

THE EFFECTS OF FOOD PROCESSING ON PLANT
CELL WALLS WITH SPECIAL
REFERENCE TO EXTENSIN

by

Linda G Wilson

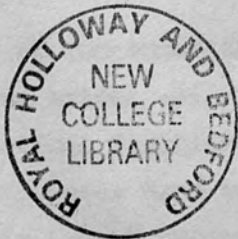
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ABSTRACT

Changes in plant cell wall composition caused by a variety of cooking and manufacturing processes have been investigated. These chemical changes have been compared with corresponding structural modifications which were assessed by light and electron microscopy. The different processing treatments have been applied to a single plant tissue, namely mung bean seedlings, thus enabling comparisons to be made between the processes. Amino acid analysis was employed to assess changes in cell wall composition. The predominant amino acids in extensin, and isodityrosine, the critical cross-linking unit in this glycoprotein, were measured. Changes resulting from separate chemical extraction of pectin and glycoprotein wall components were also examined.

An automated amino acid analysis method for isodityrosine has been established and a novel system for synthesising this dimer developed. The latter involves simple incubation of isolated cell walls with tyrosine, an arrangement which has achieved a six-fold increase in isodityrosine concentration.

A reduction in the wall content of hydroxyproline and isodityrosine was observed in samples which had been stored in sulphite solution. It is suggested that this process may degrade pectin and glycoprotein. This idea is discussed with reference to the wall structure seen by microscopical examinations. The soluble extract resulting from boiling cell walls in dilute bicarbonate solution and the water-soluble pectin fraction were both found to contain significant quantities of isodityrosine. It is proposed that these two treatments may extract extensin glycopeptides or even cross-linked extensin oligomers from the wall.

It is concluded that most of the processes damaged cell wall pectin to some extent while some also affected extensin. Results from the chemical fractionation experiments demonstrated that these two wall components tend to be co-extracted. This observation is discussed in relation to current models of cell wall structure.

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INTRODUCTION

Fruit and vegetables are preserved by freezing and sulphiting and the manufacture of products such as jams and pickles. Whether fresh or preserved, plant foods may be subjected to some type of cooking treatment before consumption. These different processing and cooking treatments change the structure of the plant tissues. The overall objective of this study was to gain a better understanding of the effects of processing on the structure and composition of edible plant materials.

In fresh plant tissue the firm, crisp texture is a result of a combination of strong cell walls, tightly bound junctions between cells and turgor pressure from the cell contents. During processing the cytoplasmic membrane is usually damaged, thus removing the contribution from turgor pressure to tissue rigidity. Hence the cellular structure and texture of processed plant tissue depend mainly on the strength and integrity of the cell walls and on the intercellular binding. Accordingly, this work concentrated on cell walls as a key factor governing the eating properties of processed fruit and vegetables.

In previous studies of the effects of processing on plant structure, different processes have been tested on the plant materials for which they are normally used. For example, the effects of pickling have been investigated using onions (Jewell, 1971) and cauliflowers (Saxton, Jewell & Dakin, 1969), while jam making studies involved strawberries (Jewell, Rantsios & Scholey, 1973). Thus it has been difficult to compare the effects of different treatments at the cellular level. In this work a range of different manufacturing and cooking processes have been tested on one plant material, namely mung bean shoots, to enable direct intercomparisons. Mung bean shoots were selected because they are readily available and have a tissue structure similar to that of the edible parts of most fruit and vegetables. Furthermore, beanshoots can be grown reproducibly in the laboratory as required, thus avoiding possible deterioration and structural changes caused by storage.

The investigation into effects of processing on cell wall physical structure was combined with analysis to assess corresponding changes in the chemical composition. Chemical analysis was specifically directed at detecting modifications of, or damage to, extensin, the glycoprotein component of the cell wall. Previous studies of effects of processing on the chemical composition of the wall have always concentrated on the polysaccharides, particularly pectin; examples include work on apples (Reeve & Leinbach, 1953) and potatoes (Keijbets, Pilnik & Vaal, 1976; Linehan & Hughes, 1969). The special reference to extensin in this work was because this component has been characterised only relatively recently and has not been the subject of such study. Furthermore, this cell wall glycoprotein has some striking compositional similarities to collagen, the major structural protein in animals. The critical role played by collagen in the properties and texture of meat is well known.

The initial practical work was directed at attempting to extract extensin from the cell wall in order to investigate effects of processing on the glycoprotein. However, extraction of extensin was not found to be feasible. The results of these preliminary experiments, and the way in which the experimental approach was subsequently modified, are discussed in detail in Chapter 2. It was decided to study processing changes in extensin as a part of the cell wall as a whole. The two main practical techniques employed were amino acid analysis to detect wall composition changes, and microscopy to assess modifications to the physical structure. These two aspects are described in Chapters 4 and 5, respectively.

Light microscopy was used to give an overall view of large groups of cells, while electron microscopy was used to provide fine detail of cell wall structure. Changes in chemical composition were detected by measuring the amino acids dissolved out of the wall by the various processes. Amino acids remaining in the insoluble wall residue were also measured and the results were compared with the composition of untreated controls. To identify changes in extensin, analyses were made of its characteristic amino acid components, particularly hydroxyproline and the critical cross-link isodityrosine. This necessitated development of an automated method to assay isodityrosine,

an investigation which involved various experiments to synthesise and isolate the dimer. This part of the work is described in Chapter 3.

A variety of manufacturing processes and cooking treatments which are normally used for fruit and vegetables were tested. Examples include storage in sulphite solution, which is used to preserve fruit for jam making; boiling in dilute sodium bicarbonate solution, a method sometimes used in catering scale cooking of green vegetables to preserve the colour; and deep frying.

In order to understand the causes of some of the observed changes in processed cell walls, further wall samples were chemically extracted to remove pectin and glycoprotein components. The amino acid data and microscopy observations from these extraction experiments were then compared with the results from the various process tests.

This approach demonstrated that the water-soluble pectin extract and the bicarbonate-soluble extract both contained significant quantities of isodityrosine. This suggests that large fragments of extensin or, possibly, cross-linked oligomers may have been extracted. Sulphite treatment was found to destroy about half the wall content of isodityrosine and this storage method resulted in a very swollen and finely layered wall structure. The changes observed after sulphiting were similar to those caused by extraction of pectin and glycoprotein from the wall.

Prior to commencing practical experiments a comprehensive survey of the literature concerning extensin was prepared. This survey was updated throughout the course of the work because the subject was developing rapidly, and the resulting extensive review is given in Chapter 1.

1. LITERATURE REVIEW

1.1 Introduction

This review concerns extensin, a glycoprotein which has recently received much attention because it is seen to be an important structural component of the cell wall. Extensin appears to form a network which is separate from, but complementary to, wall cellulose. Of particular interest is the observation that the glycoprotein is synthesised as a soluble monomer which is subsequently polymerised into an insoluble matrix. A new type of interpeptide link, isodityrosine, may be responsible for cross-linking extensin molecules in the cell wall.

In this review, the elucidation of the structure of extensin is traced through the identification of repeating peptide sequences in the backbone, and the type of carbohydrate side chains, to the secondary conformation and mode of cross-linking. The various hypotheses that have been proposed to explain the binding of extensin in the cell wall are described, including the recent "warp and weft" model. In addition, the biosynthetic pathway of extensin, which has mainly been studied by following precursors in carrot disc systems and suspension cultures, is reviewed. Because of its structural role this glycoprotein is thought to be implicated in the control of wall extensibility, and the name "extensin" has been adopted as a result. In addition to these functions, a possible role in defence is discussed. Other glycoproteins which contain hydroxyproline are mentioned in the final section; these include lectins and arabinogalactan proteins, which have, in earlier studies, been confused with extensin.

1.2 Structure of Extensin

1.2.1 Early studies

Lampert and Northcote (1960) presented the first evidence for a protein which was an integral part of the primary cell wall, and which contained virtually all of the hydroxyproline of the cell. In 1965, Lampert put

forward the argument that this hydroxyproline-rich protein must play a structural role in the cell wall, and so must inevitably be involved in cell extension. This idea, later referred to as the extensin hypothesis, led to the hydroxyproline-rich protein being given the name extensin by analogy with the structural proteins collagen and elastin. In 1967, Lamport isolated arabinosyl-hydroxyproline, a new type of carbohydrate-protein link, by partial alkaline hydrolysis of tomato cell walls. He then described extensin as a polypeptide backbone with hydroxyproline residues involved in O-glycosidic links to short arabinosides. It was pointed out that these arabinose oligosaccharides might serve as attachments for other wall polysaccharides, enabling a small amount of extensin to cross-link a "hugely disproportionate" amount of wall polysaccharide. Low concentrations of hydroxyproline residues could thus play an important part in determining the properties of the primary cell wall.

Further work on walls of cultured tomato cells, employing enzymic degradation, confirmed the presence of hydroxyproline arabinosides, and demonstrated that galactose was an additional sugar component of the glycoprotein. The major amino acids of these enzymically produced glycopeptides were hydroxyproline, serine, lysine and tyrosine (Lamport, 1969). In discussion of the short arabinose side-chains, Lamport (1970) put forward an explanation for their small size of only four or fewer sugar residues. He suggested that they might represent the beginning of much larger polysaccharide chains, which were alkali-sensitive for some reason and therefore not recovered after the alkaline treatment used to obtain the hydroxyproline arabinosides. Lamport and Miller (1971) later demonstrated that the arabinosyl-hydroxyproline linkage is widely distributed in the plant kingdom.

A second amino acid-sugar link was subsequently identified by Lamport, Katona and Roerig (1973), who showed that galactose was attached O-glycosidically to serine residues. Galactosyl-serine was identified in glycopeptides prepared from walls of cultured tomato cells by acid hydrolysis to remove the arabinosides, followed by trypsin digestion. The backbone of one of these tryptic peptides was studied, and found to

contain the sequence serine-(hydroxyproline)₄. This finding led to the proposal of the structure shown in Fig. 1 for one of the glycopeptides.

Later sequencing of the other tryptic peptides revealed that each contained at least one unit of the same peptide, namely serine-(hydroxyproline)₄ (Lampport, 1973). In discussing these findings, Lampport (1973) considered it was most likely that the major polysaccharide attachment to extensin was via serine. A possible function of the hydroxyproline arabinosides would be to stabilise the hydroxyproline-rich polypeptide backbone as a rigid rod.

Smith (1981b) has subsequently made use of this characteristic pentapeptide and has raised antibodies which recognise the sequence serine-(proline)₄ in precursors of cell wall protein which have not undergone hydroxylation of proline to hydroxyproline.

O'Neill and Selvendran (1980) studied the hydroxyproline-rich glycoprotein in cell walls of runner bean tissues, and their findings are a useful addition to the available information, which is mainly based on work with cultured cells. They employed a chemical extraction procedure with acidified chlorite, to solubilise the glycoprotein from the walls. This glycoprotein fraction, which constituted about 7% of the cell wall had hydroxyproline and serine as the most abundant amino acids. Most of the hydroxyproline residues were substituted with tri- or tetra-arabinosides, while single galactose residues were alpha-linked to some of the serine groups.

1.2.2 Carbohydrate side chains

Lampport, Katona and Roerig (1973) and Lampport (1973) developed a technique for estimating the degree of serine glycosylation in the wall, based on the finding that glycosylated serine residues were degraded when treated with hydrazine, whereas serine residues free of sugars were stable. They reported results of preliminary experiments which showed that there was a decrease in sugar-free serine residues in the cell wall with increasing age of the suspension culture. They discussed how this increase in glycosylation was consistent with the extensin hypothesis

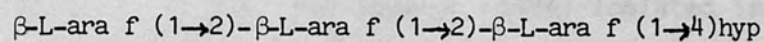
which called for increased cross-linking as cell extension decreased (Lamport, 1965). Esquerre-Tugayé and Lamport (1979), after a study of the glycosylation patterns of the hydroxyproline-rich glycoprotein which accumulates in the cell wall of infected plants, suggested more specific functions for the carbohydrate side chains. They observed that the extent of arabinosylation of hydroxyproline was higher in infected plants. In contrast, the extent of serine glycosylation did not vary significantly upon infection, but decreased with age in healthy as well as in infected plants. This led them to suggest that some of the galactosylated serine residues could provide temporary links for orienting other wall polymers, while the arabinosides attached to hydroxyproline could have a different role. This role may, perhaps, include an involvement in the disease response since the arabinose residues were known to protect the wall glycoprotein against proteolysis. In particular, proteolytic enzymes had been shown to be ineffective without prior deglycosylation of the hydroxyproline residues. The extent of arabinosylation of hydroxyproline is also reported to change during growth. Van Holst *et al.* (1980b), working on bean seedlings, observed that the degree of arabinosylation of wall-bound hydroxyproline increased when the rate of cell elongation decreased.

The structure of the tri- and tetra-arabinosides (Fig.2) has been elucidated by Akiyama and Kato (1976, 1977) and Akiyama, Mori and Kato (1980), using extracts from suspension-cultured tobacco cells.

1.2.3 Cross-links

As a result of the early studies on the structure of extensin, it was generally thought that the glycoprotein was held in the cell wall by bonding to the other polysaccharides. The most likely links were via the carbohydrate side chains of extensin. However, the identification of a component which could act as an interpeptide cross-link resulted in major changes in the ideas of how extensin was fixed in the wall. This led to the now generally accepted view that extensin may be cross-linked to itself by bonds between the peptide backbones, and is entangled with, but not covalently attached to, the cell wall polysaccharides. The evidence for this model is discussed in detail in Section 1.3.3.

a) trimer



b) tetramer

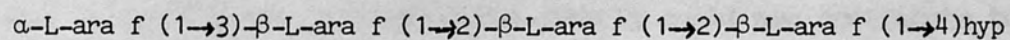


Fig.2 Structure of hydroxyproline-arabinoside (Akiyama et al, 1980)

(ara: arabinose, hyp: hydroxyproline, f: furanose)

In earlier reports, Lamport (1973, 1977) had mentioned that there was an unknown tyrosine derivative in extensin tryptic peptides from cultured tomato cells. The significance of this was not clear until Fry (1982) isolated a tyrosine dimer which he called isodityrosine. He showed that the two tyrosine units were linked by a diphenyl ether bridge, and he proposed a structure for isodityrosine, which is given in Fig. 3.

Additional evidence for the role of isodityrosine in cross-linking extensin in the wall was provided by Cooper and Varner (1983). Working with the carrot disc system (see Section 1.4.1) they showed that extensin is secreted from the cytoplasm as a soluble monomer, and that isodityrosine is formed from tyrosine as the glycoprotein is insolubilised in the wall.

Interestingly, Epstein and Lamport (1984) isolated isodityrosine from two tryptic peptides of cultured cell walls, and showed that the tyrosine dimers were actually intramolecular cross-links forming small closed loops in single extensin molecules. The isodityrosine units were typically found in sequences of tyrosine-lysine-tyrosine. Although intramolecular isodityrosine clearly could not contribute to extensin-wall binding it is now becoming apparent that isodityrosine exists in both inter- and intramolecular forms, which each have their own functions.

Stafstrom and Staehelin (1986a) have investigated cross-linking patterns in extensin by studying oligomers that can be extracted from carrot cell walls by salt elution. These small complexes are intermediates between secreted monomers and the extensively cross-linked insoluble matrix. They observed that the predominant salt-extractable fraction contained 5.3 half-residues of isodityrosine per molecule. Because this fraction is rich in monomers, i.e. has not undergone extensive cross-linking, it is reasonable to suppose that most of the isodityrosine residues are intramolecular. Stafstrom and Staehelin suggest that the presence of such links within molecules could usefully prevent formation of too many intermolecular links, and thus control the size of the 'holes' in the extensin matrix. This view carries implications for a current model of cell wall structure (Fig.4) discussed in Section 1.3.4. In general

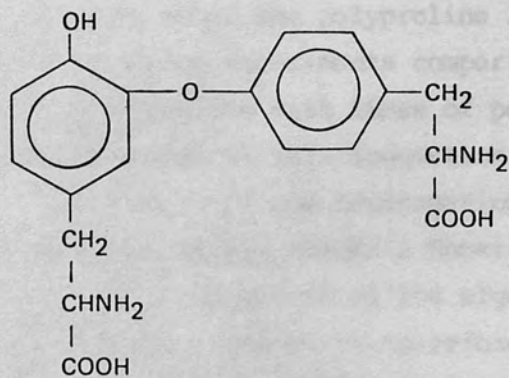


Fig.3 Structure of isodityrosine (Fry, 1982)

agreement, Fry (1983) had calculated, based on some assumptions, that there are about 9 half-isodityrosine residues per extensin precursor molecule. Stafstrom and Staehelin go on to estimate that an additional 2.5 isodityrosine cross-links could be formed intermolecularly as extensin is rendered insoluble. Of particular interest, they have observed, using electron microscopy, that cross-links in extensin oligomers are preferentially formed near the ends of molecules. Such selective positioning of the limited number of intermolecular links would form a relatively open network.

1.2.4 Secondary structure

Lampport (1977) discussed the possible secondary structure of extensin and suggested that, as extensin peptides are so rich in hydroxyproline, the polypeptide might adopt the polyproline II helix conformation. He reported that preliminary experiments comparing circular dichroism spectra of extensin peptides with those of polyproline and polyhydroxyproline supported this suggestion. A polyproline II helix was later demonstrated to be the conformation of the cell wall glycoprotein in Chlamydomonas (Homer & Roberts, 1979). This glycoprotein, the major component of the algal cell wall, has some similarities to extensin, and it is therefore used as a convenient system to study (see Section 1.3.3).

Lampport (1980) used molecular models to describe how the hydroxyproline-arabinosides could fit into this helical secondary structure. The polyproline II helix is a left-handed coil with three residues per turn and a pitch of 0.94 nm. Lampport shows beta-linked tetra-arabinosides folded back and nesting with the helix. He further proposes that three hydrogen-bonds can be formed between specific arabinose residues and carbonyl groups of the peptide backbone. This arrangement might indeed lead to a strong, stable, rod-like molecule. However, our attempt to build a model incorporating these hydrogen-bonds indicates that the configuration is not achievable (see Plate 1). Nevertheless, it is still reasonable to suppose that the arabinosides do lie parallel to the peptide backbone and stabilise the helix. Further investigations into the effects of the carbohydrate side chains on extensin structure are discussed in the next section.

**Plate 1 Molecular model of polyhydroxyproline helix with
tetra-arabinoside side chain**

Key

Atom centre colours:

hydrogen - white; carbon - black; oxygen - red; nitrogen - blue.

Bond colours:

polyhydroxyproline - grey; arabinoside - red;
hydrogen bonds (1, 2, 3) - white.

Hyp: hydroxyproline.

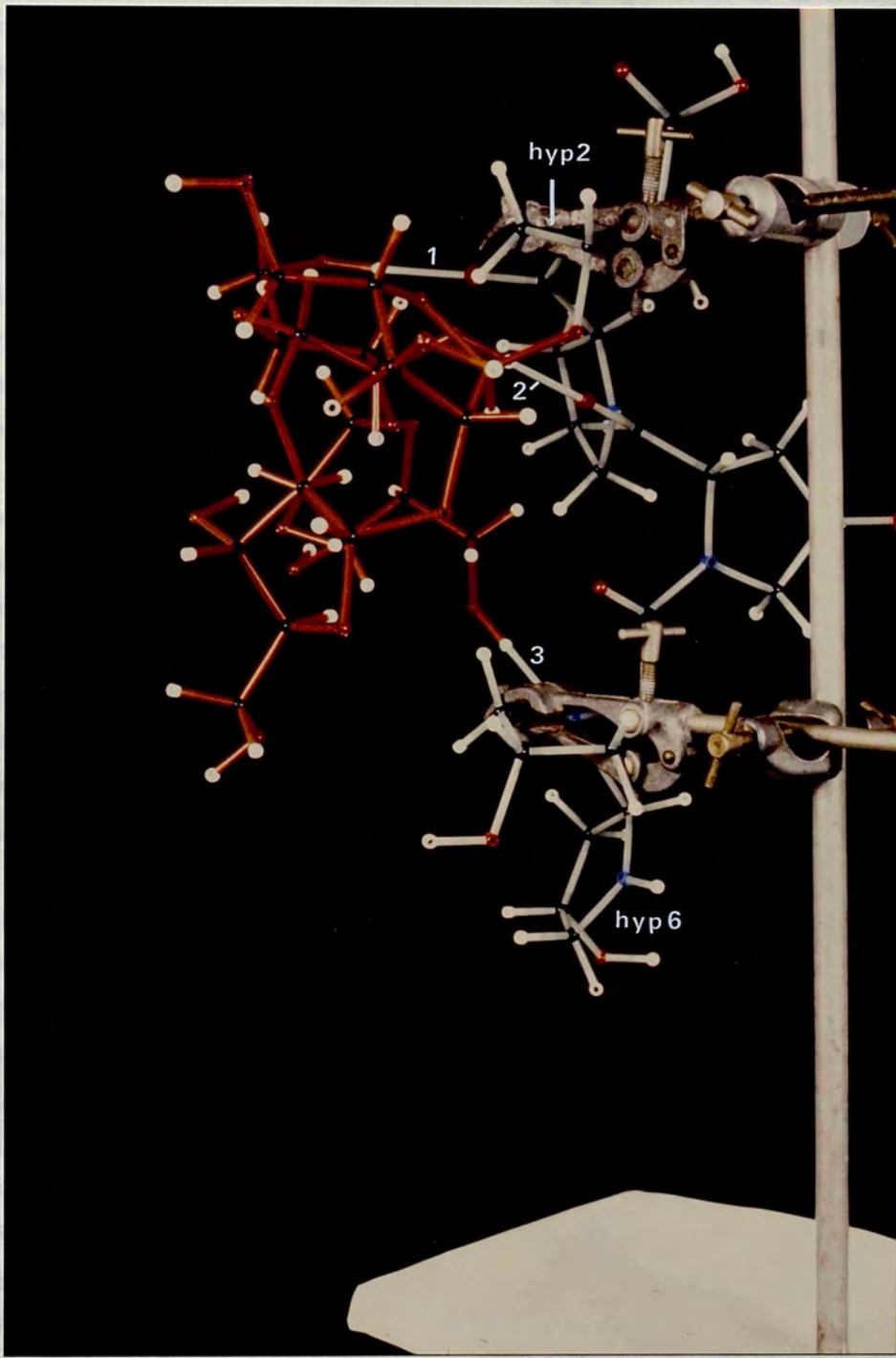
The model comprises a six-unit polyhydroxyproline helix with a tetra-arabinoside attached to hydroxyproline number 2 and hydrogen bonded to carbonyl group numbers 2, 3 and 5. It was constructed according to the drawing of Lamport (1980). The model demonstrates that the tetra-arabinoside cannot fold back and nest in parallel with the polyhydroxyproline helix as was suggested by Lamport, but instead it forms a contorted, buckled structure.

