase activity in tomato fruit six days prior to the respiratory climacteric (Poovaiah and Mukaya, 1979). These authors also observed that applied ethylene did not stimulate polygalacturonase production, and concluded that ethylene evolution, and lycopene synthesis do not occur in the absence of polygalacturonase activity.

Further support of a role for polygalacturonase in initiation of ripening comes from studies of "blotchy ripening" disorder of tomatoes. In affected tissue, ripening is retarded despite the fact that the diseased tissue is adjacent to healthy and normally ripening tissue which produces ethylene. Polygalacturonase activity in affected tissue is very low, and this is seen to be a factor contributing to
the inhibition of ripening in this tissue (Hobson, 1964). In addition, infiltrating unripe tomatoes with silver thiosulphate stimulates ethylene production but inhibits fruit polygalacturonase and lycopene synthesis (Hobson et al., 1984).

However, many experiments have produced results which are inconsistent with the hypothesis that polygalacturonase may initiate ripening. The results of Chapters 3 and 4 and of Sawamura et al. (1978) and Brady et al. (1982) show that ethylene precedes polygalacturonase activity and fruit colour development. Sawamura et al. (1978) also found that applied ethylene accelerated polygalacturonase formation and cell wall breakdown in tomatoes. Recent experiments have shown that an increase in ethylene synthesis precedes not only the appearance of polygalacturonase protein and activity by at least 20 hours, but also an increase in mRNA coding for the polygalacturonase polypeptides (Grierson and Tucker, 1983; Grierson et al., 1984; Grierson and Slater, 1984). It has also been implicated in ripening-related changes in tRNA of tomatoes (Nettler and Romani, 1976).

Ethylene stimulation of mRNA's has also been reported to occur in ripening avocados (Christofferson et al., 1982), and prior to senescence of mung beans (Grierson et al., 1982). Saltveit and McFeeters (1980) observed that in the cucumber, ethylene production also preceded a marked increase in polygalacturonase activity and loss in chlorophyll.

In addition, treatments such as low oxygen or hypobaric storage of tomatoes under which ethylene production is severely reduced inhibited the formation of polygalacturonase and lycopene synthesis (Goodenough et al., 1982; Jeffrey et al., 1984). Infiltration of
unripe tomato fruit with silver ions, stimulates ethylene synthesis but blocks the sites at which it is bound to fruit tissue preventing its action. This treatment results in inhibition of both polygalacturonase and lycopene synthesis (Hobson et al., 1984). Whether ethylene initiates these ripening events or merely accelerates the rate at which they occur is debatable, but little or no polygalacturonase synthesis appears to take place until autocatalytic ethylene production has been initiated in fruit (Hobson et al., 1984).

Not all ripening events are dependent on ethylene production. Jeffery et al. (1984) have defined ethylene-independent and ethylene-dependent biochemical changes in ripening tomatoes. Ethylene appears to have little effect on changes in sugars and acids during tomato ripening, nor do these changes rely on polygalacturonase-induced wall degradation. In contrast, fruit polygalacturonase activity and lycopene synthesis were apparently inhibited when ethylene was removed from the storage atmospheres and enhanced when fruit ethylene synthesis was allowed to resume. They suggested that ethylene-independent ripening events were regulated by pre-existing enzymes, whilst ethylene-dependent processes required transcription and translation of enzymic proteins. Some aspects of ripening can therefore proceed independently of others and they do not require the same conditions to be manifest. Thus, there may be more than one type of control mechanism operating during ripening, although there may be a single initiating event. Whether the latter is due to ethylene has to be confirmed. However, despite the close association between ethylene and polygalacturonase synthesis, there is some doubt that ethylene directly induces its synthesis (Grierson et al., 1984).
That other factors may be involved in the initiation of ripening is suggested by the following results. Treatment of tomato fruit with either ethrel or propylene, an analogue of ethylene, advanced the ripening events but did not alter the sequence in which they occurred (McGlasson and Adato, 1977). Tigchelaar et al. (1978b) also reported that treatment of nor fruits with ethylene caused an increase in respiration rate and onset of colour changes, but normal ripening did not occur and polygalacturonase was not induced. Studies of wild tomato species (Chapter 5) show that polygalacturonase activity is not always associated with a persistent increase in ethylene production and vice versa.