1.1.3 Hypoxanthine phosphoribosyl transferase

Structural investigations of yeast strains and adaptation cultures have always been limited because of the fact that hypoxanthine is essential. A

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galactose, which is found in certain strains (Günther & Varner, 1961; Cooper & Varner, 1967).

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1.2.5 Studies of extensin precursors

Structural investigations in plant tissues and suspension cultures have always been limited because the cell wall glycoprotein is insoluble. A different approach, involving studies of soluble precursors of extensin has recently provided much more information. Particularly useful have been aerated carrot root discs, which synthesise and secrete large quantities of hydroxyproline-rich glycoprotein (Stuart & Varner, 1980), although studies of extensin precursors have also been carried out using suspension cultures (Smith, Muldoon & Lamport, 1984). As a result of recent work on extensin precursors (Smith, Muldoon & Lamport, 1984; Stafstrom & Staehelin, 1986a), it is becoming apparent that extensin is probably a family of hydroxyproline-rich glycoproteins with similar, but distinct, amino acid compositions.

Much of the carrot glycoprotein can be extracted from the cell wall with salt solutions. It was thought that this glycoprotein was produced in response to wounding, and was not necessarily related to extensin. It has since been demonstrated (Van Holst & Varner, 1984) that the salt-extractable glycoprotein is indistinguishable from a glycoprotein produced in smaller quantities by unwounded carrots. These authors concluded that the glycoprotein is normally present in carrots and accumulates in larger amounts in wounded tissue.

The salt-extractable hydroxyproline-rich glycoprotein from carrot discs has a molecular weight of about 86,000 and consists of two-thirds carbohydrate and one-third protein. Hydroxyproline comprises 45% of the protein and, together with only five other amino acids, namely serine, histidine, tyrosine, lysine and valine, accounts for 95% of the polypeptide backbone. Like insoluble extensin, the predominant sugar is arabinose, which is 97% of the total carbohydrate, and this is attached to hydroxyproline residues as short side chains. Most of the hydroxyproline residues (68%) are glycosylated by tetra-arabinosides, and a further 24% have triarabinosides attached. The other sugar is galactose, which is linked to serine residues (Van Holst & Varner, 1984; Cooper, Chen & Varner, 1984).

A different approach to analysing the structure of extensin precursors has been taken by Chen and Varner (1985a). They have isolated from carrot discs DNA clones that code for peptide sequences characteristic of extensin. They have then identified clones from which the sequence

serine-(proline)₄-threonine-proline-valine-tyrosine-lysine

and the repeating units serine-(proline)₄ and (tyrosine-lysine)₂ are derived (Chen & Varner, 1985a & b). This last sequence is significant as it is reported to be the site of an intramolecular isodityrosine cross-link (Epstein & Lamport, 1984). Serine-(proline)₄ is the non-hydroxylated precursor to serine-(hydroxyproline)₄, a characteristic repeating pentapeptide of extensin which has been described in section 1.2.1. The decapeptide sequence was earlier identified by Lamport (1977) as the corresponding hydroxylated form, in cell wall tryptic peptides from cultured tomato cells.

Other similarities between tomato and carrot extensin precursors are apparent in the light of recent studies by Smith *et al.* (1986). These workers eluted soluble precursors of extensin from the cell wall of intact tomato cell suspensions. They subjected these precursors to trypsin degradation and characterised some of the resulting peptides. The same decapeptide and the short repeating sequences found in carrots were all shown to be present, and a further peptide containing intramolecular isodityrosine was also identified.

Circular dichroism studies of the carrot disc salt-soluble hydroxyproline-rich glycoprotein have shown that it is almost completely in the polyproline II conformation. Of particular interest is the observation that deglycosylation by hydrogen fluoride causes much of the helical secondary structure to be lost (Van Holst & Varner, 1984). The authors conclude that the carbohydrate is essential for the native conformation of the protein backbone. They also suggest that the changes in arabinosylation of hydroxyproline which have been observed for growing and infected tissues, discussed in section 1.2.2, may change the secondary structure of the protein.

Stafstrom and Staehelin (1986b) have used a combination of electron microscopy and gel filtration chromatography to compare the structure of carrot extensin with its deglycosylated equivalent. They demonstrated that the glycoprotein has an extended rod structure, whereas removal of the carbohydrate side-chains causes the molecule to adopt a random coil conformation. The deglycosylated protein is too thin to be resolved by electron microscopy. These authors suggest that, as polyhydroxyproline forms a stable helix, the hydroxyproline-rich areas of extensin probably do not need to be stabilised by arabinosides. Hence these areas may not be much affected by deglycosylation. In contrast, the carbohydrate's stabilising effect may be much more important in the regions which separate the hydroxyproline-rich sequences, to help confine them to a helix conformation. Stafstrom and Staehelin present a model showing how the arabinosides could interact with the protein backbone of extensin to maintain it in an extended conformation consistent with observed dimensions.

Cooper, Chen and Varner (1984) discuss the consequences of extensin existing as a narrow, rod-like molecule with the arabinose side-chains folded parallel and hydrogen-bonded to be the peptide backbone. They point out that the side chains of each amino acid would be exposed so that the serine residues would be available for galactosylation; the lysine and charged histidine groups would be available for interaction with acidic pectins; and the tyrosine residues would be available as cross-linking sites. Smith *et al.* (1986) describe how similar functions could be performed by one particular tomato extensin precursor peptide. They predict that the basic residues in the sequence

valine-lysine-proline-tyrosine-histidine-proline

are in the same plane and would interact with pectin carboxyl groups. These authors further propose that the tyrosine residue lies out of the plane and is an ideal candidate for an intermolecular isodityrosine cross-link.

1.2.6 Amount of extensin in cell walls

Quantitative information about the proportion of the total cell wall which comprises extensin is limited. The data that are available are given in Table 1. The figures show considerable differences between natural plant tissues and cultured cells. In particular, the hydroxyproline contents of suspension-cultured cells are consistently higher than those of plant tissues. Lampert (1965) comments that it almost looks as though the hydroxyproline-rich tissue culture represents a perturbed situation resulting from the removal of cells from the controls presumably present in the organised plants. Halmer and Thorpe (1976), studying tobacco callus culture, observed that friable tissue, i.e. tissue with less intercellular adhesion, has a higher wall hydroxyproline content than compact tissue. They point out that suspension cultures may be viewed as an extreme case of friability, which might explain the high hydroxyproline levels found in these cultures.

1.2.7 Current picture of extensin structure

To conclude this section, the current picture of the structure of extensin is summarised.

Extensin is a glycoprotein with a polypeptide backbone, which constitutes about one-third of the total weight, to which are attached short carbohydrate side chains. The polypeptide is rich in hydroxyproline and also contains a high proportion of serine, lysine, tyrosine, histidine and valine. Repeating short peptide sequences, particularly the pentapeptide serine-(hydroxyproline)₄ occur throughout the protein. An interpeptide link, isodityrosine, is formed between pairs of tyrosine residues in extensin molecules. Most of the hydroxyproline residues are O-glycosylated by tetra- and tri-arabinosides which are, with the exception of the terminal residue in the tetramer, beta-linked. Arabinose is the major sugar present. The other carbohydrate component, galactose, occurs as single units alpha-linked to most of the serine residues.

Table 1 Quantitative data available on the proportion of cell walls existing as glycoproteins

| Source | Amount of glycoprotein as a % of total cell wall | Amount of hydroxyproline as a % of total cell wall | % of the glycoprotein which is carbohydrate | Reference |
|---|--|--|---|-----------------------------|
| Tissue cultures (various) | - | 1.2 (2) | - | Lampert (1965) |
| Plant parts (various) | - | 0.3 (2) | - | Lampert (1965) |
| Glycopeptides from tomato suspension cultures | 14 (3) | 2 (3) | 50 (3) | Lampert (1969) |
| Cell walls from sycamore suspension cultures | 19 (4) | 2 (4) | 50 (4) | Talmadge et al. (1973) |
| Cell wall preparation from runner beans | 2.5 - 5.5 (protein only) | - | - | Selvendran (1975) |
| Acidified chlorite soluble glycoprotein fraction of bean cell walls | 7 (5) | 0.45 | 72 (5) | O'Neill & Selvendran (1980) |
| Soluble extensin precursor from carrot discs | - | - | 65 | Van Holst & Varner (1984) |

- Notes**
- (1) All % are m/m.
 - (2) Averages calculated from table of data.
 - (3) Lampert's estimates.
 - (4) These figures may be too high as they probably include contributions from the soluble hydroxyproline-rich glycoprotein secreted by the cells (see sections 1.3.1 and 1.6.2).
 - (5) Some pectin was associated with the acidified chlorite soluble glycoprotein fraction.

The glycoprotein adopts a polyproline II helix as secondary structure, stabilised by the arabinosides, which probably hydrogen-bond to the peptide backbone. This results in a rigid rod-shaped molecule.

1.3 ATTACHMENT OF EXTENSIN TO OTHER CELL WALL COMPONENTS

1.3.1 Macromolecular complex theory

The idea that all the components of the cell wall could be cross-linked to each other, mostly by covalent bonds, was first suggested by Lamport (1965). He put forward a "tentative picture of extensin in the primary wall" in which galacto-araban was a link between cellulose and protein, and different protein molecules were cross-linked by disulphide bridges. As described previously, the discovery of hydroxyproline arabinosides, and then serine galactosides, led to the suggestion that these groups could provide the attachment to the wall polysaccharides (Lamport, 1967; Lamport, 1973). Lamport (1970) commented that the hydroxyproline-rich protein of plants could be part of the cell wall protein-glycan network analogous to the peptido-glycan network of bacterial cell walls.

In 1973, the Albersheim group suggested that the primary cell walls of cultured sycamore cells could be considered as a single macromolecule. They presented a tentative structure in which xyloglucan was hydrogen-bonded to cellulose, and all the other components were interconnected by covalent bonds (Keegstra *et al.*, 1973). The wall protein was shown linked to the arabinogalactan by serine residues and not hydroxyproline, although the authors mentioned that the evidence in favour of the former link was not convincing. Nevertheless, they indicated that at least some of the pectic polysaccharides must be connected in some way to the hydroxyproline-rich protein. However, the evidence for the connection between the polysaccharides and the protein of the cell wall was based on observation of sycamore extracellular polysaccharide (SEPS). This was later shown to be a soluble hydroxyproline-rich glycoprotein, belonging to the group of arabinogalactan proteins (described in Section 1.6.2), which is secreted by the cultured cells. As SEPS is significantly different from

extensin, it cannot be used as a valid model for the cell wall (Pope, 1977; Lamport, 1978). Whilst this finding means that the carbohydrate protein links cannot be assumed from SEPS structure, the existence of connections via hydroxyproline arabinoside or serine galactoside has been neither confirmed nor refuted.

Before the findings of Pope (1977), who questioned the validity of the model proposed by Keegstra *et al.* (1973), Albersheim (1976) commented on the available evidence concerning the attachment of protein to the other wall components. He suggested that the pectic polysaccharides could be connected, in some manner, to the hydroxyproline-rich protein. However, by 1978, Albersheim had proposed a new model of the cell wall, based on a cross-linked network of polysaccharides alone. The cell wall protein was not included because of the absence of convincing evidence of a covalent connection. Darvill *et al.* (1980) went further to say that, whilst the covalent attachment of arabinose and galactose to the hydroxyproline-rich protein was accepted, the available evidence suggested that the hydroxyproline-rich glycoprotein was not covalently attached to any other of the cell wall polymers. The evidence did not rule out the existence of strong, non-covalent bonding between the hydroxyproline-rich glycoprotein and the other wall polymers.

Monro and co-workers used chemical extraction methods to study hypocotyl tissue (Monro, Penny & Bailey, 1976). They suggested an alternative model for cell wall structure, which included a more direct interaction of glycoprotein and cellulose microfibrils, but which did not involve a polyuronide bridge to bind extensin to the wall. They described wall protein and cellulose as being arranged in such a way that they could only be disentangled by breaking covalent bonds.

Preston (1979), in a well-argued paper, discussed the various suggested ways that the glycoprotein could be bound to other wall components. He concluded that the glycoprotein was somehow firmly bound in the wall, because it could only be extracted with strong reagents. The nature and distribution of the link between protein and glycan network may vary from plant to plant. However, Preston found the idea that the growing wall could be a single macromolecule unacceptable. "It is incompatible

with the known swelling and shrinkage properties of primary wall, and with extension during growth".

In conclusion, there is generally no evidence for covalent attachment between extensin and the wall polysaccharides, although a strong connection is implied because of the difficulty in extracting the glycoprotein. The earlier ideas of the wall existing as a "covalent macromolecule" have become less popular.

However, there is some evidence of a possible connection between extensin and the pectic polymers of the cell wall, which has not been disproved. Mort and Lamport (1975) reported briefly on a polyuronide arabinogalactan which may be attached to extensin via serine. In addition, Caughey and Lamport (1977) isolated a hydroxyproline-rich glycopeptide fraction with a galactose:serine ratio greater than 5, which also contained galacturonic acid. Both of these findings support the idea that extensin may be linked to pectic polysaccharides of the cell wall, via the serine residues. Similarly, O'Neill and Selvendran (1980) isolated a minor component of a crude hydroxyproline-rich glycoprotein extract, which contained a high proportion of uronic acid. However, the authors did warn that it is difficult to be sure if the uronic acid-containing material is associated with the glycoprotein in vivo or if it is co-solubilised or complexed with it after extraction.

Thus, whilst a completely covalently linked cell wall complex is unlikely, there is still some undisputed, but unconfirmed, evidence that suggests that extensin may be linked to the pectic polysaccharides via serine galactosides.

1.3.2 Lectin "glue" hypothesis

In 1974, Kauss and Glaser solubilised from mung bean hypocotyl cell walls a protein extract with lectin activity. Lectins, or haemagglutinins as they have also been called, are proteins or glycoproteins which have specific carbohydrate binding sites. Their activity can be assayed by their ability to agglutinate red blood cells, hence the name haemagglutinins, an effect brought about by binding of

the lectin to sugar residues on the erythrocyte surface. Kauss and Glaser (1974) suggested that this lectin activity could have a role in controlling the mechanical properties of the wall. They proposed that the lectin could function as a non-covalent "glue", by binding to specific polysaccharide components of the cell wall. They went on to describe how it would be possible to explain aspects of growth hormone action on the basis of a lectin "glue" in the cell wall. In particular, it is thought that treatment with auxin, a plant hormone which induces extension growth, causes the production of protons which then alter the physical properties of the wall, enabling it to extend (Rayle, 1973). It was observed that the binding capacity of the cell wall lectin was lowered at more acidic pH values. This would enable the cell wall to become more flexible under the influence of auxin, and thus capable of extending (Kauss & Glaser, 1974). The idea that the cell wall lectin may be connected with extension growth was supported by Kauss and Bowles (1976). In addition to the evidence of diminished lectin activity under acid conditions, they observed that gamma-D-galactonolactone, which is an inhibitor of growth, interacts with the lectin-binding sites. A galactoside-specific lectin would clearly find suitable, i.e. galactose-containing, polysaccharides in the cell wall. However, the involvement of lectin in cell wall binding and extension should be regarded as a working hypothesis only, and there are several other possible functions for these lectins.

Albersheim (1978) has discussed the possibility of extensin having lectin activity. He compared Kauss' lectin with that of potato (described in Section 1.6.2) but pointed out that the former had not been demonstrated to be hydroxyproline-rich. He also extended the analogy to the arabinogalactan-containing beta-lectins (also described in Section 1.6.2) but noted that, whilst these did contain hydroxyproline, they had not been demonstrated to be found in the cell walls. Kauss (1977a) similarly observed the likeness between the cell wall lectin and potato lectin, which led him to think that the cell wall lectin could actually be extensin. However, Kauss mentioned that more recent work had shown that most of the hydroxyproline in the extract could be separated from the lectin. Kauss (1977b) and Haass, Frey and Kauss (1977) subsequently confirmed that the lectin activity was clearly

separable from extensin. Nevertheless, the lectin could still play a role in wall extension growth, and Kauss (1977a) speculated on three possible functions:

- (i) new polysaccharides, synthesised intra-cellularly, could be combined with one binding site of the lectin and transported and guided to their specific site in the wall;
- (ii) lectins in the wall could allow a sliding of wall polysaccharides relative to each other, regulated by protons and/or divalent cations;
- (iii) lectins, which have also been demonstrated to occur in cellular membranes (Bowles & Kauss, 1975), could be involved in establishing contact between cell membrane and cell wall.

Haass, Frey and Kauss (1977) described how further purification of a crude extract, which exhibited lectin and galactosidase activities and contained hydroxyproline, showed that the hydroxyproline-containing proteins were completely separable from both the hydrolase and the lectin activities. More recently, Haass *et al.* (1981) demonstrated that, after extensive purification, the lectin and alpha-galactosidase activities were inseparable. They suggested that both activities might arise from a single molecular structure which incorporated sub-units with different functions. Moreover, they also found that the lectin was reversibly bound to the cell wall, independently of the lectin's sugar binding sites. These discoveries make it less likely that the lectin is directly involved as a structural component of the wall.

The cell wall lectin extracted from mung bean hypocotyls is similar to the seed lectin from the same plant. Other legume lectins also show similar properties (Hankins & Shannon, 1978; Campillo & Shannon, 1982). It has been suggested that legume lectins may, in general, be highly specific plant enzymes (Hankins & Shannon, 1978).

Elsewhere, evidence has been published which indicates that lectins may arise from the cell wall. Etzler and co-workers reported a cell wall glycoprotein found in the leaves and stems of the plant Dolichos biflorus, which may be a lectin precursor (Talbot & Etzler, 1978; Etzler, 1980; Etzler & Borrebaeck, 1980). This glycoprotein cross-reacts with antibodies against the seed lectin of the same plant although it does not itself have haemagglutinating activity. These workers suggested that the glycoprotein, which they refer to as CRM (cross-reactive material), may dissociate to give a molecule with lectin activity, and that this activation may be a response to wounding or fungal infection of the plant. The subcellular distribution of the CRM has been determined by immunofluorescence and immunocytochemistry, and compared with that of the seed lectin (Etzler et al., 1984). A significant portion of the CRM was found to be associated with, and probably non-covalently bound to, the cell wall. The seed lectin was not associated with the wall, and the authors suggest that the two lectins, although structurally related, may have different functions in different tissues. In a further study, a specific radioimmunoassay was used to follow the CRM during germination and seedling growth (Roberts & Etzler, 1984). It was found that the CRM accumulates in the seedling apex - the growing region. The observation that the highest levels of CRM were found in growing tissue and the previous finding that the lectin is associated with cell walls led these authors to suggest that CRM may be involved directly in wall extension, or that it may participate in other processes such as the transport and assimilation of cell wall polysaccharides.

1.3.3 Independent glycoprotein network hypothesis

Mort and Lampert (1977) usefully applied an old technique to the problem of studying extensin. They used anhydrous hydrogen fluoride, which specifically dissolves the polysaccharides of the cell wall whilst leaving peptide bonds intact. They found that rather than the cell wall protein dissolving as expected, it remained as an insoluble residue. This insoluble fraction, about 10% of the wall, consisted of equal amounts of wall protein and an "unknown (phenolic?) component" (Lampert, 1978). They suggested that this insoluble residue must

contain some other, as yet unidentified, cross-links. In addition, it may be cross-linked to itself independently of any possible links to cell wall polysaccharides. However, Lampport (1978) pointed out that proof of this idea required the isolation of cross-linked peptides and the identification of components involved at the cross-link region. To expand his earlier suggestion, Lampport (1980) put forward the concept of two semi-independent cell wall networks - protein and carbohydrate. He suggested that the tyrosine derivatives, which he had earlier been unable to identify (Lampport, 1977), could be possible candidates for the cross-links, but the isolation of cross-linked peptides was still needed for proof.

In 1975, Selvendran, Davies and Tidder had reported that hydroxyproline-rich glycoprotein could be solubilised from the cell wall by treatment with warm sodium chlorite and acetic acid, a reagent traditionally used for delignification. Later, O'Neill and Selvendran (1980) suggested that the glycoprotein may be held in the cell wall by "phenolic cross-links", which would account for the effectiveness of the acidified chlorite extraction method.

As mentioned in Section 1.2.3, the final evidence came from Fry (1982), who isolated, from cell walls, the compound isodityrosine, which is indeed both phenolic and a derivative of tyrosine. Fry reported that the amount of isodityrosine in cell wall hydrolysate was proportional to the amount of hydroxyproline, and suggested that it was a component of extensin. Significantly, he observed that the diphenyl ether bridge of isodityrosine was split by acidified chlorite, the treatment used to solubilise extensin. Fry thought that isodityrosine was the uncharacterised tyrosine derivative reported by Lampport (1977) and this was subsequently confirmed (Lampport & Epstein, 1983). He pointed out that this dimer might be formed in the cell wall by peroxidase and would act as an interpeptide cross-link holding the glycoprotein in the wall.

It is now accepted that the glycoprotein is cross-linked by isodityrosine, and thus may be insolubilised in the cell wall in a way which does not involve the carbohydrate side chains. The concept of a

separate extensin network has been developed further by Lamport, and his new model is discussed in Section 1.3.4.

The idea of an independent glycoprotein network has been extended to a possible glycoprotein-phenolic complex containing lignin. Whitmore (1978a) suggested that an early stage of lignification may involve cross-linking of the protein during polymerisation of lignin monomers. In experiments with lignin precursors together with various proteins and polysaccharides, he observed bonding of polyphenolic substances to proteins, especially those containing hydroxyproline, catalysed by peroxidase. In further experiments, using tissue culture cell walls incubated with coniferyl alcohol and hydrogen peroxide, Whitmore (1978b) demonstrated the formation of lignin, which was bound to carbohydrate and hydroxyproline-containing proteins. Whitmore (1982) extended his previous argument for lignin-protein structural bonds by comparing the amino acid distributions of proteins associated with lignin and those of the whole cell wall. He suggested that, when polymerising, lignin links covalently with cell wall glycoprotein, and that the bonds may be formed preferentially with hydroxyproline.

Whilst it is possible that these findings of lignin-protein complexes are artifacts of the lignin extraction method, the idea of a lignin-glycoprotein complex is consistent with the finding of the insoluble residue left after hydrogen fluoride treatment of cell wall (Mort & Lamport, 1977). Lamport and Catt (1981) discussed the possibility that extensin, which may itself be cross-linked by a peroxidase-catalysed tyrosine coupling, could be involved in further linkage to lignin. This would mean that the two interacting but semi-independent wall networks mentioned before (Lamport, 1978) could consist of a glycoprotein-phenolic network, and a polysaccharide one. Further evidence in support of the involvement of peroxidases in cell wall cross-linking includes the observation that peroxidases tend not to occur in regions of highest growth rate (Lamport & Catt, 1981).

A glycoprotein complex separate from a polysaccharide network in the cell walls of higher plants is a feasible arrangement when compared with the cell walls of some simple algae. The glycoprotein of Chlamydomonas

has been much studied (Hills *et al.*, 1974; Catt, Hills & Roberts, 1976, 1978; Roberts, 1979), owing to similarities with extensin, its ease of extraction compared with the problems of isolating glycoproteins from the cell walls of higher plants, and in its own right as a self-assembling wall component. The major glycoprotein fraction of *Chlamydomonas* cell wall accounts for about 70% of the wall and has high levels of hydroxyproline, arabinose and galactose (Catt, Hills & Roberts, 1976). This glycoprotein is capable of reversible self-assembly into a crystalline lattice, characteristic of the native cell wall, and it has been shown that the sugar residues are important for accurate reassembly (Catt, Hills & Roberts, 1978). Homer and Roberts (1979), by studying the circular dichroism spectra of peptides of this glycoprotein, established that the glycoprotein exists as about 50% polyproline II conformation. They point out the relevance of these findings to an understanding of the structure of extensin, owing to some similarities between the amino acid and sugar composition of the two glycoproteins. In view of the presumed parallels between these glycoproteins, Lamport (1977; 1980) has described how the cell wall component of higher plants could well have evolved from primitive protists such as *Chlamydomonas*.

In conclusion, the concept of a glycoprotein, or glycoprotein-lignin network, separate from a polysaccharide complex, seems a credible explanation for the attachment of extensin in the cell wall. The evidence of a hydrogen fluoride insoluble residue, and the finding of a protein-protein link in isodityrosine, supports this idea. The notion that glycoprotein might form a semi-independent network is both the most recent and the most promising model.

Other, longer-standing, suggestions have generally been disproved or, at least, are not much talked about.

1.3.4 "Warp and weft" hypothesis

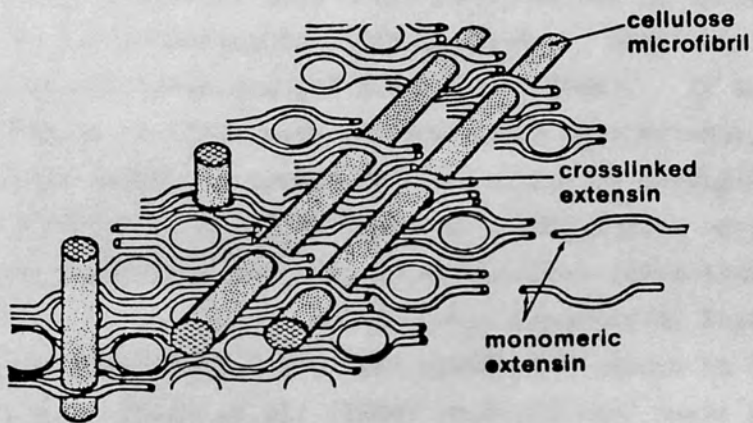
In the light of all the evidence previously described, Lamport and Epstein (1983) have put forward a new working hypothesis for the structure of the wall. Referred to as the "warp and weft hypothesis", this proposes, more precisely than the independent glycoprotein network model, the arrangement of the wall polymers.

The basis of the hypothesis is that the primary cell wall is a woven structure "consisting of two concatenated polymers : cellulose microfibrils penetrating the mesh of an extensin net, suspended in a hydrophilic pectin-hemicellulose gel". That is the cellulose and extensin networks, without being covalently joined, are interlinked by the formation of closed loops of extensin around the microfibrils (see Fig. 4). Lamport (1985) has added that such an organised cell wall model suggests a highly engineered mechanical coupling of load-bearing polymers leading to stress distribution throughout the wall.

In this woven structure, the microfibrils are referred to as a "warp", which runs parallel to the plane of the wall, while the most likely orientation of the extensin "weft", at least initially, is perpendicular to this plane. The extensin mesh, it is predicted, could orient and control the cellulose microfibrils. To enable extensin to weave throughout the cellulose network, it is suggested that the predominantly rod-like structure has non-helical kinks at intervals. This hypothesis fits the requirements for a cell wall that is strong and yet able to expand in a controlled manner. The authors suggest that the xyloglucans which are hydrogen-bonded to the cellulose component are also a part of the same structure and have a role in cell extension. For example, the xyloglucans could act as "latches" which can be opened during xyloglucan turnover to allow microfibril slippage through the extensin mesh, thereby permitting extension growth.

Lamport and Epstein point out that there are two requirements for this new hypothesis. The first is that the extensin network must be capable of being assembled in the wall from monomers. As described in Section 1.4, it is now accepted that the insoluble extensin matrix is polymerised in this way. The second requirement is that the mesh size of cross-linked extensin must approximate to the dimensions of the cellulose microfibrils. The experimental evidence for this is discussed below.

Lamport and Epstein (1983) estimate that there are approximately thirty amino acid residues between isodityrosine links. This follows from Fry's (1982) observation of a hydroxyproline : isodityrosine ratio of



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**Fig.4 Hypothetical extensin-cellulose network -
"warp and weft" model (Lampert & Epstein, 1983)**

15 : 1 and an extensin hydroxyproline content of 50%. They go on to calculate that, in a polyproline II helix, about seventy-six residues would be needed to wrap around a cellulose microfibril. However, at least some of the isodityrosine units form intramolecular links between close tyrosine residues in the same extensin monomer. These clearly cannot act as interpeptide cross-links and Lampport and Epstein's proposed function for the intramolecular isodityrosine groups is to lock the peptide conformation and stabilise the protein. Formation of such tyrosine dimers within one extensin molecule results in longer amino acid chains between the intermolecular isodityrosine groups. On the basis of the minimum length needed between intermolecular cross-links, they have concluded that there could be two to three times as much intra- as intermolecular isodityrosine. Note this is similar to the estimate of Stafstrom and Staehelin (1986a). If sufficient isodityrosine links were intramolecular the extensin 'mesh' size could be large enough to accommodate cellulose microfibrils. Alternatively, if the inter- and intra- types of isodityrosine were unevenly distributed, such that the intermolecular cross-links were near the ends of extensin molecules, as has been suggested by Stafstrom and Staehelin (1986a) (Section 1.2.3), this would also result in large 'holes' in the network. Smith *et al.* (1986) observed that there are marked repeated peptide sequences in tomato extensin precursors. They suggest that such a periodic structure, with regularly repeated cross-links, would be likely to create an extensin network with a controlled pore size. The precise porosity of the extensin network, however, still remains to be defined (Lampport, 1985).

Further insight into the possible roles of intramolecular isodityrosine has been provided by Stafstrom and Staehelin (1986a). They have confirmed that there are kinks in extensin monomers which are visible by electron microscopy. They have also shown that these kinks coincide, both in number and position, with blocks of tyrosine-lysine-tyrosine. The location of such blocks have been predicted from DNA sequences by Chen and Varner (1985b). Stafstrom and Staehelin suggest that the kinks are visible manifestations of intramolecular isodityrosine cross-links. It is interesting that most of the tyrosine residues in the central region of extensin occur in sequences of

tyrosine-lysine-tyrosine which have been demonstrated to form intramolecular cross-links (Epstein & Lamport, 1984). Most of the tyrosine residues near the ends of extensin molecules, where intermolecular links occur, are in different sequences. Further, Stafstrom and Staehelin propose that intramolecular isodityrosine could function by making extensin more rigid and it could bend extensin to help interaction with other wall components.

In conclusion, the warp and weft hypothesis is a useful refinement to the general idea of an independent glycoprotein network. This most recent model assigns importance to an interpeptide link, isodityrosine, while being consistent with the evidence for other interconnections between different cell wall polymers.

Figure 5 shows a model for the cell wall which incorporates the warp and weft idea, and includes the other major wall polymers. This diagram represents our current understanding of the way in which the wall components are interconnected. The cellulose mesh is shown with xyloglucans forming "latches" between fibrils. Looped around the cellulose fibrils are extensin molecules which are mostly in a helix conformation, but contain some non-helical sections which could allow the glycoprotein to bend. Extensin molecules are cross-linked by intermolecular isodityrosine groups. The pectins may simply form an amorphous matrix surrounding the extensin and cellulose networks, and these have therefore been represented as random threads in the style of Cooper, Chen and Varner (1984).

1.4 Biosynthesis of Extensin

1.4.1 Systems which have been studied

The biosynthesis of extensin has been fairly thoroughly investigated because it has been thought by many workers that isolating precursors and following their incorporation into the cell wall might help towards an understanding of the structure of extensin itself. Two different model systems have been preferred:

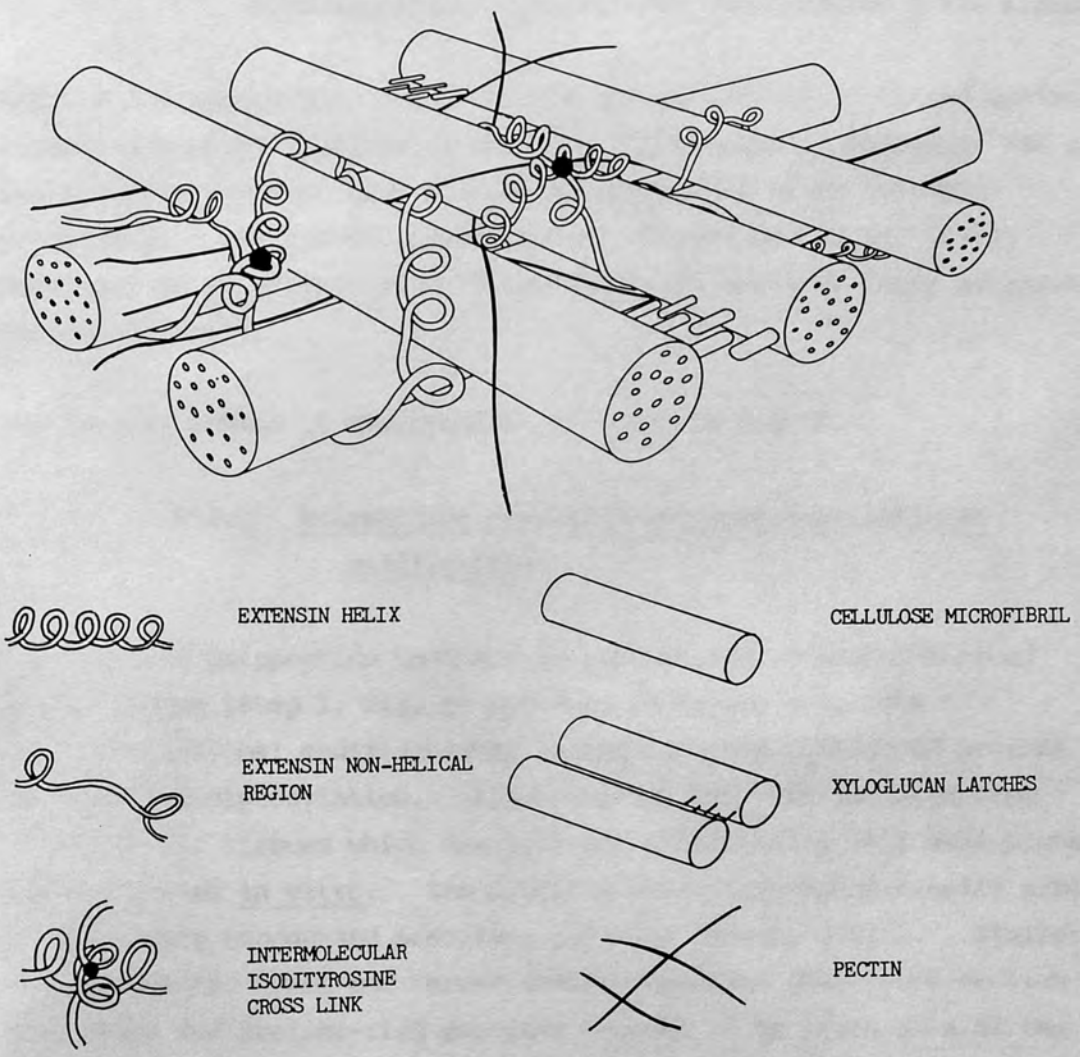


Fig.5 Diagram of a current view of polymer arrangement in the plant cell

- (i) "aged" carrot parenchyma discs, which are injured tissues that synthesise and secrete large amounts of hydroxyproline-containing glycoproteins; this system was originally developed by Chrispeels and co-workers.
- (ii) suspension cultures, which also form larger amounts of hydroxyproline-glycoproteins than ordered plant tissues.

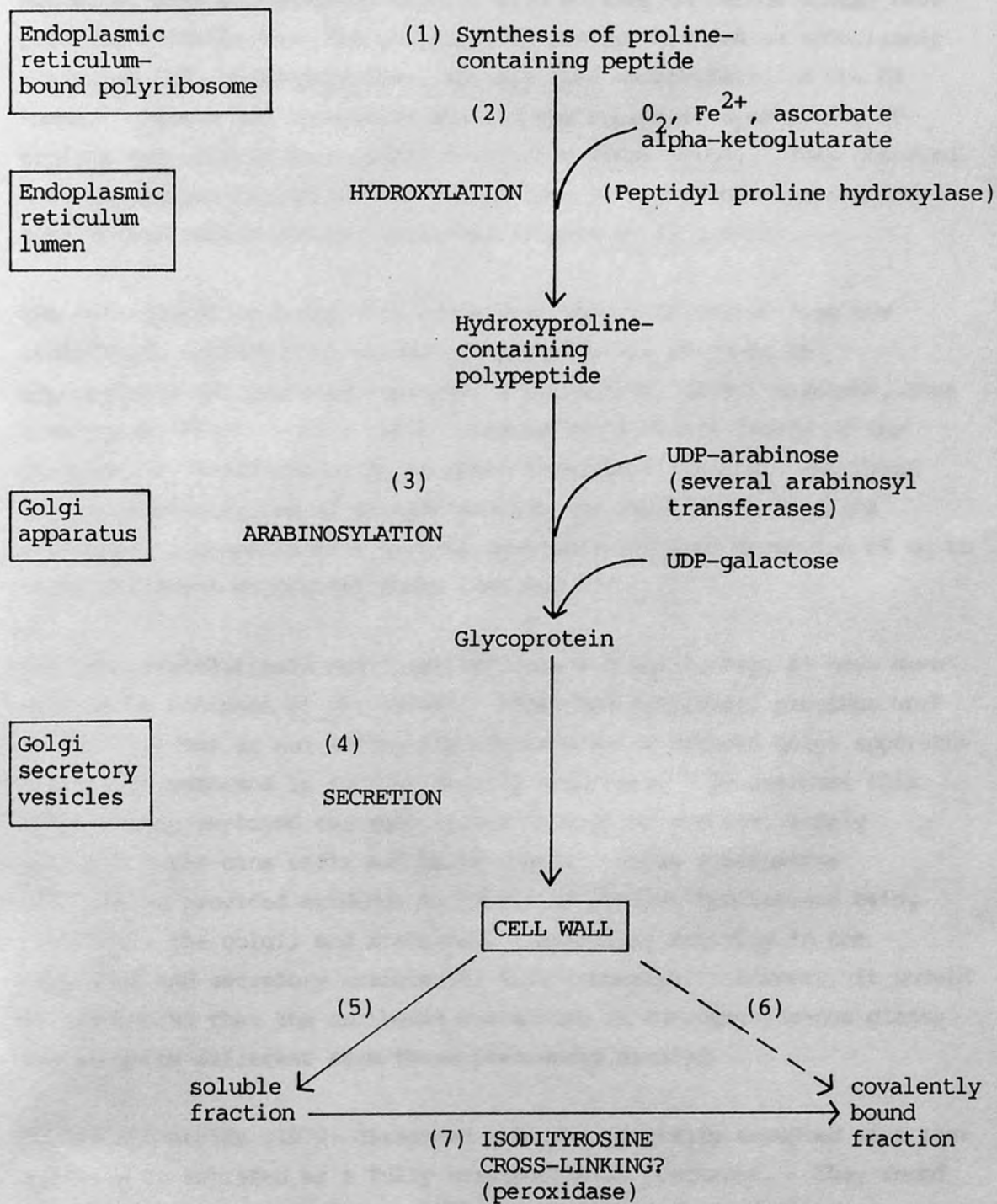
Alternative approaches, involving the use of antibodies raised against a repeating peptide sequence of extensin and studies of messenger RNA and translation products, have also been employed to study extensin precursors. For recent investigations, Cooper and Varner (1984) developed an in vitro system of isolated cell walls to study extensin insolubilisation.

The general scheme of biosynthesis is given in Fig. 6.

1.4.2 Polypeptide synthesis and post-translational modification

The extensin polypeptide backbone is synthesised on membrane-bound polyribosomes (Step 1, Fig. 6) and then undergoes extensive post-translational modifications, including hydroxylation of proline residues and glycosylation. Messenger RNA has been isolated from carrot discs, tissues which are actively synthesising cell wall protein, and translated in vitro. The products were electrophoretically similar to previously recognised precursor proteins (Smith, 1981a). Similar work by Stuart, Mozer and Varner (1982) indicated that cytosine-rich mRNA codes for proline-rich peptides thought to be precursors of the hydroxyproline-rich glycoprotein.

Most of the proline residues in the polypeptide are hydroxylated by the action of peptidylproline hydroxylase (Step 2, Fig. 6) (Chrispeels, 1970). This enzyme, which requires molecular oxygen, iron, ascorbate and alpha-keto-glutarate (Sadava & Chrispeels, 1973b; Lampert, 1977) is similar to an enzyme involved in collagen biosynthesis.