Likewise, salt and ethrel treatment of non-ripening mutant fruit (Chapter 6) showed that increased ethylene production alone could not account for the changes in colour observed and did not stimulate polygalacturonase production. Salt treatment must have stimulated the production of metabolites which enabled the tissue to respond to ethylene. However, salt treatments did not markedly enhance polygalacturonase activity as they did colour production. Thus other intermediate steps between ethylene action and the induction of polygalacturonase must have remained blocked.

A model for ripening has been suggested by Brady et al. (1982) that places intermediate events between ethylene action and polygalacturonase induction, and allows that the completion of ripening, including full colour development, is dependent on polygalacturonase action. This hypothesis seems to fit the results discussed here,
Thus although ethylene may stimulate protein synthesis and ripening processes, it appears that other metabolites may be involved with both the initiation of ripening events and the sequence of events leading from ethylene binding to polygalacturonase and lycopene synthesis.

There is increasing evidence that tissue sensitivity to ethylene is more important to the initiation of ripening than actual levels of ethylene production by fruit (Chapter 1, Trewavas, 1981). Tomatoes show a great resistance to ethylene during maturation indicating the presence of ethylene antagonists in fruit tissue (Lyons and Pratt, 1964; McGlasson and Adato, 1977). In addition, tomato ripening is enhanced by detachment of fruits from the plant (Chapter 3 and 4; McGlasson and Adato, 1977). This points to the involvement of substances contributed by the plant that inhibit ripening e.g. auxins, cytokinins and gibberellins. Evidence to support this comes from the fact that the threshold levels of ethylene required to stimulate ripening are higher in attached than detached fruits. Ethylene production is dependent on the induction of enzymes, in particular, ACC synthase. Auxin and other growth regulators may exert their effect by stimulating the synthesis or degradation of these ethylene forming enzymes or by regulating the activity of the ethylene forming system (McGlasson et al., 1978).

Likewise, gibberellins are known to retard senescence and thus antagonise the effects of ethylene. Colour changes are particularly susceptible to the influence of gibberellins. Treatment of tomato fruit with gibberellins delays both loss of chlorophyll and increase in carotenoids. Both processes are involved in the change of chloroplasts to chromoplasts. Gibberellin treatment caused
retardation of tomato polygalacturonase activity and softening (Babbitt et al., 1973). Ethylene was antagonistic to the effects of gibberellin on tomato colouring and softening. In the tomato gibberellin did not affect the onset of colour (Babbitt et al., 1973), or the increase in softening, but retarded the rate of development of these processes. McGlasson et al. (1978) suggest that it is unlikely that gibberellins regulate ethylene production and the initiation of ripening (Dilley, 1969). But they may interfere indirectly by retaining the tissue in a relatively juvenile state. Liberman et al. (1977) suggested that cytokinins, rather than auxins, influenced fruit ethylene production.

Metabolites other than growth regulators could induce ripening. Roberts and Osborne (1981) found that ethylene production of fruit was not under the control of auxins, but possibly regulated by the increase, or release of, membrane-confined cofactors of ethylene biosynthesis. If cofactors are compartmentalised within a membrane-bound organelle, it follows that any treatment regulating membrane integrity would affect the cofactor release and hence ethylene production.

Mukherjee and Choudhuri (1981) observed that water stress increased activity of oxidative enzymes in Vigna seedlings. IAA oxidase, peroxidase and glycolate oxidase were all stimulated. Thus, the ripening effect of salt treatments on tomatoes could be due to stimulation of oxidative metabolism, which as discussed in Chapter 1, may be involved in regulation of many ripening processes (Frenkel, 1972, 1975 and 1978). Such stimulation of peroxide levels in the cell wall could also account for some wall degradation due to peroxidation of pectins independent of enzymic activity (Kertesz, 1943).
They may also be involved in regulating those ripening processes that Jeffery et al. (1984) determined were not regulated by ethylene.