For explanation of numbered steps, see text.

Fig.6 Scheme for the biosynthesis of extensin

Wienecke, Glas and Robinson (1982), also working on carrot discs, have provided evidence that the polypeptides are synthesised on endoplasmic reticulum (ER)-bound polysomes, and are then hydroxylated in the ER lumen. Tanaka and co-workers studied the substrate specificity of proline hydroxylase in suspension-cultured Vinca rosea. They observed that the enzyme recognises the polyproline II helix conformation and hydroxylates tetra-proline sequences (Tanaka et al., 1982).

The hydroxyproline-containing protein is then transferred from the endoplasmic reticulum to the golgi apparatus, in which it is glycosylated and secreted (Gardiner & Chrispeels, 1975; Wienecke, Glas & Robinson, 1982). Karr (1972) studied the arabinosylation of the polypeptide, which occurs by transfer from UDP-arabinose. He found that a complex system of several enzymes was required to form the arabinose oligosaccharides as this synthesis involves formation of up to three different glycosidic bonds (see Fig.2).

The post-translational modifications (steps 2 and 3, Fig. 6) have been studied by Robinson et al. (1984). They had criticised previous work on dicotyledons as not adequately distinguishing between golgi apparatus and plasma membrane in sucrose density gradients. To overcome this problem they employed two monocotyledonous plant systems, namely cultured sugar-cane cells and maize roots. These alternative experiments provided evidence in favour of proline hydroxylase being located in the golgi, and arabinosyl transferase activity in the cisternal and secretory vesicles of this organelle. However, it should be remembered that the synthesis mechanisms in monocotyledonous plants may be quite different from those previously studied.

Tanaka and Uchida (1979) disagreed with the generally accepted view that extensin is secreted as a fully arabinosylated precursor. They found extensin precursors which were heterogeneous in size and charge in suspension-cultured Vinca rosea protoplasts. It was suggested, and later confirmed (Tanaka et al., 1982), that this heterogeneity was due to the isolation of precursors at different stages of glycosylation, an observation not reported elsewhere. This apparent discrepancy presumably stems from their preparation method, which extracts all partly glycosylated precursors in intracellular organelles.

1.4.3 Secretion and insolubilisation in the cell wall

Early studies using pulse-chase experiments with ^{14}C -labelled proline led Chrispeels (1969) to identify hydroxyproline-containing macromolecules associated with membranous organelles of the cytoplasm as precursors of cell wall hydroxyproline. Brysk and Chrispeels (1972) continued these studies and suggested that a hydroxyproline-containing glycoprotein that could be extracted from the cell wall by salt solutions was a precursor of the covalently bound glycoprotein. Carbohydrate analysis of the secreted glycoprotein showed the presence of large amounts of arabinose and only traces of other sugars. This observation led to the speculation that the salt-extractable precursor could become covalently attached to the cell wall (step 7, Fig. 6) by the addition of galactose.

Cooper and Varner (1983) have carried out similar pulse-chase experiments to label carrot hydroxyproline-rich glycoprotein. It was found that the pool of soluble glycoprotein became non-extractable over 24 hours. The experiments of Brysk and Chrispeels were too short to demonstrate this insolubilisation. As described in Section 1.2.5, there is now sufficient evidence that this carrot hydroxyproline-rich glycoprotein is a true precursor of extensin, and not a wound-related protein.

Pope (1977) re-examined Chrispeels' data in the light of his own results from suspension-cultured sycamore cells. He concluded that the wall protein is transferred directly from a particulate fraction to the wall, where it is immediately incorporated without passing through a salt-extractable pool. He criticised Chrispeels' procedure as not adequately demonstrating that the salt-extractable fraction behaved as a pool.

However, subsequent work by Smith, Muldoon and Lamport (1984) indicates that sycamore cells are unsuitable for studying extensin precursors. Cultured tomato cells were employed as an alternative, and salt solutions used to elute extensin precursors from the cells' surfaces. Two monomeric precursor glycopolypeptides were identified. Also

found was an unexpectedly large pool of precursors, calculated to be sufficient for 9 hours' growth, and a concomitantly slow pool turnover.

Smith (1981a) used the carrot disc system in more detailed experiments with radiolabelled proline and arabinose. His results showed that one hydroxyproline-rich glycoprotein species accounted for most of the protein incorporated into the cell wall. In addition, synthesis in the presence of alpha,alpha'-dipyridyl (an inhibitor which prevents proline hydroxylation) resulted in a protein which appeared to be a non-hydroxylated, non-glycosylated form of the same glycoprotein. Moreover, this could still become tightly bound to the cell wall. This provided evidence that the hydroxylation and arabinosylation steps are not required for covalent attachment to the wall. Also, the finding that the underglycosylated precursor was actually secreted to the cell wall indicates that, unlike collagen, the structure required for secretion does not depend on post-translational modification.

Stafstrom and Staehelin (1986b) point out, however, that secretion and even cross-linking of unglycosylated extensin does not necessarily mean that the protein may be functioning properly in the wall. It is conceivable that without carbohydrate, which has been demonstrated to be essential for maintaining an extended helix conformation, the protein, despite being cross-linked, is a non-rigid, weak structure. Certainly, the warp and weft model demands that the glycoprotein has an elongated rod conformation with accurate spacing of cross-links to provide the necessary strength (Section 1.3.4).

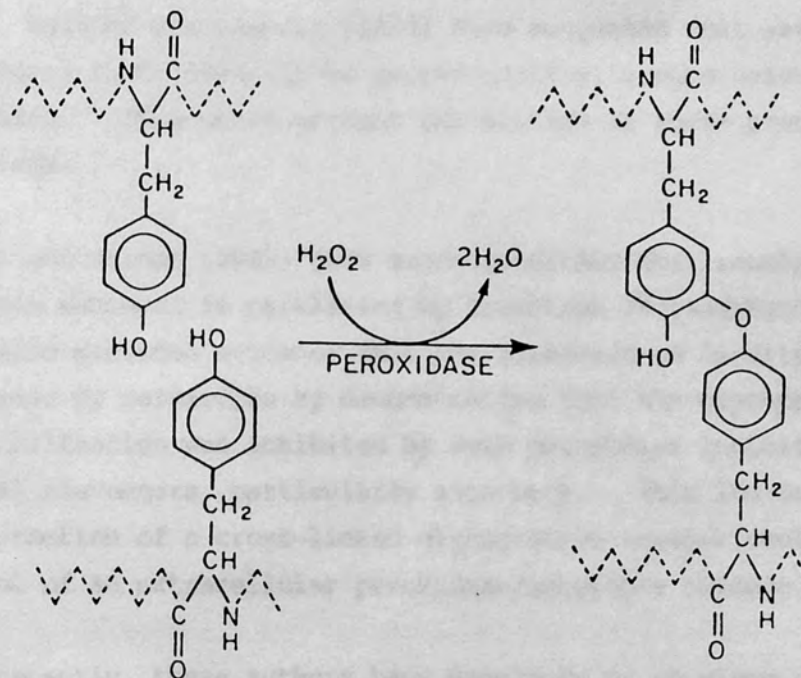
In comparison with the particular requirements of the warp and weft model, other authors have suggested alternative means of attaching extensin to the wall. Smith (1981a) cites results of Chrispeels and co-workers and contrasts the observation of Brysk and Chrispeels (1972) that arabinose is the only sugar detected in the salt-extracted glycoprotein, with the findings of Chrispeels (1969) and Cho and Chrispeels (1976) that the glycoprotein covalently bound to the cell wall has galactose attached. Smith advanced a model to explain the covalent attachment of glycoprotein to the cell wall. He proposed that

a single cytoplasmic precursor is secreted via two alternative pathways. In the first, the protein has arabinose added to it and enters the cell wall as the salt-soluble fraction (step 5, Fig. 6). In the second, the protein acquires galactose in addition to arabinose, enabling this glycoprotein to be covalently attached to the cell wall (step 6, Fig. 6).

However, some of the studies on which this model is based have been questioned. Brysk and Chrispeels (1972) report a precursor rich in glycine, but, as described in Section 1.2.7, this amino acid is not a significant component of extensin. The isoelectric point of their material is correspondingly lower than that reported by Cooper, Chen and Varner, (1984). It is probable that both these anomalies were caused by a contaminant. Thus Van Holst and Varner (1984) occasionally found amino acid compositions comparable with the results of Brysk and Chrispeels (1972), and these were due to a contaminant that tended to co-elute with the glycoprotein.

It seems likely, therefore, that Brysk and Chrispeels were studying extensin precursors but in an impure preparation, and with experiments that were too short to indicate true turnover. Nevertheless, Smith's hypothesis for steps 5 and 6 (Fig. 6) is still considered to be valid. It appears that none of the specific criticisms of Brysk and Chrispeels' work is relevant to the results on which Smith's model is based. Although this model has not been disproved, a different version is now widely accepted, namely that extensin is secreted to the wall as a fully glycosylated precursor which is subsequently fixed there by the mechanism described below.

In a short communication, Cooper and Varner (1981) reported on investigations into cell wall protein cross-linking. They found that removing hydrogen peroxide and inhibiting peroxidase increased the proportion of cell wall protein remaining soluble. The significance of this was not apparent until Fry (1982) identified isodityrosine as an interpeptide cross-link in cell wall glycoprotein (see Section 1.3), and proposed that this dimer is formed in the wall by peroxidase, by the mechanism in Fig. 7.



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Fig.7 Proposed mechanism for the formation of peptidyl isodityrosine, by the oxidative coupling of two peptidyl tyrosine residues, thereby cross-linking the peptides (Fry, 1983)

It now seems likely that extensin is rendered insoluble by the formation of isodityrosine links between the peptide backbones and not via the carbohydrate side chains as was originally thought.

Smith, Muldoon and Lamport (1984) have suggested that extensin precursors bind ionically to pectin carboxyl groups before isodityrosine formation. This would account for elution of these precursors by salt solutions.

Cooper and Varner (1983) have since confirmed that insolubilisation of extensin monomers is paralleled by formation of isodityrosine. They have also provided evidence that the formation of isodityrosine is catalysed by peroxidase by demonstrating that the glycoprotein insolubilisation was inhibited by both peroxidase inhibitors and free radical scavengers, particularly ascorbate. This led to the idea that the formation of a cross-linked glycoprotein network could be under the control of an extracellular peroxidase/ascorbate oxidase system.

More recently, these authors have developed an in vitro system of isolated cell walls for experiments on extensin cross-linking (Cooper & Varner, 1984). By incubating labelled salt-soluble extensin with this system in neutral buffer, they showed that the isolated cell walls have the capacity to insolubilise extensin, probably through the formation of isodityrosine. Further, analysis of the mix of salt-soluble wall proteins before and after incubation demonstrated that the insolubilisation was specific for extensin. This cross-linking of soluble extensin catalysed by wall enzymes was found to be inhibited by acidic pH conditions, an observation which led Cooper and Varner to speculate that the solubility of extensin could be regulated by auxin-induced hydrogen ion secretion. They propose two means by which wall pH might regulate extensin solubility. Firstly, the activity of the enzyme catalysing the cross-linking would be affected by pH, and, secondly, the structure of extensin would be directly affected. This latter mechanism, brought about by protonation of histidine residues, could, in turn, have two effects. Thus, as histidine dipeptides occur repeatedly adjacent to tyrosine residues, the local environment of the tyrosine side chains could be changed. This might alter the affinity of the peroxidase cross-linking catalyst or change the stability of

tyrosyl free radical intermediates. In addition, histidine protonation should also increase the affinity of the polycationic extensin for polyanionic pectin with the result that protein-pectin interactions might regulate the availability of tyrosine residues for oxidation.

Following the observations that inter- and intramolecular isodityrosine links appear to be formed from tyrosine residues in different sequences (discussed in section 1.3.4), Stafstrom and Staehelin (1986a) suggested that variation of the proportions of the two dimers could also provide a means of controlling extensin solubility. It is quite feasible that different peroxidases catalyse formation of the two links, and differential expression of the enzymes could regulate the number of cross-links between different molecules.

1.4.4 Current picture of biosynthetic mechanism

From the evidence detailed above, the biosynthesis of extensin, as currently understood, can be summarised as shown below.

The polypeptide backbone is synthesised first, and then undergoes extensive post-translational modifications, namely hydroxylation of proline residues and glycosylation. Formation of oligoarabinosyl side chains is the glycosylation step which has been studied. Galactosylation has not been investigated, but this sugar is only a minor component of the glycoprotein.

The fully formed soluble glycoprotein is then secreted to the cell wall. Before it is insolubilised there, it can be extracted by salt solutions, and it has been suggested that the glycoprotein is, at this stage, ionically bound to pectin. Surprisingly, it has been shown that proline hydroxylation and glycosylation are not required for secretion and insolubilisation.

It is thought that the glycoprotein becomes bound in the cell wall by the formation of interpeptide isodityrosine links. The synthesis of this unusual phenolic link between tyrosine residues is catalysed by peroxidase, and the reaction is inhibited by acidic pH. This has led

to the suggestion that cross-linking could be regulated by auxin-induced hydrogen ion secretion.

1.5 Possible Roles of Extensin

1.5.1 Structural

It is generally accepted that the hydroxyproline-rich glycoprotein must have a structural role in the cell wall - as a physical building component which contributes to the strength of the wall. Specific arguments for this structural function are discussed below.

Compositionally, extensin is exceptionally rich in hydroxyproline and has a repeating peptide periodicity which, as described by Lamport and Catt (1981), typifies a structural rather than an enzymic macromolecule. This composition leads to a regular helical conformation which is stabilised by the carbohydrate side chains into a rigid rod-like molecule, which is further evidence for a structural polymer.

The current view of the arrangement of extensin in the cell wall is of an interpeptide-linked glycoprotein which forms a separate network that complements the cellulose mesh. In this way, it contributes to the strength and rigidity of the cell wall. Further, indirect evidence for the structural function of extensin arises from the tenacity with which it binds to the other wall components. Thus, it is generally very difficult to extract the glycoprotein from the cell wall without using degradative treatments (Brown & Kimmins, 1981; Lamport, 1969, 1980; Monro, Bailey & Penny, 1974). Even treatment with anhydrous hydrogen fluoride leaves the glycoprotein and other phenolic material as an insoluble "skeleton" (Mort & Lamport, 1977). Extensin can only be extracted by conditions which break phenolic links (O'Neill & Selvendran, 1980).

Cooper (1984) carried out experiments with 3,4-dehydroproline, a specific inhibitor of proline hydroxylase, to investigate the function of hydroxyproline-rich glycoproteins in the cell walls formed during

wall regeneration by tobacco cell protoplasts. He observed that when hydroxyproline synthesis was inhibited the cell wall was not properly formed. This result led him to conclude that hydroxyproline-rich glycoproteins are important for the structural integrity of the primary cell wall and for the correct assembly of other wall polymers.

Lamport (1977) and Ashford and Neuberger (1980) have specifically pointed out the similarities between collagen, a structural protein of animals, and extensin: both are extracellular hydroxyproline-rich glycoproteins, which adopt, at least in part, a polyproline II helical conformation. The most significant resemblance between the two proteins is that their biosynthesis involves molecular oxygen, iron, alpha-ketoglutarate and ascorbate. The likeness between extensin and collagen led Lamport (1977) to pose the question of whether these proteins have a common evolutionary origin. This idea is supported by McConville, Fisher and Clarke (1982), who present evidence that older species of marine algae are devoid of hydroxyproline, whereas more recently evolved algae, higher plants and animals contain significant levels of this imino acid. They suggested that hydroxyproline evolved as an oxygen atmosphere became established and the capacity for hydroxylation of proline developed. However, the idea that extensin and collagen have common evolutionary precursors is refuted by Chen and Varner (1985b) in the light of recent information about extensin DNA and amino acid sequences.

One particular species of alga, *Chlamydomonas*, has already been described because of the relevance of its cell wall composition to studies of extensin. This organism has a wall which contains no cellulose, but comprises mainly a hydroxyproline-rich glycoprotein with a carbohydrate composition and helical structure with some similarities to extensin.

Stemming from an acceptance that extensin is a structural component of the cell wall, it has been suggested that this glycoprotein may be the specific component that regulates the degree of wall extensibility. The next section will describe evidence for this specific function of extensin.

1.5.2 Extension growth control

Lampport, in a major paper (Lampport, 1965), in which he gave the hydroxyproline-rich cell wall glycoprotein the name extensin, proposed that this glycoprotein must inevitably play some role in cell extension. He envisaged extensin as providing the "knots" in the cell wall, that could be loosened to enable extension to occur. The ability of the cell wall to extend would depend on the amount of extensin and the state of its cross-links to the other wall components.

There is a consensus that the hydroxyproline-containing proteins increase markedly in the cell wall during the transition of rapidly elongating tissue into non-elongating mature tissue (Cleland & Karlsnes, 1967). There is some debate, however, about the exact timing of this accumulation of extensin in relation to the stopping of elongation (Sadava, Walker & Chrispeels, 1973; Vaughn, 1973; Klis, 1976; Van Holst *et al.*, 1980b).

Sadava and Chrispeels (1973a) carried out growth experiments with excised pea epicotyls. They demonstrated that in the presence of a growth hormone there was no increase in wall hydroxyproline. On addition of growth inhibitors, however, there was a three-fold increase in hydroxyproline. After further experiments with alpha,alpha'-dipyridyl to block the hydroxyproline increase, they concluded that the accumulation of a hydroxyproline-rich component was necessary to render the walls inextensible. However, Lang (1976) questioned the assumption of such a specific effect of alpha,alpha'-dipyridyl. He pointed out that this iron chelator, which blocks proline hydroxylation, can cause an acceleration of stem elongation which is unrelated to its effect on hydroxyproline biosynthesis. Lang explained that his results do not disprove the hypothesis that there is a causal relationship between the cessation of growth and the deposition of extensin, but they indicated that care was needed in interpreting results based on the use of alpha,alpha'-dipyridyl.

The problems of using inhibitors to study the mechanism of wall growth have been pointed out by Huber and Nevins (1981). They warned that the inhibitors may act non-specifically or indirectly and proposed a new approach, involving antibodies raised against cell wall protein, as a potential method for specifically inhibiting growth. In initial studies with corn seedlings, they found that antibodies against protein associated with the cell wall inhibited growth of the isolated cell walls.

Hoson and Wada (1980) compared the elongation rate and amino acid content of rice coleoptiles grown under and on the surface of water. They concluded that the lower hydroxyproline content caused by the lower oxygen tension in water is one of the factors promoting the growth of rice coleoptiles under water. However, their results show that the hydroxyproline-rich cell wall protein cannot be regarded as the final growth cessation factor in rice coleoptiles.

The references which have been discussed in the preceding paragraphs all report increases in the quantity of hydroxyproline in the cell wall as growth slows or stops. Other results, discussed below, have pinpointed the binding of hydroxyproline-rich glycoprotein to the wall as being the cause of reduced growth.

Fujii, Suzuki and Kato (1981) suggested that a growth inhibitor is one of the factors that cause differential growth between upper and lower halves of geotropic zea roots, causing them to curve downwards. The inhibitor selectively increases the concentration of hydroxyproline-containing proteins rigidly bound to the cell walls in the lower halves of the roots, thereby reducing the growth. Further support for the proposed role of the hydroxyproline-rich glycoprotein in stopping cell elongation was provided by Monro, Penny and Bailey (1974). They used sequential extraction procedures to compare elongating and non-elongating hypocotyl sections and found that there was more bonding of the glycoprotein in the wall of non-elongating tissue.

In the light of recent information on the probable cross-linking of soluble extensin by isodityrosine, it is now clear that a reduction in

wall extensibility is more likely to be related to the amount of hydroxyproline rigidly bound in the wall than to the total secreted to the wall.

A current view of extensin's role is that it provides a second network which restricts movement between cellulose microfibrils (Lampert & Epstein, 1983). To achieve this requirement of a separate network, extensin is secreted to the wall in a soluble form and is then locked in position around the cellulose skeleton by the formation of isodityrosine cross-links. Lampert no longer describes extensin as functioning as a "knot" that can be "tied and untied" to permit growth. It is assumed that the insolubilisation of extensin in the wall is an irreversible process. Lampert and Epstein now suggest that the xyloglucan component of the wall may function as reversible bonds or "latches", which could be broken to permit growth. The xyloglucans are hydrogen-bonded to the cellulose fibrils, and these authors explain how xyloglucan turnover, which is enhanced by auxin, could open the latches and allow the microfibrils to slip through the extensin mesh and permit cell extension.

It has been mentioned previously (Section 1.3.2) that auxins induce extension growth via hydrogen ion secretion. Cleland and Rayle (1978) have also proposed that wall loosening, which is essential for growth, may involve breakage of bonds to xyloglucans.

Shannon, Flood and Wain (1984) investigated the role of extensin in auxin-induced cell enlargement in potato tuber discs. They found that alpha,alpha'-dipyridyl inhibited hydroxyproline synthesis and auxin-induced cell expansion, and that these inhibitions could be reversed by Fe^{2+} ions. But it was also found that this expansion could be halted without inhibiting hydroxyproline synthesis. It was argued that extensin biosynthesis is necessary for auxin-dependent cell expansion, but the extent of this expansion is regulated by factors other than the amount of extensin.

Shannon and co-workers' observations are consistent with the view that it is not the total amount of extensin in the wall that controls extensibility, but rather the degree of cross-linking of extensin.

They found that cell expansion requires extensin synthesis, but presumably such expansion requires synthesis of all the cell wall polymers to build new wall.

Of particular relevance, Cooper and Varner (1984) found that wall-catalysed cross-linking of soluble extensin was inhibited, in an in vitro system, by acidic conditions. This observation indicates a means by which auxins could act to permit growth. Thus, auxins induce acid secretion, which may suppress cross-linking of soluble extensin during wall growth. When growth hormone production ceases, extensin can cross-link to harden the new wall. In support of this argument there is separate evidence that extensin cross-linking may not take place until growth is complete, or nearly complete. In particular, Lamport and Catt (1981) report that peroxidases, which catalyse the formation of the cross-links, tend not to occur in regions of highest growth rate.

In conclusion, the original idea, that extensin provides links which can be reversibly broken to allow growth, has been contradicted by recent evidence. The current picture of the role of extensin is as a structural polymer which becomes fixed in the wall and prevents cell extension. Synthesis and secretion of soluble extensin precursors, however, do not necessarily inhibit growth.

1.5.3 Defence

Lamport (1980) pointed out that involvement of extensin in extension growth need not exclude an additional role for this glycoprotein in disease resistance. The levels of the hydroxyproline-rich glycoprotein have been shown to undergo a ten-fold increase in melon seedlings infected with the fungus Colletotrichum lagenarium (Esquerré-Tugayé & Lamport, 1979). This increase in extensin is correlated with resistance to anthracnose, the disease caused by the fungal infection (Esquerré-Tugayé & Toppan, 1976). Esquerré-Tugayé et al. (1979) concluded that the accumulation of glycoprotein acts as a defence mechanism, which is effective if started early in the host. Conversely, inhibiting glycoprotein synthesis in diseased plants results

in an accelerated and more intense colonisation of the host by the pathogen. Toppan, Roby and Esquerré-Tugayé (1982) found indirect evidence that ethylene regulates this defence mechanism. They showed that in the presence of specific inhibitors of ethylene biosynthesis, both ethylene and ^{14}C -hydroxyproline deposition in the cell wall of infected tissue was significantly lowered. Furthermore, treatment of healthy tissues with a natural precursor of ethylene stimulated both the production of the hormone and incorporation of ^{14}C -hydroxyproline into cell wall protein. Work with in vitro and in vivo experiments is reported to demonstrate that the increased synthesis of hydroxyproline-rich glycoprotein caused by fungal infection is mediated by ethylene (Esquerré-Tugayé et al., 1980).

The increase in cell wall hydroxyproline content observed in aged carrot discs has also been suggested to occur as part of a wound response in the excised tissue (Chrispeels, 1969). Chrispeels, Savada and Cho (1974) pointed out that the increase in extensin biosynthesis may be part of the plant's defence mechanism against invading pathogens. Fukuda and Kagimoto (1982) similarly observed an increase in the cell wall and subcellular particle hydroxyproline levels on ageing sections of sweet pepper fruits. They thought the increase in hydroxyproline during ageing derived from the biosynthesis of the hydroxyproline-rich protein and its precursor in response to wounding of the tissues. Kimmins and Brown (1975) also found that the cell wall glycoprotein was synthesised in response to wounding in bean leaves. It is possible that production of hydroxyproline-rich glycoprotein in each of these stress situations may be controlled by ethylene, as wounding, ageing and infection are all known to cause plants to release large amounts of ethylene (Toppan, Roby & Esquerré-Tugayé, 1982).

However, more recent work has shown that in the melon-Colletotrichum lagenarium system, the increase in ethylene production occurs too soon after inoculation to be a wound-stress response (Toppan & Esquerré-Tugayé, 1984). These authors found that ethylene levels were raised before the pathogen had invaded the tissues and before the onset of symptoms. They have now demonstrated that the production of ethylene is stimulated by glycopeptides or "elicitors" from the fungal

cell walls. Toppan and Esquerré-Tugayé suggest that the early increases in ethylene observed after inoculation may be occurring as a response to cell-surface interaction between host, possibly via special receptors, and pathogen. They also found that this interaction is not species specific; elicitors from different fungi can induce ethylene synthesis in non-host plants. Furthermore, these elicitors have been shown to stimulate the synthesis of both ethylene and the hydroxyproline-rich glycoproteins (Esquerré-Tugayé *et al.*, 1985). It has been suggested (Mauch, Hadwiger & Boller, 1984) that ethylene and fungal elicitors are actually separate signals which can both induce the biochemical defence reaction, of which synthesis of hydroxyproline-rich glycoproteins is a part.

In view of the possible existence of a glycoprotein-phenolic cell wall network containing lignin (described in Section 1.3.3), it is significant that phenolic compounds have also been observed to accumulate during infection. Glazener (1982) demonstrated that lignin-like materials are synthesised by young tomato fruits after infection by *Botrytis cineria*, and has suggested that the formation of a polyphenolic layer around the infection helps limit the spread of the fungus. Grand and Rossignol (1982) described changes in lignification involved in systemic protection of melons to *Colletotrichum lagenarium*. It appears that an initial inoculation with the fungus stimulates the enzymes required for lignin synthesis so that in a later exposure to the pathogen rapid lignification occurs and restricts the infection.

Hammerschmidt, Lamport and Muldoon (1984) studied the resistance of varieties of cucumber to the fungus *Cladosporium cucumerinum*. Their results support Esquerré-Tugayé's view that the increases in hydroxyproline-rich glycoprotein are a factor in disease resistance, and provide additional evidence for the involvement of lignin. In particular, resistant cultivars of cucumber showed a larger and more rapid increase in wall-bound hydroxyproline-rich glycoprotein and lignin than susceptible cultivars. The timing of the hydroxyproline enhancement indicates that, in resistant varieties, hydroxyproline increase is caused by the triggering of a resistance mechanism, while,

in susceptible cultivars, the enhancement occurs as a result of damage to the tissue caused by the pathogen. These workers also put forward that there must be some association between lignin deposition and extensin accumulation.

Further evidence for the possible role of extensin in the defence mechanism of plants can be found in the structural similarity of this glycoprotein to bacterial agglutinins. Many different plants produce glycoproteins which bind to infecting bacteria. Sequeira, Gaard and De Zoeten (1977) demonstrated that tobacco leaf cells can agglutinate avirulent cells of the bacterial wilt pathogen Pseudomonas solanacearum. The bacteria become attached and then enveloped by the plant cell walls. In contrast, virulent bacteria are not bound, but remain free to multiply in the intercellular spaces.

Potatoes also produce a bacterial agglutinin which was at first thought to be the same as the potato lectin isolated by Allen and Neuberger (see Section 1.6.2). Working with potatoes Sequeira and Graham (1977) showed that there was a correlation between virulence and the presence of a bacterial extracellular polysaccharide which protects the cells from becoming bound.

Mellon and Helgeson (1982) isolated a glycoprotein agglutinin from tobacco callus tissue cultures. They found that, like extensin, the protein was basic and rich in hydroxyproline, while the carbohydrate was predominantly arabinose. The glycoprotein also had a high content of lysine and histidine with a low proportion of acidic residues. This resulted in its being positively charged at physiological pH. The avirulent bacterial cells, which lack an extracellular polysaccharide, are negatively charged and hence agglutination could be caused by simple ionic interaction. The tobacco glycoprotein also agglutinated zoospores of both compatible and incompatible races of the fungus Phytophthora parasitica. This led to the conclusion that the agglutinins may not have any race-specific role and, if they have any function in recognition, they may merely be non-specific sensors of foreign invaders.

Leach, Cantrell and Sequeira (1982a) have isolated and characterised a similar agglutinin from potatoes, and have demonstrated that while this glycoprotein is very similar to other hydroxyproline-containing glycoproteins such as extensin and carrot cell wall glycoprotein, it is clearly distinct from potato lectin. In particular, the potato lectin and agglutinin have different biological activities. Thus the agglutinin strongly binds avirulent strains of P. solanacearum but has weak haemagglutinating activity, whereas the lectin agglutinates erythrocytes but not bacteria.

Using immunofluorescence techniques, Leach, Cantrell and Sequeira (1982b) have shown that potato agglutinins are located in the plant cell wall. They propose that, in general, agglutinins play a role in the initial interaction with bacteria, but conclude that it is not yet clear whether this interaction is specific, or if it leads to any particular host response.

Van Holst and Varner (1984) have noted the similarities in structure and activity between these agglutinins of tobacco and potato and carrot cell wall glycoproteins. This is illustrated by the observation that carrot glycoprotein will agglutinate avirulent cells of P. solanacearum. Cooper, Chen and Varner (1984) also comment that such activity as a non-specific bacterial agglutinin could be simply explained by the high isoelectric point - a consequence of the predominance of basic amino acid residues in this glycoprotein. Similarly Smith, Muldoon and Lampert (1984) point out the parallels between tomato extensin precursors and these agglutinins and suggest that structural and agglutination roles are not mutually exclusive.

In conclusion, extensin is clearly involved in the defence mechanisms of some plants to wounding and infection. There is evidence that the enhanced extensin levels may function by strengthening the cell wall, perhaps in a lignin-glycoprotein complex, or as bacterial agglutinins. Results of work with the cucumber-Cladosporium cucumerinum system for which, unlike the melon-Colletotrichum lagenarium system, resistant varieties are available, have shown that hydroxyproline-rich glycoprotein, and probably lignin, are also involved in a resistance mechanism.

1.5.4 Other roles

A number of other possible functions have been proposed for extensin. For example, Albersheim (1978) indicated that it could act as a carrier protein, with the function of transporting polysaccharides from the cytoplasm to the wall. Kauss and Glaser (1974) pointed out that the glycoprotein could guide the polysaccharides into their "right" position in the cell wall. An alternative idea involves extensin's acting as a primer for the synthesis of the wall polysaccharides, in a similar mechanism to the polymerisation of starch and glycogen (Brown & Kimmins, 1977).

1.6 Other Cell Wall and Hydroxyproline-rich Glycoproteins

1.6.1 Other cell wall glycoproteins

Some of the glycoproteins associated with the plant cell wall are enzymic in nature. Peroxidase is a glyco-enzyme which is found in the cell wall, where it is involved in lignin polymerisation. Lampert (1980) discussed how peroxidase was a typical example of extracellular enzymes which commonly have carbohydrate constituents which confer increased solubility, thermal stability and resistance to proteases. Suspicion that the cell wall peroxidase contained hydroxyproline, important in view of confusion with extensin, were finally answered by Liu and Lampert (1974), when they demonstrated that horseradish cell wall peroxidase iso-enzymes do not contain hydroxyproline.

Other protein components of the cell wall have been shown to play a role in protecting the plant against infection. Proteins which inhibit endo-polygalacturonase enzymes secreted by fungal pathogens have been extracted from bean hypocotyls, tomato stems and other dicotyledonous plants (Albersheim & Anderson, 1971; Anderson & Albersheim, 1972). These inhibitors have several properties which indicate that they may be part of the group of glycoproteins which have phytoagglutinating activity. These possible glycoproteins are specific inhibitors for polygalacturonases secreted by a variety of different fungi, but they do not affect other fungal enzymes, for example cellulase. This response

to different pathogens led Albersheim and Anderson-Prouty (1975) to propose that the polygalacturonase inhibitor system represents a general, rather than specific, mechanism for resistance. Recent work on closely related strains of beans with different resistances to anthracnose has shown that there are significantly higher levels of inhibitor bound to the walls of resistant lines than to the walls of susceptible ones (Lafitte *et al.*, 1984). This glycoprotein inhibitor is specific for endopolygalacturonase but, as found before, is inactive to other cell wall degrading enzymes. The presence of this inhibitor in cell walls was shown to protect against enzymic degradation, and is therefore likely to be effective in reducing infection. However, the levels of inhibitor do not change after infection, and hence the authors concluded that this only forms a passive resistance mechanism.

In this section on cell wall glycoproteins other than extensin, it is important to mention that, while developing extraction techniques for the hydroxyproline-rich glycoprotein, Selvendran (1975) also isolated a relatively hydroxyproline-poor glycoprotein fraction which was associated with the wall hemicellulose. These hydroxyproline-poor glycoprotein(s) may, like extensin, be structural; alternatively, they may be enzymes. Brown and Kimmins (1978) also observed hydroxyproline-poor glycoproteins produced after wounding of leaves.

1.6.2 Other hydroxyproline-rich glycoproteins

There are two other major types of secreted glycoproteins which have some similarities to extensin (Lamport, 1980), namely potato lectin and the arabinogalactan proteins. These glycoproteins are discussed briefly, and compared with extensin.

Potato lectin was first purified by Allen and Neuberger (1973) and shown to be a hydroxyproline-containing glycoprotein, with a specificity towards N-acetylglucosamine oligosaccharides. The glycoprotein was water-soluble, and contained 11% hydroxyproline, 3% galactose and 47% arabinose (Allen *et al.*, 1978). Potato lectin resembles extensin in that it contains both serine residues with the unusual alpha-linked galactose and hydroxyproline arabinosides. This lectin may be

representative of a class of soluble glycoproteins which would include precursors of extensin (Allen *et al.*, 1978). Muray and Northcote (1978) commented that potato lectin was an unusual example of a soluble glycoprotein in that it contained only short oligosaccharide sidechains.

The arabinogalactan proteins are a more recently discovered group of compounds and their location and function are not yet completely understood. They are found to occur intra- and extra-cellularly in seeds and plant tissues and are secreted by suspension-cultured cells (Pope, 1977; Selvendran & O'Neill, 1982). Sycamore extracellular polysaccharide (SEPS) is an arabinogalactan protein from cultured sycamore cells, which was at one time mistakenly thought to be an extensin precursor. Structurally, although having some similarities to the composition of extensin, these complexes contain a much higher proportion of alanine and highly branched carbohydrate. The latter is a 3,6-linked arabinogalactan, which is attached to the protein by hydroxyproline-galactose, a glycopeptide link which does not occur in extensin (Lampert, 1978; Clarke, Anderson & Stone, 1979). Interestingly, van Holst and Fincher (1984) have found that part of the protein of an arabinogalactan protein also exists in the same polyproline II conformation as extensin and *Chlamydomonas* cell wall glycoprotein.

Some of the arabinogalactan proteins are known as the "all beta-lectins" because they are less specific than classical lectins, like that of potato described above, and will bind to beta-anomers of different sugars. The beta-lectins are not haemagglutinins, presumably because each molecule only has a single carbohydrate binding site (Lampert & Catt, 1981).

Of particular interest, in view of confusion with extensin, is a report of a beta-lectin ionically bound to the walls of cultured bean cells (Van Holst *et al.*, 1980a).

1.7 Future Possibilities

Until recently, the insolubility of extensin has been the major obstacle to its study. Although, for some time, it has been accepted that

extensin plays a role in controlling growth and is involved in disease resistance, the inability to extract this glycoprotein from the cell wall has restricted knowledge of its structure.

A better understanding of the composition, sequence and secondary conformation has only emerged since the discovery of soluble extensin precursors. These precursors can be readily eluted from the wall and represent fully formed extensin molecules which have not yet been cross-linked. More recently, information on extensin structure has come from studies of its biosynthetic intermediates at a stage even earlier than soluble precursors. Part of the peptide sequence of extensin has been predicted from the RNA, and - most recently - the DNA, which code for this protein.

No doubt, the complete primary sequence of extensin will be elucidated eventually by these techniques. From such information the conformation of the polypeptide backbone and the arrangement and interaction of carbohydrate side chains should become clearer.

A major advance will be to locate all the sites of isodityrosine residues and identify which are intra- and intermolecular. It will be most interesting to confirm if these two types of links do indeed occur in different sequences. This will surely provoke investigations into the mechanisms which control cross-linking of extensin in the cell wall. A better understanding of how the formation of an insoluble matrix of extensin is regulated should explain the role of this glycoprotein in controlling growth. It should also clarify extensin's involvement in disease resistance, which currently appears rather confusing.

Following on from an elucidation of the structure and cross-linking of extensin, its relationship with other wall polymers remains to be confirmed. Advances in this area should greatly improve knowledge of the cell wall structure, in particular the question of whether extensin and cellulose really do exist in a woven network.

2. EXTRACTION OF EXTENSIN

2.1 Background and Objectives

At the start of this project, much of the information about extensin, which has been reviewed in the previous chapter, was not available. In particular, the existence of isodityrosine was not known, and hence the binding of extensin in the wall was not understood. The idea that the glycoprotein was attached to wall polysaccharides via its carbohydrate side chains was still being considered.

An objective at this stage was to extract extensin from the cell wall in order to characterise the composition and properties of this glycoprotein. It was intended to compare these characteristics with those of the same glycoprotein extract isolated after the cell wall had been treated by different food processing conditions. It was also planned to correlate any changes in the biochemical properties of the glycoprotein with corresponding mechanical or structural changes in the wall.

Preliminary work involved selecting a suitable plant tissue to study, and then developing a method to separate cell walls from this tissue. Such isolated cell wall fragments were used for all subsequent experiments. Early experimental work was directed at extracting extensin, or at least extensin glycopeptides, from the cell wall. The various methods that were employed in attempts to isolate extensin are discussed below.

As a result of these preliminary extraction experiments it was concluded that this particular approach was not satisfactory. Separation of extensin from the cell wall in order to investigate any changes which had been caused by processing was not feasible. It was therefore decided to modify the approach. The experimental plans that were adopted in the light of these early experiments, and with the benefit of additional information from the literature, are discussed in the final section of this chapter.

2.2 Preparation of Cell Wall Fraction

Mung bean seedlings Phaseolus mungo were selected for this study because their cellular structure is typical of the edible parts of most fruit and vegetables. Beanshoot tissue is largely composed of parenchyma cells, which make up the fleshy parts of plant foods, and the structure is described in more detail in Chapter 5. The shoots can be grown when required, to provide a living system which is directly comparable with fresh fruit and vegetables. The ability to provide a continuous sample of fresh material from seed avoided the need for storage and consequently prevented problems of deterioration that would have been encountered with material such as apples or carrots. In particular, as it was intended to study the effects of freezing, this method could not be used to preserve raw material.

An advantage of using young tissue, like seedlings, is that they do not contain starch storage granules which, if present, necessitate a more thorough washing of cell walls during preparation. In addition, young tissue has only undergone very limited lignification, restricted to the vascular cells. Secondary thickening with lignin would further complicate cell wall structure and would probably hinder glycoprotein extraction. Beanshoots were grown from seed, when required, in the dark under constant temperature and humidity conditions. Details of this method are given in Appendix A.1.

In developing a cell wall preparation method the aim was to homogenise tissue and separate clean fragments of wall in such a way that the final sample was free of cytoplasmic contamination, but otherwise as unchanged as possible. Published methods sometimes employ tissue homogenisation in solvents, and the available methods invariably involve a final alcohol or acetone washing and drying stage. It was thought that this treatment might precipitate intracellular proteins on to the wall, and it was thought that drying would certainly affect the physical structure of the wall.

The method that was chosen involved homogenisation in buffer followed by washing with sodium dodecyl sulphate (SDS) solution to remove membranes

and intercellular proteins (Bailey & Kauss, 1974). This resulted in a preparation of fine white cell walls which was used in subsequent experiments without being dried. The efficiency of tissue homogenisation was monitored by examination of samples with a light microscope.

Kauss and Bowles' method involved grinding seedlings with a pestle and mortar. This was time-consuming for large quantities of material and an ultrasonic homogeniser was tested as an alternative. This device was found to be very effective at breaking the tissue into small clumps of cells, but unfortunately it could not achieve further comminution. Consequently, the hand method for grinding was retained. The routine procedure that was used for preparing cell walls is described in Appendix A.2.

2.3 Enzyme Digestion

The main structural polymers of young plant cell walls are, apart from extensin, all carbohydrate. It should be possible therefore, to treat cell walls with a mixture of polysaccharide hydrolysing enzymes and render the glycoprotein soluble by dissolving away all the other wall components.

Commercial preparations of cellulase, pectinesterase and polygalacturonase were checked for absence of proteolytic activity. These enzymes were then used to digest cells walls (Appendix A.3). Each enzyme was added in approximately the same proportion as its polysaccharide substrate occurs in the wall.

The soluble extract resulting from cell wall digestion was separated by gel permeation chromatography (GPC) on Bio-gel P-10 (Bio-Rad). An acrylamide resin, such as this, was employed because agarose and dextran gels would not be stable to the polysaccharide degrading enzymes present in the extract. Bio-gel P-10 has a suitable molecular weight operating range such that the added enzymes were above the exclusion limit of 20,000, and hence were eluted in the void volume. GPC was performed in 0.1M Tris buffer at pH 7, containing 0.005% thimerosal as preservative.

The column was calibrated with albumin, alpha-lactalbumin, cytochrome C and histidyl-tyrosine as molecular weight standards.