Whilst ethylene, in combination with other growth regulators and/or oxidative metabolism, initiates and stimulates ripening processes in tomatoes, it is doubtful that it regulates directly the rate of subsequent ripening processes. Ethylene production by slow-ripening mutant hybrid tomatoes and some wild Lycopersicon species did not reflect their colour development or polygalacturonase activity (Chapters 4, 5 and 6). A closer relationship between colour development and polygalacturonase activity was observed in this work than between ethylene production and rates of fruit ripening (Chapters 3, 4 and 5).

In mutant hybrid lines reduced polygalacturonase activity was associated with low lycopene levels (Chapter 4). Brady et al. (1982) suggest that whilst polygalacturonase is not involved with initiating ripening, it may have a catalytic role in subsequent processes. The activity of polygalacturonase may release substances from cell walls, which lead to further increases in ethylene and to other intracellular responses of ripening, including chloroplast to chromoplast transitions.

There are several mechanisms by which polygalacturonase could influence other ripening events. Polygalacturonase may produce its effect on ripening parameters by causing the release from cell walls of proteins. That the enzyme is capable of doing so has been shown by Strand et al. (1976) and Hobson et al. (1983b). Some of these wall-bound proteins have enzymic activity (Strand et al., 1976;
Kaur-Sawhney et al., 1981; Hobson et al., 1983b). In particular, wall-located peroxidases may be involved in ripening by their effects on oxidation of auxins and free-radical associated ethylene production (Frenkel, 1975; Legge and Thompson, 1983). However, Hobson et al. (1983b) observed that no significant in vivo release of proteins occurred until ripening was well advanced. Thus they are unable to influence cytoplasmic processes until late in ripening.

Other wall-located substances may affect ripening. Albersheim et al. (1983) have identified oligosaccharides from plant cell walls that act as regulatory molecules, these could be released by polygalacturonase.

Degradation of the middle lamella by PG1 may also release calcium from the cell wall, thereby increasing cytoplasmic levels of this ion. Calcium regulates many metabolic processes, including respiration at low calcium concentrations (Wills and Rigney, 1979). Rigney and Wills (1981) demonstrated a drop in wall calcium levels during fruit ripening.

Mattoo and Vickery (1977) postulated that changes in the degree to which enzymes are bound to membranes comprise one of the mechanisms by which enzyme activities are controlled during ripening. Polygalacturonase could influence membrane integrity by weakening the cell wall structure or altering the extracellular environment such that fluxes of metabolites across membranes are altered.

However, the experiments on the effect of salt treatment of non-ripening mutant tomato fruit show that under some circumstances colour development of fruit may proceed to levels similar to those of
Fig. 7.1. A model of the roles of ethylene \((\text{C}_2\text{H}_4)\) and polygalacturonase (PG) in tomato fruit ripening

- Direct effects
- Possible indirect effect
- Site of inhibition

\[\text{ACC} \xrightarrow{\text{Oxygen}} \text{C}_2\text{H}_4 \rightarrow \text{Silver ions} \rightarrow \text{Increase in C}_2\text{H}_4 \text{ attached to receptor site} \rightarrow \text{metabolism}

Change in tissue sensitivity to \(\text{C}_2\text{H}_4\)
(Altered by fruit maturity and salt treatments)

Inhibited by some genotypes

Chloroplast Activation of mRNA's

FRUIT Accumulation PG1

RIPENING PG2 Accumulation Pectinesterase,

IN OTHER wall-bound enzymes. Changes in calcium and pH in intercellular environment.