The soluble extract was applied to the column and the fractions were assayed for protein using a modification of the Lowry method (Hartree, 1972). This indicated the presence of a small quantity of non-enzyme protein with a molecular weight of 2-3000. These non-enzyme protein fractions were subjected to isoelectric focusing at pH 3.5-10, but no proteins were resolved. Recent information, however, indicates that the conditions employed were not suitable for focusing extensin-like proteins. The isoelectric point of soluble carrot extensin is reported to be between 10 and 12 (Cooper, Chen & Varner, 1984). Hence it is possible that extensin glycopeptides were indeed isolated from the cell wall, but were not purified by the methods used.

Hydroxyproline content of wall fractions is a useful indication of the content of extensin. Accordingly the soluble enzyme digest, the insoluble residue and the added enzymes were assayed for hydroxyproline using a colorimetric method (B.S.4401, 1979). This assay involves acid hydrolysis and neutralisation followed by oxidation of hydroxyproline by chloramine-T and reaction with p-dimethylaminobenzaldehyde. Hydroxyproline forms a red product which is quantified, compared with standards, by measurement of the absorbance at 558 nm. Unfortunately, it was found that the hydroxyproline-content of the enzymes was much higher than that of the cell wall. Hence it was not possible to quantify glycoprotein extraction by hydroxyproline content.

It was concluded that digesting the cell walls with carbohydrate hydrolases may be, in principle, a feasible method of rendering extensin soluble. However, attempts to identify or quantify the extract had proved unsuccessful. As it was not possible to determine the effectiveness of this extraction method it was not investigated further.

2.4 Acidified Chlorite Extraction

A chemical method using sodium chlorite and acetic acid was reported by O'Neill & Selvendran (1980) to be effective at extracting glycoproteins

from runner-bean cell walls. This treatment, adapted from a delignification process, offered a means of extracting hydroxyproline-rich glycoproteins without using severe degradative conditions, which had previously been the only alternative.

Acidified chlorite is now known to split isodityrosine and this is probably how it effects extensin release from the cell wall. At the time the method was published, however, the mechanism was unknown and its authors could only propose that the reagents acted on some type of phenolic cross-link.

In their published procedure, O'Neill & Selvendran (1980) treated cell walls to remove pectin prior to extracting the glycoprotein. This pretreatment required extensive heating compared with the short glycoprotein extraction stage. In view of the objective of this work, to investigate the effects of processing, including different types of cooking, on the glycoprotein extract, it was felt that this depectination stage was undesirable and should be omitted if possible. Accordingly, the extraction method was tried in full, and with the pretreatment omitted.

Samples of cell wall were heated with 0.12% acetic acid and 0.3% sodium chlorite at 70°C for 30 minutes to extract glycoprotein. (Appendix A.4 Stage 2). Additional samples were depectinated by extraction with water at 80°C for four hours and with ammonium oxalate at 80°C for two hours, prior to glycoprotein extraction (Appendix A.4 Stages 1 and 2). The glycoprotein fractions were dialysed and freeze-dried.

Hydroxyproline contents of the acidified chlorite-soluble fractions were determined using the colorimetric assay (B.S.4401, 1979), outlined in the previous section. This assay is intended for meat products which contain much higher levels of hydroxyproline than the samples under test, and was replaced by a micromethod (Drozd, Kucharz & Szyja, 1976) in later experiments. Nevertheless, the hydroxyproline results obtained give an indication of the efficiency of glycoprotein extraction.

Hydroxyproline was measured as free imino acid after hydrolysis, and represents the quantity of non-dialysable hydroxyproline-containing polymers. It was found that more hydroxyproline was extracted from the cell wall by acidified chlorite when the depectination treatment was omitted. It was thought that the reduced levels of hydroxyproline found in the glycoprotein fraction prepared after depectination could be accounted for if the treatment extracted extensin in addition to pectin. In a later acidified chlorite extraction experiment (Section 4.3.3) the pectin fractions were also assayed, confirming that hydroxyproline was indeed extracted by the pretreatment.

Further glycoprotein fractions were prepared by the acidified chlorite method without the depectination stage, for electrophoresis. Samples were run on gradient polyacrylamide slabs and cellulose acetate strips in both TRIS-Boric acid-EDTA buffer at pH 8.4 and TRIS-Glycine buffer at pH 3.7. Amido black and Coomassie blue R. were used to stain protein, and Toluidine blue and Alcian blue to stain carbohydrate. Electrophoresis demonstrated that while the extracts were rich in carbohydrate, no protein components were apparent.

It was concluded, at the time, that despite the earlier observation that glycoprotein extracted directly from cell walls rich in pectin yielded a higher recovery of hydroxyproline, this extract probably also contained contaminating carbohydrate, which presented problems in electrophoresis of the protein. It is now known that extensin has unexpected electrophoretic behaviour under standard conditions of electrophoresis. Stuart & Varner (1980) discuss these characteristics of carrot extensin and suggest that they are a consequence of the high carbohydrate, hydroxyproline and basic amino acid content.

As a result of this experiment, it was decided, regardless of the electrophoresis results, that acidified chlorite was a potentially useful way of extracting extensin from the cell wall, and it was used in later work.

2.5 Salt Extraction

Stuart & Varner (1980) described a method by which hydroxyproline-rich glycoproteins could be extracted from carrot discs by treatment with calcium chloride solution. This procedure was tested on beanshoot cell walls as it appeared to offer a quick, simple means of extracting the glycoprotein by a cold process.

The extraction system used 0.2M calcium chloride and 5mM dithiothreitol, a disulphide-bond breaking agent. In addition, polyvinylpyrrolidone, at a concentration of 4.5%, was included, presumably to complex plant phenols and prevent their binding to the glycoprotein being isolated.

It was found that this method was not at all effective at extracting glycoprotein material from beanshoot cell walls. The reason for this is apparent from recent literature. Aerated carrot discs are actually injured tissues which synthesise large quantities of extensin. This extensin is readily extracted, by salt solutions, unlike the cross-linked glycoprotein in normal cell walls.

2.6 Discussion and Plans for Future Work

It was indicated, at the beginning of this chapter, that an early practical objective was to extract extensin from cell walls. It was planned to compare the properties of this glycoprotein extract from control and processed tissue, to see the effects of food-processing conditions. However, the results of these initial experiments, and the literature that had since become available, indicated that extensin was a much more integral component of the wall than was first thought. It had become apparent that it was not feasible to extract this glycoprotein without degrading the polymer or splitting critical cross-links. It was therefore decided at this stage, to revise the approach to look at the effects of processing on extensin as a part of the cell wall as a whole.

The position regarding the three different extraction methods which were investigated can be summarised as follows. Enzymic digestion of cell wall carbohydrate is a novel biochemical approach which may have been successful in extracting extensin, but it is not possible to tell with the analyses used. Salt extraction is a published method which, according to recent literature (see Section 1.2.5), is effective only with certain types of tissue and would not be expected to work with beanshoot cell walls. Recent publications (see Section 1.2.3 and 1.3.3) have also provided a mechanism for the effectiveness of acidified chlorite. It is now thought that this technique does not really extract the glycoprotein in its native state, but that the reagents split isodityrosine cross-links which hold the polymeric network in the wall. This technique is still a uniquely useful way of removing glycoprotein from the wall, albeit in the form of extensin monomers or partly cross-linked oligomers.

Various ways of measuring the mechanical or physical properties of the cell wall, in order to correlate these with changes in the chemical structure, were considered at this stage in the project. Differential scanning calorimetry (DSC) was contemplated to assess molecular conformation changes induced by heat and possibly other processes. Collagen, the animal structural protein, which has some structural similarities to extensin, is known to undergo characteristic thermal transitions which can be detected by DSC. Initial tests, however, indicated that the prepared extracts contained too complex a mix of polymers to study with the equipment available. It was also clear that this technique was not suitable for testing whole cell walls.

Several methods are available for measuring the texture of plant tissues. An Instron, or the Stevens-LFRA texture analyser could have been used to compare the mechanical properties of control and processed beanshoots. It was considered, however, that the texture changes observed would actually be related to the state of turgor of the cell contents rather than the strength of the cell wall. As it is only practically feasible to measure whole tissue, these techniques cannot provide quantitative information on cell wall strength.

An alternative, and workable, means of looking at changes in the structural properties of the cell wall is microscopy. A combination of light and electron microscopy of whole tissue shows the arrangement of groups of cells. This indicates the state of coherence of the tissue and shows any separation or compression of cells. Electron microscopy of isolated cell walls provides even more detail on the degree of damage such as fracture or layering occurring within the wall.

In the light of experience gained in this early stage it was decided to adopt a combination of microscopy, to look at structural changes, and amino acid analysis, to investigate chemical changes caused to the whole cell wall as a result of processing. Determination of amino acids which are destroyed by processing or which are dissolved out of the wall, provides an indication of the effect on wall proteins. In particular, as extensin is unusually rich in hydroxyproline, and is the only wall polymer likely to contain isodityrosine, analysis of these two components gives information on the specific effects on this glycoprotein. It has already been described above how microscopy can be used to determine how food processing affects cell wall structure. Changes in the appearance of the wall caused by different processes can also be compared with changes observed after chemical treatments to extract specific wall constituents such as pectin or glycoprotein.

A combined approach using amino acid analysis and microscopy was used to investigate the effects of processing on cell walls. The methods employed and the results obtained are described in Chapters 4 and 5, respectively. Prior to investigating changes in amino acid composition it was necessary to establish a routine technique for quantifying isodityrosine. The methods used to develop this assay and the steps taken to isolate and synthesise isodityrosine to use as a reference standard are described in the next chapter.

3. ISODITYROSINE

3.1 Introduction

Isodityrosine is a new type of tyrosine dimer, which has been identified in extensin. This tyrosine derivative can form inter-peptide links which are thought to polymerise the glycoprotein into an insoluble matrix in the cell wall. The history of the identification of isodityrosine is traced from earlier reports of an "unknown tyrosine derivative" in Section 1.2.3. The idea that isodityrosine enables the formation of a glycoprotein network which is independent of wall polysaccharides is discussed in Sections 1.3.3 and 1.3.4.

In view of the critical role played by isodityrosine it was important to investigate the effects of processing on this particular amino acid dimer. Any process which caused isodityrosine to split would weaken the binding of the glycoprotein and hence reduce the strength of the wall. Initial work was therefore aimed at establishing an automated amino acid analysis procedure for quantifying isodityrosine. The first section in this chapter describes the sequence of experiments carried out to develop an isodityrosine assay. A preliminary investigation indicated that isodityrosine could be one of three peaks in the amino acid chromatogram of cell wall hydrolysates. Subsequent work included various attempts to isolate and synthesise isodityrosine to confirm which of the three peaks corresponded with this compound.

The remainder of this chapter describes experiments undertaken with the objective of preparing sufficient isodityrosine to calibrate the amino acid analysis method. A procedure for isolating naturally occurring isodityrosine from cell wall hydrolysates is reported. Several different syntheses which were also employed to prepare isodityrosine are described.

3.2 Development of Automated Analysis for Isodityrosine

3.2.1 Investigation of the amino acid profile of cell walls

Isodityrosine is reported to be stable to 6N hydrochloric acid hydrolysis, (Appendix A.5) (Fry, 1982), a method routinely used to produce free amino acids for analysis. The tyrosine dimer is, however, split by acidified chlorite (Fry, 1982), and advantage was taken of this property in the first step of developing an assay.

A whole cell wall preparation was subjected to acid hydrolysis to release free amino acids and any isodityrosine. Part of this hydrolysate was then treated with acidified chlorite. The amino acid profiles of the two samples were measured using an LKB automated analyser (Appendix A.6.1). The chromatograms are shown in Fig.8, and the changes observed after acidified chlorite treatment are summarised in Table 2. It is apparent from the traces that there are three peaks, referred to as A, B and C, which might be isodityrosine. Of these A is much larger than B or C, and is at the beginning of the chromatogram where di- and tripeptides often elute.

The peaks with retention times of 440 and 542 seconds correspond to some unknown ninhydrin-positive components which are stable to acidified chlorite. To try to identify these components a number of unusual amino acids and related compounds were analysed. It was also hoped to find standards which coeluted with potential isodityrosine peaks, thus helping to eliminate them. Throughout the project a range of amino acids and physiological components were tested, as available, in an effort to identify the unknown peaks in chromatograms of cell wall hydrolysates. In particular, a number of tyrosine derivatives, such as 3-amino tyrosine, were tested for coelution with potential isodityrosine peaks. Where similarities appeared in retention times, samples of cell wall hydrolysate were spiked with the known compounds and re-analysed, to help confirm identity.

The peaks at 440 and 542 seconds have not yet been identified, although a few unusual amino acids, namely homoserine, alpha-amino adipic acid,

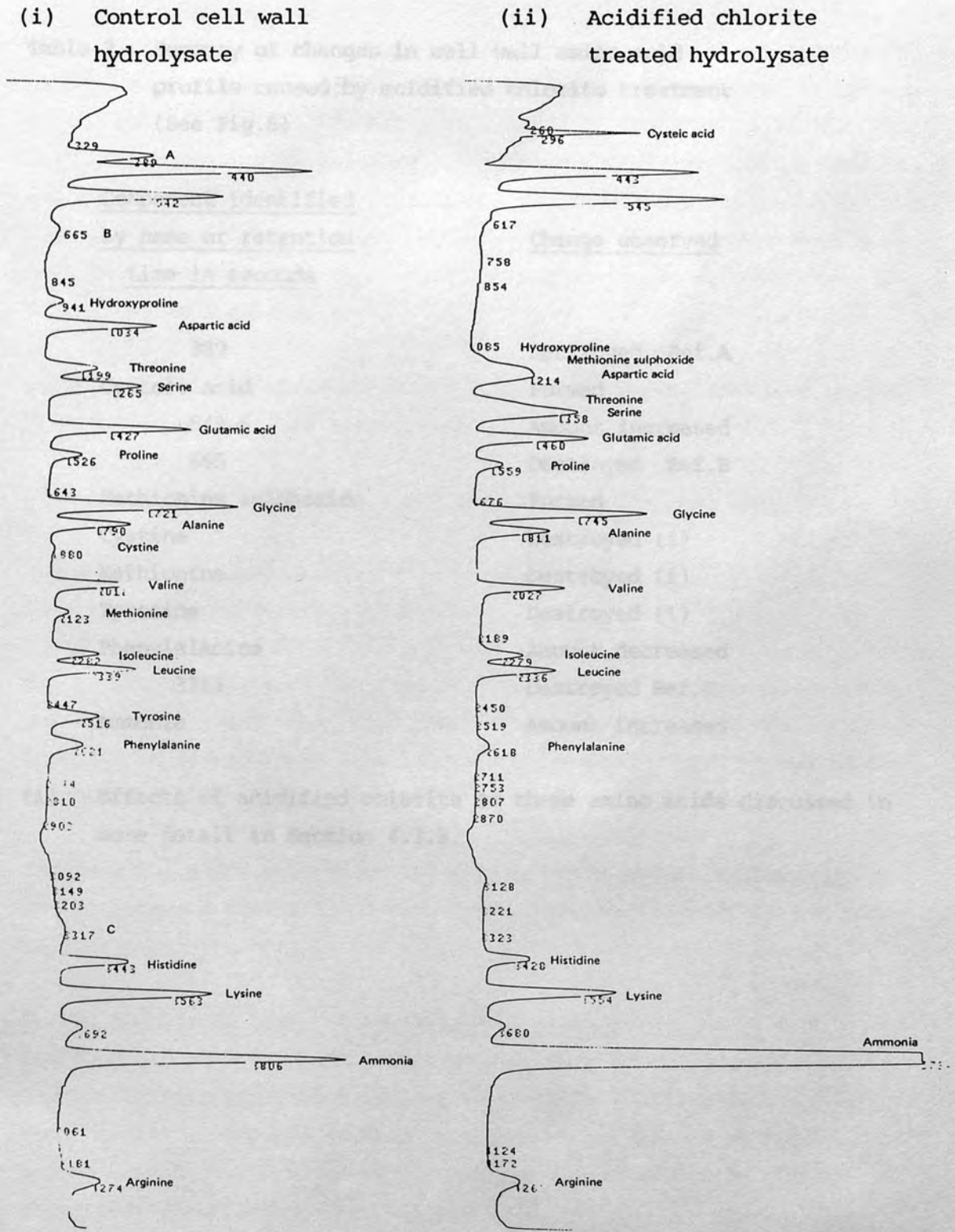


Fig. 8 Chromatograms showing effect of acidified chlorite on cell wall amino acid composition

(The significance of peaks A, B and C which are present in (i) but absent in (ii) are discussed in Section 3.2.1).

Table 2 Summary of changes in cell wall amino acid profile caused by acidified chlorite treatment
(See Fig.8)

| <u>Component identified</u> <u>by name or retention</u> <u>time in seconds</u> | <u>Change observed</u> |
|--|------------------------|
| 389 | Destroyed Ref.A |
| Cysteic acid | Formed |
| 542-5 | Amount increased |
| 665 | Destroyed Ref.B |
| Methionine sulphoxide | Formed |
| Cystine | Destroyed (i) |
| Methionine | Destroyed (i) |
| Tyrosine | Destroyed (i) |
| Phenylalanine | Amount decreased |
| 3317 | Destroyed Ref.C |
| Ammonia | Amount increased |

(i) Effects of acidified chlorite on these amino acids discussed in more detail in Section 4.3.3.

cysteic acid and methionine sulphoxide, have been positively identified as occurring in samples derived from cell wall hydrolysates. The size of the peak at 440 seconds has been noticed to increase in samples which have been subjected to repeated washing and rotary evaporating, and it has also been found, in other work at this laboratory, to occur in hydrolysates of seeds. This indicates that the compound producing the peak at 440 seconds may be a breakdown product or an unusual amino acid found only in plant material.

A table of all the amino acids and related compounds tested, with their corresponding retention times, is given in Appendix B.

3.2.2 Amino acid analysis of isodityrosine samples

Two samples of isodityrosine were kindly supplied by Steven Fry, who was then working in Colorado, U.S.A.

Sample 1 was an acetone solution of isodityrosine isolated from potato callus cell walls. Analysis gave a single small peak at 413 seconds, which is in between peak A and the chlorite-stable unknown peak at 440 seconds.

Sample 2 was a dry portion of synthetic isodityrosine prepared by Fry. Analysis gave a single large peak centred at 3314 seconds in the region of peak C.

It was considered that the isolated sample was possibly the true compound and that the synthesised sample could have been modified, for example by decarboxylation, during the lengthy purification. Decarboxylation would produce a more basic compound and consequently a longer retention time. This possibility was investigated by an experiment described in the next section.

During amino acid analysis of sample 2 it was observed that the integrator trace showed a negative peak at about 390 seconds. Further investigation showed that this peak appeared in most runs and, because it coincided with the position of peak A, it upset quantification of this component. Thiodiethanol, which is added to all ready-prepared

buffers used for amino acid analysis was found to be the cause of the negative peak, and for all subsequent analyses buffers were made up without this ingredient.

3.2.3 Isodityrosine synthesis

The chemical synthesis method used by Steven Fry employed oxidation of tyrosine with ferricyanide (Fry, 1984). This procedure was undertaken with the purification steps omitted to see if peaks A, B or C were present in the chromatograms of the crude mix. Amino acid analysis of the synthesis product indicated that it contained components eluting in the regions of both peaks A and C.

In a further experiment, thin-layer chromatography (TLC) was employed to separate the larger polymers of tyrosine in order to simplify the amino acid chromatogram. The resulting sample yielded a broad peak at 3305 seconds, in region C, but there were no peaks in regions A and B.

Experimental details of the synthesis and TLC methods are given in Appendix A sections 7 and 8, respectively.

3.2.4 Attempt to adapt assay procedure to isolate isodityrosine

An assay for isodityrosine (Fry, 1982; 1984), involving paper electrophoresis followed by descending chromatography was scaled up in an attempt to isolate naturally occurring isodityrosine from cell wall hydrolysate (Appendix A.9). The initial stage alone was tested, and a sample eluted from the electrophoretogram gave a clear peak with a retention time of 443 seconds and a small peak at C. The sample contained a very high concentration of ammonia, used for elution, and it was suspected that this may have raised the pH enough to interfere with the normal separation.

Attempts to wash out ammonia from this sample, and attempts at isolation using both steps of the method yielded very low levels of amino acids and no peaks of interest.

3.2.5 Observation of the secondary amine character of components giving peaks A and B

Proline, hydroxyproline and other secondary amino acids yield a yellow product with ninhydrin whereas all primary amino acid products are blue. Amino acid analysis of a cell wall hydrolysate was carried out while the absorbance of the ninhydrin product was monitored at both 440nm and 570nm. It was observed that some of the components - those causing peak A, the peak at 440 seconds and peak B - absorbed more strongly at 440nm and were therefore behaving as secondary amines. This indicated that the peaks at A and B could not be isodityrosine. For comparison, the isolated sample (Number 1) provided by Fry, which gave a retention time of 413 seconds, was analysed under similar conditions. It was observed that this component also reacted like a secondary amine.

Further inspection of the traces showed that the smaller peak centred at 3314 seconds, peak C, yielded a blue product like other amino acids and was, therefore, still a possible candidate for isodityrosine.

3.2.6 Summary of evidence for isodityrosine peak in cell wall hydrolysate chromatograms

Comparison of chromatograms of normal and chlorite-treated cell wall hydrolysates indicates that three alternative peaks, identified as A, B and C (Fig.8) could correspond to isodityrosine. The chromatogram of an isolated sample of isodityrosine, supplied as a gift, did not correspond exactly to any of these three peaks, but was close to peak A. In contrast, analysis of a synthetic sample, similarly supplied, indicated that isodityrosine eluted at peak C. It was thought, however, that this synthetic sample may have been modified during purification. Subsequent preparation of synthetic isodityrosine, using the same method but omitting the purification stage, and analysis of the crude mix revealed that both peaks A and C were present. Significantly, simple TLC purification to remove larger tyrosine polymers resulted in a sample containing only peak C. The evidence in favour of A or C was, however, not much further clarified by the next experiment to isolate naturally occurring isodityrosine from cell wall

hydrolysates. This product contained compounds which eluted at C as well as close to A. A final observation ruled out the possibility that either peaks A or B could correspond to isodityrosine. It was found that the amino acids giving these peaks behaved as secondary amines, whereas isodityrosine (see Fig.3) is a primary amine.

In view of this inference that peak C corresponded to isodityrosine, experiment 3.2.1 was repeated on a larger scale to confirm that this small peak was indeed lost after acidified chlorite treatment.

Another, larger sample of isodityrosine was later received from Steven Fry. This material was checked by TLC in comparison with tyrosine and synthetic dityrosine and found to agree with published R_f values. Methods for dityrosine synthesis and TLC are given in Appendix A parts 10 and 8, respectively. Amino acid analysis of this sample showed it to contain a single peak which coeluted exactly, in spiking tests, with peak C in a cell wall hydrolysate. For subsequent analysis peak C was taken to correspond to isodityrosine.

A typical chromatogram of cell wall hydrolysate is shown in Fig.9. The isodityrosine peak is unusually broad compared with other amino acids, and this was found to be a characteristic shape for this component.

A sufficiently large sample of isodityrosine was not available to prepare a quantitative reference standard, to enable calculation of the molar response of isodityrosine in the ninhydrin detection system employed. The value for tyrosine, which would be expected to be similar to isodityrosine (on a weight basis) was therefore used in all quantitative estimations of isodityrosine.

3.3 Preparation of Isodityrosine - Isolation from Cell Walls

3.3.1 Background

Attempts to isolate isodityrosine from cell wall hydrolysates using a combination of paper electrophoresis and chromatography were described previously (3.2.4). Further efforts to scale up this technique resulted

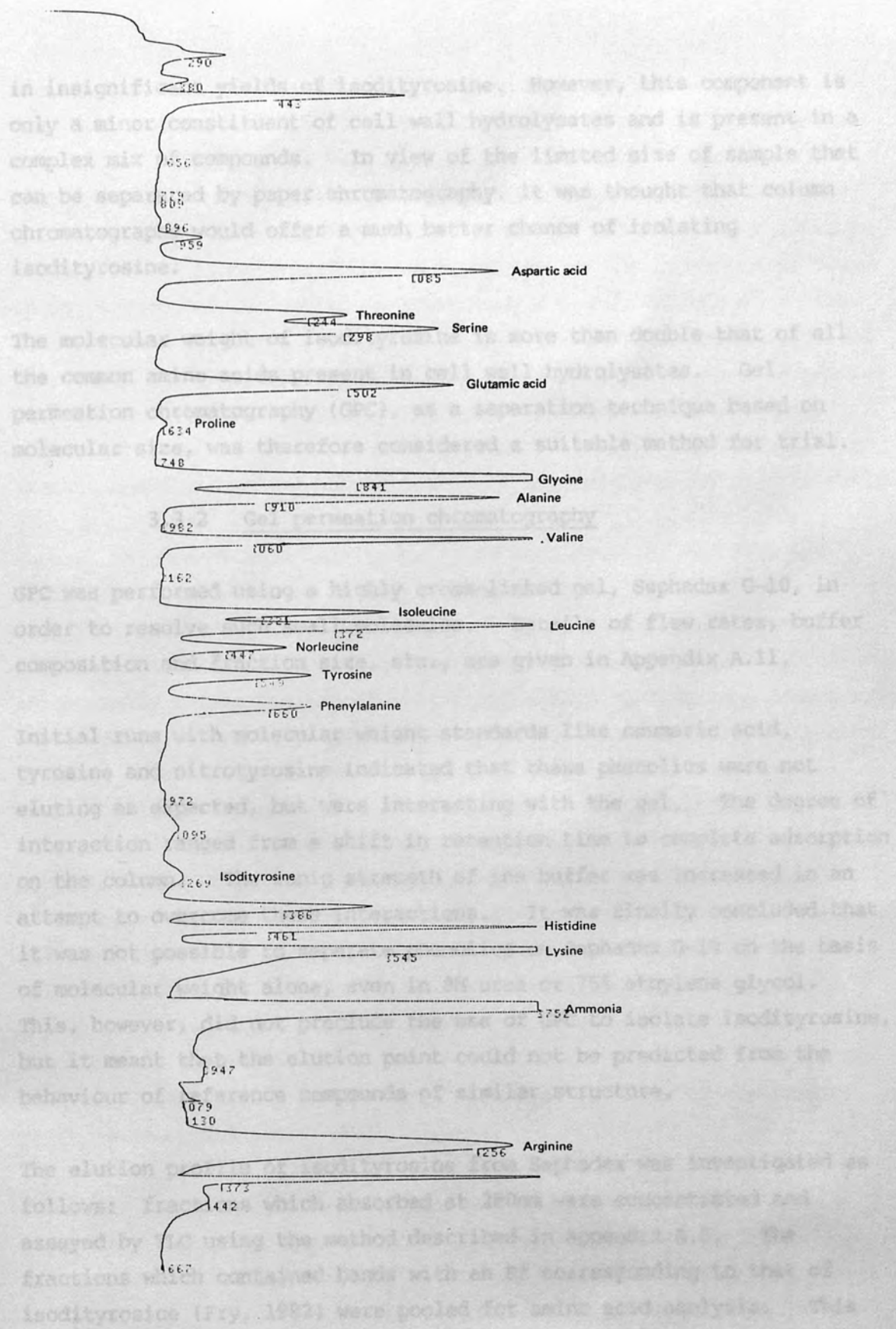


Fig. 9 Chromatogram of cell wall hydrolysate, LKB Analyser

in insignificant yields of isodityrosine. However, this component is only a minor constituent of cell wall hydrolysates and is present in a complex mix of compounds. In view of the limited size of sample that can be separated by paper chromatography, it was thought that column chromatography would offer a much better chance of isolating isodityrosine.

The molecular weight of isodityrosine is more than double that of all the common amino acids present in cell wall hydrolysates. Gel permeation chromatography (GPC), as a separation technique based on molecular size, was therefore considered a suitable method for trial.

3.3.2 Gel permeation chromatography

GPC was performed using a highly cross-linked gel, Sephadex G-10, in order to resolve such small molecules. Details of flow rates, buffer composition and fraction size, etc., are given in Appendix A.11.

Initial runs with molecular weight standards like coumaric acid, tyrosine and nitrotyrosine indicated that these phenolics were not eluting as expected, but were interacting with the gel. The degree of interaction ranged from a shift in retention time to complete adsorption on the column. The ionic strength of the buffer was increased in an attempt to overcome these interactions. It was finally concluded that it was not possible to separate phenolics on Sephadex G-10 on the basis of molecular weight alone, even in 8M urea or 75% ethylene glycol. This, however, did not preclude the use of GPC to isolate isodityrosine, but it meant that the elution point could not be predicted from the behaviour of reference compounds of similar structure.

The elution profile of isodityrosine from Sephadex was investigated as follows: fractions which absorbed at 280nm were concentrated and assayed by TLC using the method described in Appendix A.8. The fractions which contained bands with an R_f corresponding to that of isodityrosine (Fry, 1982) were pooled for amino acid analysis. This technique confirmed the presence of isodityrosine.

Accordingly, it was decided to scale up the GPC isolation of isodityrosine by combining fractions from several runs. Prior to this, the following refinements were applied to the method. The optimum sample loading and flow rate to achieve maximum yield and best resolution from phenylalanine and tyrosine, the two components eluting either side of isodityrosine, were investigated. A procedure to clean up the sample before loading on the column was also introduced. The cell wall was extracted with ethyl acetate to remove phenolic acids and the sample was then run through mini-columns of Sephadex G-10 to remove compounds which adsorb to this resin. The total eluate was then concentrated and applied to the main column. These modifications were checked and found not to reduce the yield of isodityrosine significantly.

Several batches of cell wall hydrolysate were prepared and each was chromatographed in two or three portions, with the intention of pooling fractions containing isodityrosine. During the course of these isolations amino acid facilities were not available. When it subsequently became possible to carry out analysis, fractions from a few representative runs were checked for the exact position of isodityrosine elution. It was found that the yield of this component varied considerably. A number of possible causes of this variation were considered in detail. The age of hydrolysate and the length of storage at each of the stages of preparation and the immediately previous treatment of the column such as cleaning or storage in preservative have all been ruled out. It appeared that the yield of isodityrosine was related to batch of cell wall hydrolysate more than any other single factor. In the light of data accumulated from processing experiments described in Chapter 4, it is now clear that the amount of isodityrosine does vary considerably between cell wall hydrolysates, an observation which is discussed later (3.5).

A further batch of cell wall hydrolysate was chromatographed and the concentrations of isodityrosine in the sample loaded on to the column was analysed in addition to the amounts in different fractions to check for recovery. This hydrolysate contained the equivalent of 0.19 mg isodityrosine per gram dry cell wall which is now known to be low

compared with all the data collected for controls in the processing tests (see Appendix C).

The amount of isodityrosine recovered in the fraction containing most of this component was found to be approximately 27% of that loaded. The previous fraction also contained isodityrosine but this was not resolved from another component by amino acid analysis and hence could not be quantified. The weights of isodityrosine loaded and recovered are listed in Table 4 for comparison with later experiments. Copies of chromatograms of the starting hydrolysate and the major isodityrosine-containing fraction are given in Fig.10. It is relevant to note that this fraction, whilst only containing 27% of the isodityrosine loaded, does not contain any significant quantities of impurities. This degree of purification was not found in subsequent isolations.

3.4 Preparation of Isodityrosine - Synthesis

3.4.1 Adaptation of a method for dityrosine

Dityrosine is a tyrosine derivative which is found in some animal proteins such as collagen. This compound has a biphenyl structure in contrast to isodityrosine, which has an ether link between the two tyrosine residues. The structures of di- and isodityrosine are given in Fig.11. A number of syntheses for dityrosine have been published, and the method of Gross and Sizer (1959) was employed to synthesise a sample for a reference marker for TLC. This synthesis involved incubation of tyrosine with horseradish peroxidase and hydrogen peroxide at pH 9.2. It was considered that lowering the incubation pH to approximate to that prevailing in the cell wall might result in synthesis of isodityrosine.

This hypothesis was tested. The method for synthesising dityrosine (Appendix A.10) was followed, and incubations were set up at both pH 4 (adjusted by the addition of hydrochloric acid) and pH 9.2.

It was observed that after the enzyme was precipitated with alcohol and filtered, the filtrate at pH 9.2 was yellow whereas the one at pH 4 was colourless.

(i) Cell wall hydrolysate

10 μ l hydrolysate diluted 1:1 with loading buffer.

Sample loaded on to GPC contained 400 x as much isodityrosine as this analysis.

Loaded 0.25 μ g norleucine in this analysis sample.

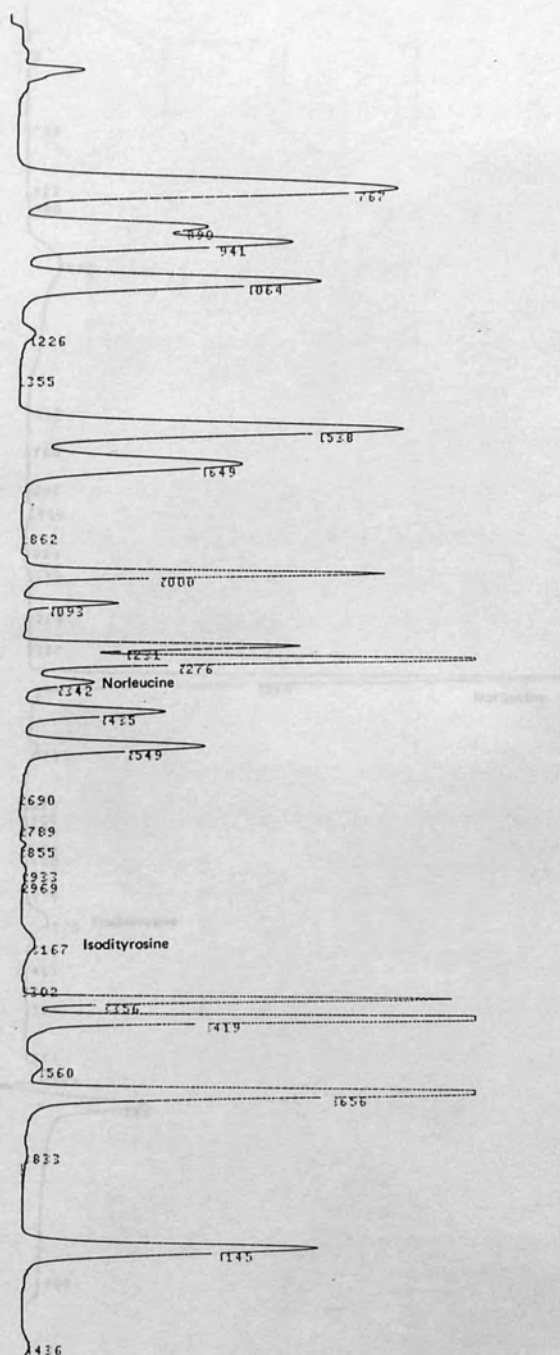


Fig. 10 Amino acid chromatograms of cell wall hydrolysate used for GPC, and major isodityrosine containing fraction

(ii) Fraction 27

80 µl fraction 27 diluted 1:1 with loading buffer.

Total fraction (4.35 ml) contains 109 x as much isodityrosine as this analysis.

Loaded 2 µg norleucine in this analysis sample.

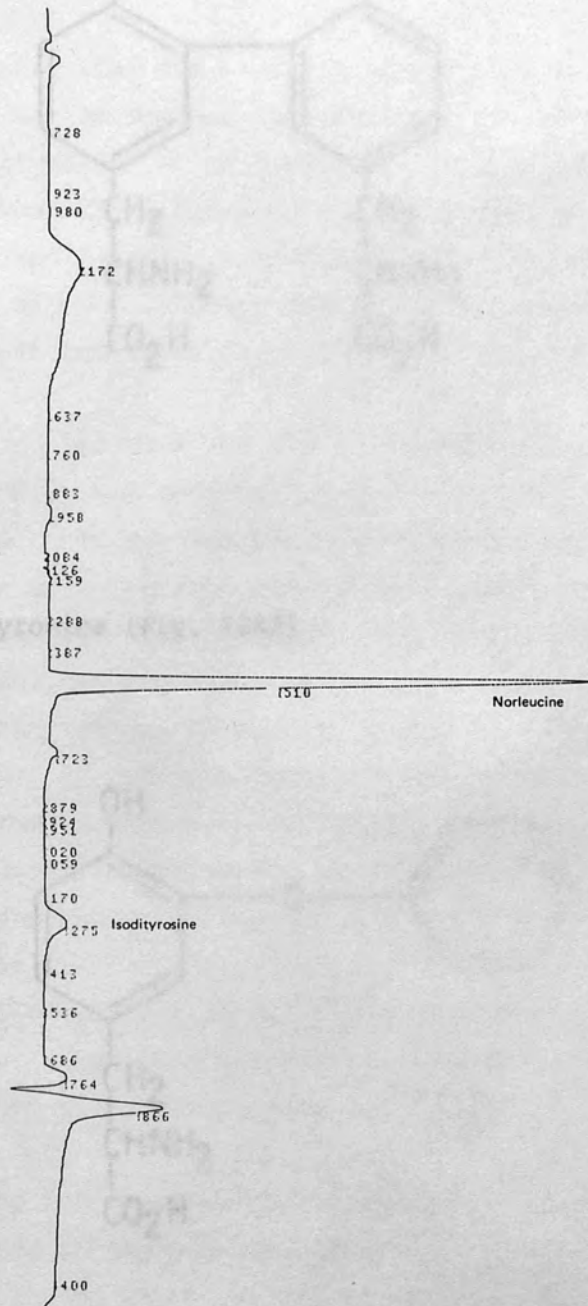
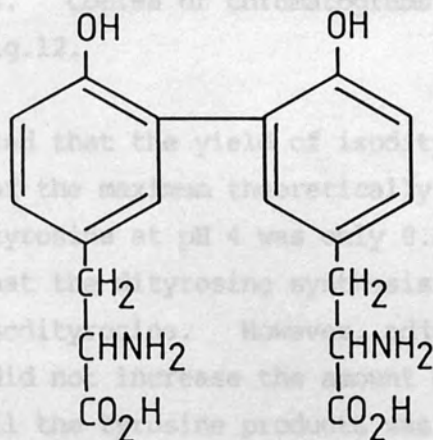


Fig. 10/Continued

(i) Dityrosine

... of the incubation mix was concentrated and dissolved in loading buffer for amino acid analysis. Standards of isodityrosine and dityrosine (supplied by Steven Fry) were run for comparison of retention times. Values of chromatograms of the incubation products are shown in Fig.11.

It was calculated that the yield of isodityrosine from the incubation at pH 9.2 was 14% of the amount theoretically possible from tyrosine. The yield of isodityrosine at pH 4 was 1.1% of maximum. This has demonstrated that the tyrosine system is also capable of synthesizing isodityrosine. However, at the pH 4 system, the cell wall did not make the amount of isodityrosine produced, and the yield of all the tyrosine products were reduced in the pH 4 system.



In an attempt to increase the yield of isodityrosine, the incubation method, at pH 9.2, was modified by replacing the tyrosine by 3-iodotyrosine. It was thought that tyrosine and isodityrosine might react together more readily than tyrosine alone because being

(ii) Isodityrosine (Fry, 1982)

the reaction between two tyrosine residues. In addition, the position of the iodine group should direct the attack of tyrosine towards carbon number 3, and thus preferentially form isodityrosine, rather than other tyrosine products. In the synthesis of isodityrosine, the iodine group was used to direct the synthesis of isodityrosine. It was calculated, based on the yield of isodityrosine plus tyrosine. The inclusion of isodityrosine in the incubation yield above that of tyrosine alone.

A sample of the isodityrosine synthesis product was purified by the GC method described in the previous section. It was found that all of the isodityrosine loaded on to the GC column was recovered in three fractions, although the fraction containing the isodityrosine contained another component of the synthesis as well. It could theoretically be possible, because of the 1:1 ratio of tyrosine and isodityrosine

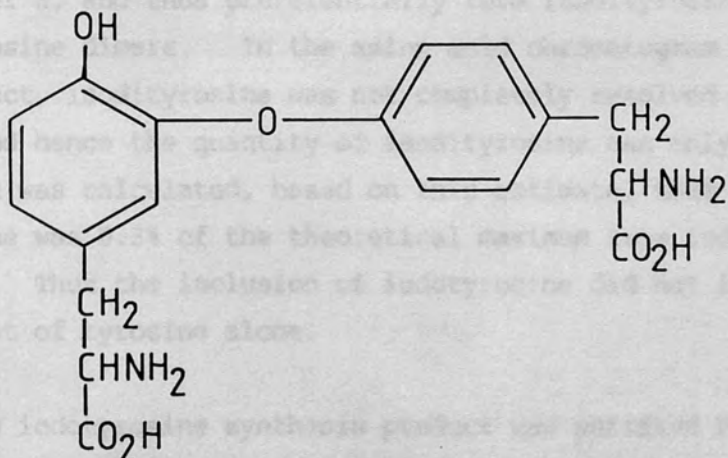


Fig.11 Comparison of the structures of di- and isodityrosine

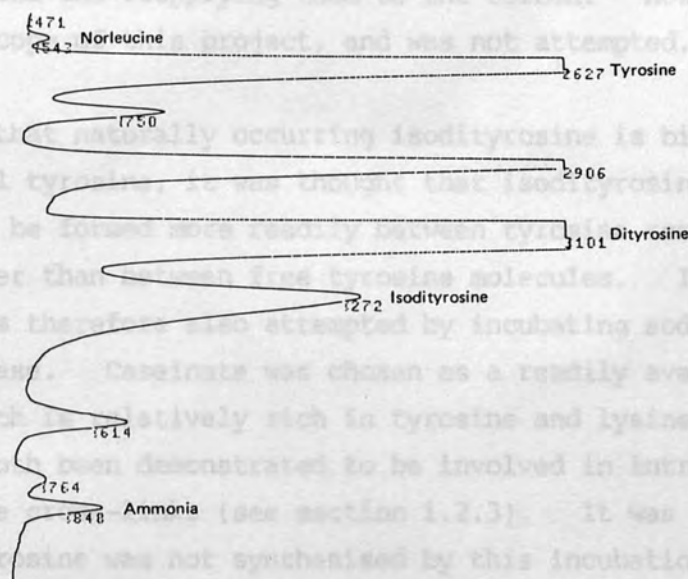
A portion of each of the incubation mixes was concentrated and dissolved in loading buffer for amino acid analysis. Standards of isodityrosine and dityrosine (supplied by Steven Fry) were run for comparison of retention times. Copies of chromatograms of the incubation products are shown in Fig.12.