SOFTENING OF Middle lamella dissolution

PERICARP

TISSUE
normal ripening fruit with only minimal polygalacturonase activity. In *L. cheesmanii* fruit polygalacturonase levels were negligible but some carotenoids were produced. Conversely, *L. chmielewskii* fruit contained high polygalacturonase activity but remained green. Thus there probably exist many intermediate events between the action of polygalacturonase on cell wall metabolism and its effects on cytoplasmic processes. However, this does not preclude the existence of a close interaction between polygalacturonase and some ripening parameters, such as colour, under normal ripening conditions.

In conclusion, it appears that the normal ripening process in tomatoes is dependent on high levels of both ethylene and polygalacturonase production to initiate and regulate the complex series of events that constitute fruit ripening (Fig. 7.1). In particular, fruit colour production is seen to be closely regulated by an interaction between ethylene and polygalacturonase activities. However, these reactions are influenced by other metabolites and/or physiological processes, the nature of which has yet to be elucidated.
REFERENCES


ALBERSHEIM, P., DARVILL, A. G., McNEIL, M., VALENT, B. S., SHARP, J. S.,
NOTHMEIL, E. A., DAVIS, K. R., YAMAZAKI, N., COLLIN, D. J.,
YORK, W. S., DULMAN, W. F., DARVILL, J. E. AND DELL, A.
(1983). Oligosaccharides: Naturally occurring
carbohydrates with biological regulatory functions. In
"Structure and Function of Plant Genomes". Ciferri, O.
pp. 293-312.

at the electron microscope level. Amer. J. Botany 50,
732-745.

Stained pectin as seen in the electron microscope.

Macquarie University, New South Wales, Australia.

of the polygalacturonases of tomato fruits. Aust. J.
Plant Physiol. 9, 155-169.

USDA Visual Aid TM-L-1. The John Henry Co., Lancing,
Michigan, USA.

ANON. (1977). Tomato colour chart. Agricultural Training Board,
Horsham, West Sussex.


the non-ripening tomato mutant nor. Physiol. Plant. 52, 213-217.


of isoenzymes in fruits of a normal cultivar of tomato
and of the rin mutant at two stages of development.
Plant Physiol. 60, 496-498.

Factors influencing the quality of tomato products.
1. Surface localized pectic enzymes inactivated by
blanching. Food Tech. 6, 197-199.

heat-resistant pectolytic factor from tomatoes.
Arch. Biochem. Biophys. 17, 197-199.

ripened unripe fruit. Food Res. 12, 530-534.

of Fruits and their Products" Vol. 1. Hulme, A. C. (Ed.).

of abscisic acid in fruits of normal and Nr, rin and nor
mutant tomatoes during growth, maturation and senescence.


Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. Phytochem. 
(In press.)

Plant Physiol. 52, 759-763.

Proc. West Virginia Acad. Sci. 43, 141-145.


HortSci. 2, 33-35.


The sequence of events collectively known as ripening is the single most dramatic event in the life of climacteric fruit. It is quite clear that in the tomato, ribonucleic acid (1) and protein synthesis (see 2) are involved in the process, and a great deal of study has gone into an elucidation of the sequence of changes, especially in terms of precise alterations in enzymic components of various fruits (3-7). While specific proteins play a part in promoting ripening, it is possible that increased turnover, activation and transfer of proteins across membranes can best explain the process, possibly with ethylene being concerned with the initiation and co-ordination of many of the separate events. Some of the more obvious and easily followed changes are illustrated in Figure 1.

FIGURE 1. The rate of change of four parameters as mature green tomato fruit ripen. The rise in ethylene precedes that for the enzyme polygalacturonase (PG) by 24-48 h (8, 9).

How can we use the techniques at our disposal to understand more about the role of ethylene in the ripening of climacteric fruit such as the tomato, and how can the rate of ethylene synthesis be manipulated...
to give some control over the postharvest life of the fruit?

One of the first events that heralds the onset of ripening is an increase in ethylene production and respiration from a low steady level. Polygalacturonase (PG), an enzyme that appears to be intimately concerned with texture changes (8) and whose natural substrate is pectic acid, is not active in green tomatoes but is synthesized soon after rises in ethylene are detected (9, 10). The enzyme exists in several multimolecular forms, with component 1 appearing first and having about twice the molecular weight of two more components, known as 2A and 2B (10). There is evidence that these isoenzymes are all variants of the same polypeptide (9). Pectinesterase activity appears not to be limiting throughout ripening, but its action is a pre-requisite for efficient degradation by PG. While induction of PG by ethylene has not been demonstrated, the view can be held that until the autocatalytic phase of ethylene production is seen, little or no PG synthesis takes place. Also an increasing number of cases can be quoted where the ratio of maximum ethylene production to the basal rate is positively correlated with PG activity at full development (see 7).

There is convincing evidence from low-pressure storage experiments with tomatoes that below a critical level of ethylene and oxygen (see 7), where ethylene-binding sites are altered (11, vide infra), or where tissue from parts of fruit is not appropriately conditioned to respond to ethylene through chilling injury or physiological disorder (12), ripening is either inhibited or irreversibly prevented. A number of single gene mutations

![CONTROL OF RIPENING](image)

**FIGURE 2.** A representation of the inhibition of polygalacturonase (PG) and other enzymes involved in ripening by 'non-ripening' genes or silver thiosulphate (AgTS). The signs $\sim$ and $\times$ indicate mutants such as Spanish Winter, Longkeeper and Alcobaca which may be modifications at the non-ripening locus.
of tomato plants have the effect of preventing ripening from taking place at the usual time for full development (13). Similarly, the introduction of about 1 mmole of silver thiosulphate (AgTS) through the transpiration stream into intact tomatoes interferes with the ability of parts of the walls of the fruit to ripen (14). The situation is illustrated in Figure 2.

Neither the genetic nor the chemical inhibition of ripening can be overcome by exposure to physiological levels of ethylene (see 13). However, attached fruit containing the 'ripening inhibitor' (rin) gene can be partly ripened by exposure to excess ethylene (15), while detached fruit of this line require both ethylene and oxygen (16). Following the work of Mizrahi and his group (17), we have confirmed that salt stress can overcome to some extent the block to ripening by some of the mutant genes, especially in combination with application of an ethylene-producing chemical. Typical effects on PG activity, colour, ethylene evolution and firmness of the fruit are shown in Table 1. The stimulation of ripening

<table>
<thead>
<tr>
<th>Genotype and treatment</th>
<th>Units of PG</th>
<th>Tomato colour index</th>
<th>Ethylene (nl g⁻¹ h⁻¹)</th>
<th>Firmness index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcobaca (non-isogenic with cv. Ailsa Craig)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48.4</td>
<td>-15.51</td>
<td>47</td>
<td>9.09</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>60.5</td>
<td>15.51</td>
<td>97</td>
<td>6.29</td>
</tr>
<tr>
<td>0.5% NaCl + 'ethrel'</td>
<td>51.2</td>
<td>16.57</td>
<td>288</td>
<td>5.68</td>
</tr>
<tr>
<td>Spanish Winter (non-isogenic with cv. Ailsa Craig)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>114.4</td>
<td>7.61</td>
<td>337</td>
<td>4.65</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>214.8</td>
<td>43.69</td>
<td>511</td>
<td>3.77</td>
</tr>
<tr>
<td>0.5% NaCl + 'ethrel'</td>
<td>184.7</td>
<td>41.19</td>
<td>404</td>
<td>3.57</td>
</tr>
<tr>
<td>Non-ripening nor nor (isogenic with cv. Ailsa Craig)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.4</td>
<td>-14.91</td>
<td>-</td>
<td>6.75</td>
</tr>
<tr>
<td>1% NaCl</td>
<td>52.9</td>
<td>15.01</td>
<td>-</td>
<td>4.42</td>
</tr>
<tr>
<td>1% Na₂SO₄</td>
<td>8.2</td>
<td>18.10</td>
<td>-</td>
<td>5.03</td>
</tr>
</tbody>
</table>

^aSee (18); mg galacturonic acid released /h/100 g fresh wt.
^bSee (19)
^cReciprocal of the compression in cm under a load of 1 kg for 5 sec.
is not confined to sodium chloride since several other salts that were taken up by the fruit were also effective. In general, salts affected the colour and firmness much more than either PG or ethylene levels. In Alcobaca and nor nor lines, only PG1 was found; Spanish Winter showed a small amount of PG2A and 2B as well. The very long shelf-life and general resistance to deterioration shown not only by salt-treated non-ripening mutants but also by hybrids containing one or more non-ripening alleles (7, 13) appear to be a reflection of PG activity that is much lower than normal. Ethylene levels are also attenuated, and in one

![Graph](image-url)

**FIGURE 3.** 1-aminocyclopropane-1-carboxylic acid (ACC) levels in cv. Ailsa Craig (++) and in an F₁ hybrid containing the rin allele (rin +) (1977A) instance (Figure 3) has been shown to be matched by reduced levels of ACC (Mordy Atta Aly, unpublished). In summary, the ability of homozygous non-ripening lines to enter the autocatalytic phase of ethylene production is clearly inhibited. In some cases, the block to further development may be overcome but whatever the stimulus used, PG activity and ethylene levels are much below normal.

Of the well-known ethylene antagonists that we have infiltrated into mature green tomato fruit detached from the plant immediately before treatment, 2,5-norbornadiene (20), α-aminooxyacetic acid (21), α-amino-
isobutyric acid (22) and AgTS, the silver salt was by far the most effective in preventing ripening. In further experiments using unpicked green tomatoes, infiltration of 1 μmole of AgTS into the vascular system in part of the peduncle leading to a fruit truss caused a proportion of the outer walls to fail to change colour. The composition and pectic enzyme activities in both the green and red walls of treated fruit (unpublished data) can be compared with normal tissue having similar colour. In essence, the composition of all wall tissue following silver infiltration was abnormal, and was generally similar to that in fruit showing a ripening disorder known as 'blotch' (23). As indicated in Table 2, the green tissue from infiltrated fruit failed to change colour much, and

Table 2. Characteristics of outer locule wall tissue from fruit infiltrated with silver thiosulphate (cv. Sonatine)

<table>
<thead>
<tr>
<th>Source of tissue</th>
<th>Units of PG⁴</th>
<th>Tomato Colour Indexᵇ</th>
<th>Ethylene (nl g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control fruit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature green</td>
<td>0</td>
<td>-24.43</td>
<td>0.4</td>
</tr>
<tr>
<td>Red</td>
<td>6531</td>
<td>46.18</td>
<td>8.6</td>
</tr>
<tr>
<td>Silver-treated fruit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>226</td>
<td>-17.48</td>
<td>4.4</td>
</tr>
<tr>
<td>Red</td>
<td>4753</td>
<td>51.61</td>
<td>11.6</td>
</tr>
</tbody>
</table>

⁴See (18); mg galacturonic acid released/h/100 g fresh wt.  
ᵇSee (19)
although apparently producing adequate amounts of ethylene, less than 5% of the PG activity of red tissue from the same fruit was shown. In order to prevent ripening, the silver treatment must be given prior to the autocatalytic phase of ethylene production.

The green tissue from infiltrated fruit contained particles that were electron-dense when sections were examined by electron microscopy. The deposits were found in phloem cell walls and were particularly associated with the middle lamella. In addition, the particles were observed in the intercellular spaces of parenchyma, bordering the lumen, as is shown in Figure 5.

![FIGURE 5. Particles (arrowed) containing concentrations of silver and sulphur deposited throughout the cell wall of phloem, but more concentrated in the middle lamellae and intercellular space (S), in tomato tissue infiltrated with 20 mM silver thiosulphate. Lower concentrations of the silver complex resulted in particles being found more exclusively in the middle lamellae. Bar mark = 2 \( \mu \text{m} \); unstained.](image)

Figure 5. X-ray microanalysis has confirmed that the particles contained concentrations of silver and, to a lesser extent, sulphur (unpublished data).

Since AgTS infiltration resulted in a failure by the green tissue to synthesize PG in normal quantities, we have attempted to show that the tissue was still capable of making protein. No evidence of silver-containing deposits were found in the cytosol. Preliminary work indicates
that an inducible enzyme such as nitrate reductase was synthesized in green tissue from silver-treated fruit. Nevertheless, conclusive proof must await further tests to show that amino acids can be incorporated into protein in this tissue.

CONCLUSION

Silver is a particularly potent ion in preventing the ripening of tomato tissue. The metal has strong anti-ethylene properties (see 24), and if it binds to those sites that are concerned with increasing sensitivity towards, or with the increased production of the hormone, this could prevent normal ripening through inhibiting the synthesis of PG and other proteins concerned with completion of ripening. A direct effect of silver on the protein synthetic mechanism is not thought likely.

In normal ripening, rising ethylene levels are closely followed by selective protein synthesis, of which the enzyme PG is an important example. Whether PG is induced by ethylene is at present an open question. However, it is clear that the ripening mechanism is extremely sensitive to absolute levels of ethylene and to an activation mechanism for recognition of the hormone. Further investigations into the biochemical causes for natural (e.g., 'blotch', non-ripening mutants) or deliberate interference with the ripening sequence may help to pinpoint the controlling steps in the sequence of changes. A more complete knowledge of the processes involved should diminish spoilage of tomato fruit, and contribute towards economic benefit for both producers and consumers.

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REFERENCES


23. Winsor GW, Massey DM. 1959. The composition of tomato fruit. II. Sap expressed from fruit showing colourless areas in the walls. J. Sci.
FACTORS DETERMINING THE FIRMNESS LOSS BY RIPENING TOMATOES

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The extent to which the activity of polygalacturonase (PG) and the percent loss of cell wall material during ripening determine the loss of firmness by tomato fruit have been studied. Five cvs., Alisa Craig (intrinsically soft), Bellina, Sonatine, Money-maker and Potentate (firm) were grown, and fruit at five standard colour stages were analysed for the above factors. In all these cvs., PG activity increased up to the orange stage of ripeness due to the synthesis of only PG I, except for cv. Sonatine that had a little PG II as well. The firmness loss up to this point depended not so much on total PG activity as on the rate of its synthesis. Firmness changes between the orange and red stages were not closely related to the rate of production of PG IIA and IIB. The rate of PG I production is seen as having overriding importance in determining the firmness of the ripe fruit, and the content of cell wall material is considered as subsidiary to the activity of this enzyme.

THE EFFECT OF SALT-TREATMENT ON THE RIPENING OF SLOW AND 'NON-RIPENING' TOMATO MUTANTS


Four of the 'non-ripening' characters known as Spanish Winter (SW), Longkeeper (L), Alcobaca (A) and non-ripening (nor) are being incorporated into standard tomato cvs. In their present backgrounds, SW and L ripen very slowly, while A and nor are even more extreme. Addition of 500 ml 0.5% NaCl per day was made to the peat-based compost in which the four lines were growing, and the fruit assessed for colour, firmness, polygalacturonase (PG) isoenzymes and ethylene levels. The salt-treatment increased the colour (a/b ratio) and the rate of softening of A and nor, but only the colour of SW and L. No significant increases in PG were observed with any genotype, but PG I often rose in A and nor, while PG IIA and IIB in SW and L fruit became more prominent. Salt-treatment enhanced ethylene output in SW and A, the only ones so far tested. The conclusion is that the farthestance of ripening by salt-treatment of certain non-ripening mutants has been confirmed, but this appears not to be due to an increase in the total PG activity.