It was calculated that the yield of isodityrosine from the incubation at pH 9.2 was 1% of the maximum theoretically possible from tyrosine. The yield of isodityrosine at pH 4 was only 0.1% of maximum. This has demonstrated that the dityrosine synthesis system is also capable of synthesising isodityrosine. However, adjusting the pH to that found in the cell wall did not increase the amount of isodityrosine produced, and the yield of all the tyrosine products was reduced in the pH 4 system.

In an attempt to increase the yield of isodityrosine, this incubation method, at pH 9.2, was modified by replacing half the tyrosine by 3-iodotyrosine. It was thought that tyrosine and iodotyrosine might react together more readily than tyrosine alone because iodine, being more electronegative than hydrogen, should be easier to eliminate during the reaction between two tyrosine residues. In addition, the position of the iodo group should direct the attack of tyrosine hydroxyl groups to carbon number 3, and thus preferentially form isodityrosine, rather than other tyrosine dimers. In the amino acid chromatogram of this synthesis product, isodityrosine was not completely resolved from iodotyrosine and hence the quantity of isodityrosine can only be estimated. It was calculated, based on this estimate, that the yield of isodityrosine was 0.3% of the theoretical maximum from iodotyrosine plus tyrosine. Thus the inclusion of iodotyrosine did not increase the yield above that of tyrosine alone.

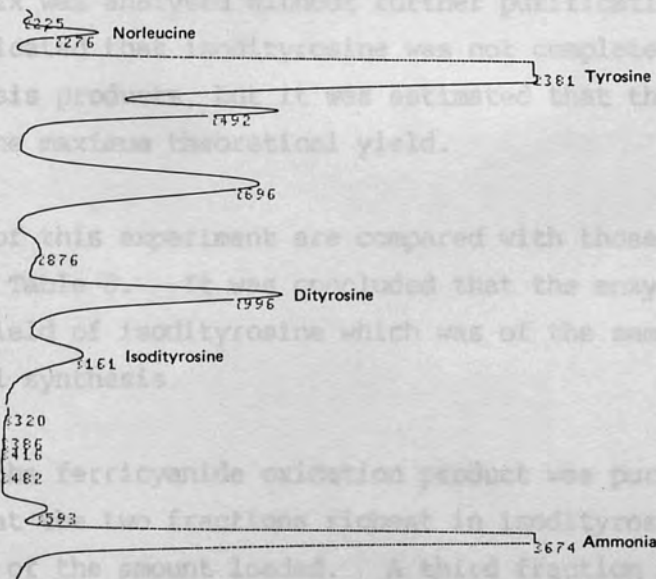
A sample of the iodotyrosine synthesis product was purified by the GPC method described in the previous section. It was found that 68% of the isodityrosine loaded on to the GPC column was recovered in three fractions, although the fraction containing the most isodityrosine contained another component of the synthesis mix as well. It should theoretically be possible, because of the relative elution patterns of this impurity and isodityrosine, to improve resolution by concentrating

(i) At pH 9.2 (Dityrosine synthesis method)



Half total incubation mix dried, dissolved in 3 ml loading buffer containing 0.15 mg norleucine. Portion diluted 5 times. Loaded 10 μ l (i.e. loaded 0.1 μ g norleucine)

(ii) At pH 4



Half total incubation mix dried, dissolved as above. Loaded 5 μ l (i.e. loaded 0.25 μ g norleucine)

Fig. 12 Amino acid chromatograms of incubation products resulting from tyrosine incubation with peroxidase

these fractions and reapplying them to the column. However, this was beyond the scope of this project, and was not attempted.

Considering that naturally occurring isodityrosine is biosynthesised from peptidyl tyrosine, it was thought that isodityrosine cross-links may possibly be formed more readily between tyrosine residues in a protein rather than between free tyrosine molecules. Isodityrosine synthesis was therefore also attempted by incubating sodium caseinate with peroxidase. Caseinate was chosen as a readily available raw material which is relatively rich in tyrosine and lysine, amino acids which have both been demonstrated to be involved in intramolecular isodityrosine cross-links (see section 1.2.3). It was found, however, that isodityrosine was not synthesised by this incubation method.

Fry's (1984) isodityrosine synthesis method involving ferricyanide oxidation of tyrosine, which had been employed in experiment 3.2.3, was repeated quantitatively for comparison with the enzymic syntheses. Details of the method are given in Appendix A.7, and a portion of the incubation mix was analysed without further purification. Amino acid analysis indicated that isodityrosine was not completely resolved from other synthesis products, but it was estimated that this method resulted in 0.6% of the maximum theoretical yield.

The results of this experiment are compared with those of the previous syntheses in Table 3. It was concluded that the enzymic methods produced a yield of isodityrosine which was of the same order as that from chemical synthesis.

A sample of the ferricyanide oxidation product was purified by GPC. It was found that the two fractions richest in isodityrosine contained a total of 22% of the amount loaded. A third fraction also contained isodityrosine but this was not resolved by amino acid analysis and could not be quantified. All of these fractions also contained dityrosine and the elution patterns of the two tyrosine dimers are probably too similar to enable further resolution.

Table 3 Summary of yields of isodityrosine from different syntheses

| <u>System</u> | <u>Yield as a % of theoretical maximum</u> |
|---|--|
| Tyrosine + peroxidase pH9.2 | 1.0 |
| Tyrosine + peroxidase pH4 | 0.1 |
| Tyrosine + iodotyrosine + peroxidase | 0.3* |
| Tyrosine + ferricyanide | 0.6* |

* Calculation used estimate of isodityrosine peak area because of poor resolution in amino acid analysis.

Quantitative data on the amounts of isodityrosine loaded and recovered from these syntheses products are given in Table 4 for comparison with other samples.

3.4.2 Synthesis using cell wall systems

Initial experiments

It was considered that the particular conditions present in the cell wall, for example presence of polyanionic pectin, may provide an environment uniquely conducive to isodityrosine synthesis. An experiment was therefore set up to see if the concentration of isodityrosine could be increased. Cell walls were incubated with peroxidase and hydrogen peroxide for 24 hours (details in Appendix A.12).

The incubation mix was acid-hydrolysed and hydrolysates of cell wall and enzyme alone were prepared for comparison of amino acid profiles. Of particular interest, it was found that isodityrosine naturally present in the control was absent from the incubation product. It thus appeared that isodityrosine had actually been destroyed during the incubation.

In order to confirm this observation, additional controls comprising cell walls alone, and cell walls incubated with hydrogen peroxide but without enzyme, were prepared. Analysis of hydrolysates of these samples confirmed that isodityrosine was destroyed by hydrogen peroxide treatment. It was also found that some methionine, phenylalanine, histidine and tyrosine were lost, presumably oxidised by the conditions used.

The reaction between tyrosine moieties in cell wall glycoprotein to form isodityrosine is an enzymic reaction, proposed to be catalysed by peroxidase (Fry, 1982; 1984). It was thought that addition of tyrosine, a reactant in this system, might help to shift the equilibrium in favour of formation of product, isodityrosine. This idea was investigated in the experiment described below. It should be noted that

Table 4 Recovery of isodityrosine from different experimental systems by GPC

| Sample (GPC experiment reference) | Isodityrosine loaded mg | Isodityrosine recovered mg | % recovered | Number of fractions containing this amount isodityrosine |
|--|-------------------------------|----------------------------------|----------------|--|
| Cell wall hydrolysate (3.3.2) | 0.11 | 0.03 | 27 | 1 ⁽ⁱ⁾ |
| Iodotyrosine synthesis (3.4.1) | 0.22 | 0.15 | 68 | 3 ⁽ⁱ⁾ |
| Ferricyanide synthesis (3.4.1) | 0.41 | 0.09 | 22 | 2 ⁽ⁱ⁾ |
| Cell wall + tyrosine incubation 3 (3.4.2) | 0.15 | 0.008 | 5 | 1 ⁽ⁱⁱ⁾ |
| Cell wall + tyrosine incubation 4 (3.4.2) | 0.08 | 0.004 | 5 | 4 ⁽ⁱⁱ⁾ |

Notes:

- (i) 100 drop fractions
- (ii) 50 drop fractions

this was carried out in parallel with the previous experiment and the adverse effect of hydrogen peroxide on isodityrosine was, consequently not known.

Two test systems were set up corresponding exactly to the previous experiment. In the first system cell walls were incubated with peroxidase and hydrogen peroxide as before, but with tyrosine added. It was calculated that the addition of 0.02% tyrosine to the incubation mix would give a reasonable excess over the concentration of cell wall tyrosine. The second test involved incubation of cell walls just with tyrosine to see if any endogenous cell wall enzymes could catalyse the formation of isodityrosine. The incubation mixes were hydrolysed and the amino acid compositions compared with that of a corresponding cell wall control.

It was found that no isodityrosine was present after cell walls were incubated with hydrogen peroxide. However, of particular significance, a relatively large peak corresponding to isodityrosine was found in the second test sample. Chromatograms of this test incubation and a control are given in Fig.13. The identity of the isodityrosine peak was confirmed by spiking with authentic dityrosine and isodityrosine.

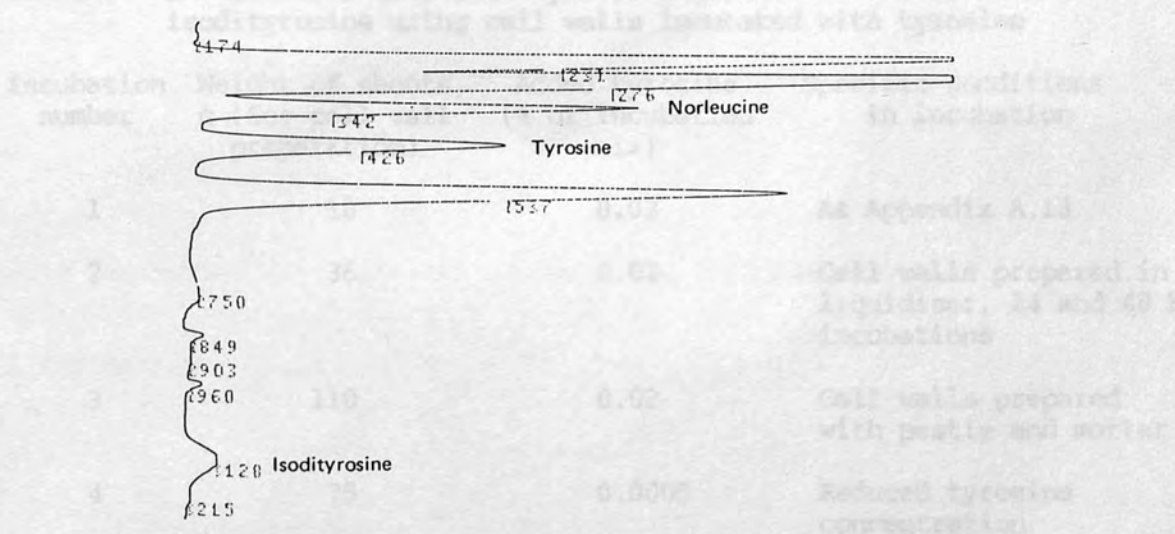
It was calculated that the concentration of isodityrosine was increased to more than six times that of the control. The quantitative results are given in Table 5, incubation number 1 (this formed the first in a series described below).

It was concluded from this experiment in which isodityrosine was successfully synthesised by incubating cell walls with tyrosine alone, that this could be a suitable method for preparing isodityrosine. It was decided to scale up the incubation to provide a larger sample suitable for purifying isodityrosine by gel permeation chromatography.

Further developments

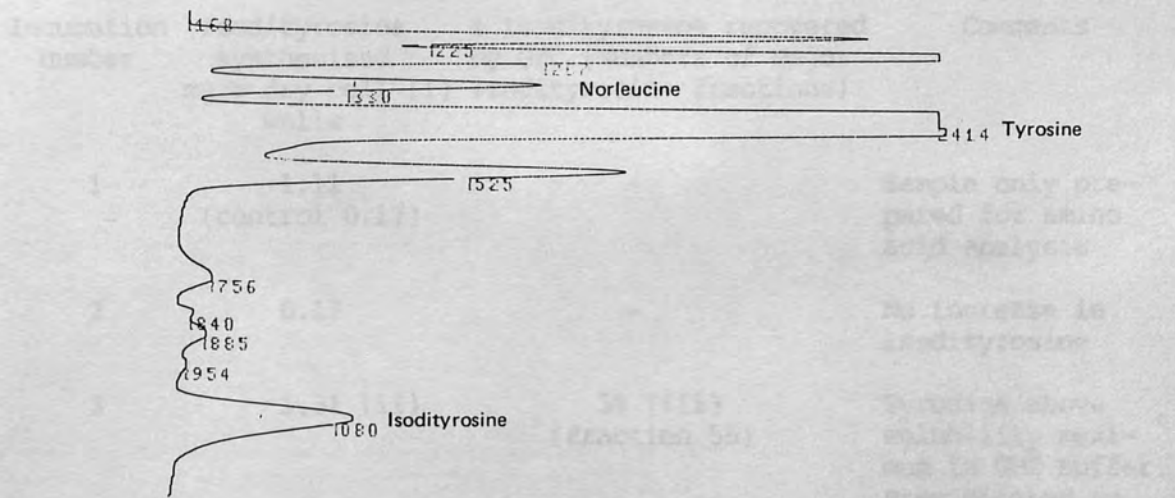
The cell wall and tyrosine system for synthesising isodityrosine is described above. A number of modifications to the initial procedure

(i) Cell wall control



Contains 0.17 mg isodityrosine per g dry cell wall

(ii) Cell wall incubated with tyrosine



Contains 1.11 mg isodityrosine per g dry cell wall

Fig. 13 Amino acid chromatograms of hydrolysates of cell walls incubated with tyrosine (Section 3.4.2) and control

Both samples contain same amount of norleucine.
Note increased size of isodityrosine peak in (ii)

Table 5 Quantitative data from syntheses and isolations of isodityrosine using cell walls incubated with tyrosine

| Incubation number | Weight of shoots, g (for cell wall preparation) | Added tyrosine (% of incubation mix) | Specific conditions in incubation |
|-------------------|---|--------------------------------------|--|
| 1 | 10 | 0.02 | As Appendix A.13 |
| 2 | 36 | 0.02 | Cell walls prepared in liquidiser, 24 and 48 h incubations |
| 3 | 110 | 0.02 | Cell walls prepared with pestle and mortar |
| 4 | 75 | 0.0005 | Reduced tyrosine concentration |
| 5 | 75 | 0.01 | Intermediate tyrosine concentration |
| 6 | 10 | 0.02 | Refrigerated after incubation |

| Incubation number | Isodityrosine synthesised mg/g dry cell walls (i) | % Isodityrosine recovered by GPC (Numbers of major isodityrosine fractions) (ii) | Comments |
|-------------------|---|--|--|
| 1 | 1.11 (control 0.17) | - | Sample only prepared for amino acid analysis |
| 2 | 0.17 | - | No increase in isodityrosine |
| 3 | 1.31 (ii) | 5% (iii) (fraction 55) | Tyrosine above solubility maximum in GPC buffer. Precipitated on column and in fractions |
| 4 | 0.65 | 5% (iii) (fractions 53-56) | Fractions stored |
| 5 | 0.17 | Not possible to quantify (present in fractions 53-56) | No increase in isodityrosine |
| 6 | 0.33 | - | Sample only prepared for amino acid analysis |

For foot notes (i) - (iii), please see over
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Footnotes to Table 5

- (i) Weight dry cell wall used for incubation converted from weight of shoots using average equivalent dry weight calculated from all processing samples (as Section 4.2.5).
- (ii) Estimate, isodityrosine not resolved from adjacent peaks.
- iii) See also Table 4.

were tried in the course of increasing the scale of the incubation. This sequence of modifications and the consequent observations are discussed below. The resulting yields of each synthesis and the recoveries from GPC, with a summary of the conditions used, are given in Table 5. In this table, for comparison, the experiment described in the previous section is referred to as incubation 1.

In order to speed up the preparation of a larger quantity of cell walls, in Experiment 2 the grinding was carried out using a liquidiser rather than a pestle and mortar. Cell walls prepared in this way were incubated with tyrosine for periods of 24 and 48 hours. The longer the incubation was included to check if this improved the yield of isodityrosine. After hydrolysis of the incubation products it was found that the amount of isodityrosine present in both samples was only the same as that found in the control in Experiment 1. It was concluded that this experiment had not resulted in any synthesis of isodityrosine and it was thought that the change of grinding method was the cause. It is possible that homogenising tissue in a liquidiser inactivated the enzyme responsible for catalysing isodityrosine formation, for example by causing rupture of cellular organelles and release of some inhibitor. Alternatively, the enzyme which exists in the cell wall (Lampert, 1980), may have been washed out of the wall fragments which are more finely ground by this method. Detailed investigation of these possibilities was beyond the scope of the project, and use of the liquidiser was abandoned.

Accordingly, in incubation 3, the cell walls were ground using a pestle and mortar. The preparation was incubated with tyrosine and the products were acid hydrolysed. Portions of the resulting hydrolysate were prepared for both amino acid analysis and for GPC. Amino acid analysis indicated that isodityrosine had been synthesised in even greater quantities than in the first experiment. It was found, however, that tyrosine was precipitated on the GPC column and in some fractions, and caused severe practical problems in purifying this sample. Fractions were recovered from only one run and these were found to contain just a small proportion of the isodityrosine loaded on

to the column. In view of the problems encountered in this experiment, it was decided to reduce the tyrosine concentration in the next trial.

In incubation 4 the tyrosine addition was reduced to one-quarter of that used in previous incubations, and it was found that isodityrosine was synthesised, although at about half the final concentration of the first experiment. The total product from incubation 4 was separated by GPC, and all the fractions stored. However, with a recovery of only 5%, this did not produce sufficient isodityrosine to use to calibrate the amino acid analysis method. It was decided to repeat the incubation, and risk increasing the tyrosine concentration in the hope of synthesising more isodityrosine. Accordingly, experiment 5 was set up, containing an intermediate level of tyrosine. It was found, however, that the incubation, like number 2, did not synthesise any isodityrosine above the level found in controls.

It had been noticed that the incubation products from experiment 1 were stored in a refrigerator over a weekend, and the possible effects of this were tested as a final experiment. It was concluded that refrigerating the incubation mix did improve the yield a little, but this was not the critical factor causing the high yield of experiment 1.

At this point it was concluded that this route to synthesising isodityrosine was not as useful as it had appeared from Experiment 1. The yields were very variable and the possible causes of this were considered. The factors which may affect isodityrosine synthesis are debated below.

3.5 Discussion

It will be recalled that, owing to the critical role of isodityrosine in cross-linking extensin, it was desirable to assay this component. An automated method for quantifying isodityrosine was therefore needed, and the first part of this chapter describes how such a method was developed. The peak corresponding to isodityrosine in the amino acid chromatogram of cell wall hydrolysates was identified, and confirmed using a synthetic reference sample shown to have the correct

chromatographic behaviour by other methods. Calculations of isodityrosine concentration, however, had to be based on a calibration factor for tyrosine, although the factors for tyrosine and isodityrosine would be expected to be similar. A larger quantity of isodityrosine was needed to prepare a solution of known concentration in order to determine the specific response factor for this compound.

To attempt to provide sufficient isodityrosine for calibration of this analysis method, both isolation of naturally occurring isodityrosine and preparation of synthetic material were used. Isodityrosine was isolated from cell wall hydrolysates using GPC. This technique was employed to separate isodityrosine from other amino acids on the basis of molecular weight difference. Although it was found that the elution pattern of phenolic compounds like isodityrosine was not related to molecular weight, under the conditions used, this technique was nevertheless calibrated and used to isolate isodityrosine.

Synthesis of isodityrosine initially involved adapting a method which used peroxidase coupling of tyrosine to produce dityrosine. This method was modified by adjusting pH conditions and substituting iodotyrosine as substrate. The yields resulting from these different modifications are shown in Table 3. The effect of different substrates on peroxidase synthesis of isodityrosine was investigated further, and cell wall-bound tyrosine was tested. Of particular interest, a system using cell walls incubated with tyrosine, but without any added enzymes, was found to synthesise isodityrosine successfully. This synthesis was presumably catalysed by endogenous wall enzymes. The novel cell wall synthesis system was scaled up with the intention of isolating isodityrosine by GPC. A number of modifications to the incubation method were tried but it was found that the yields of isodityrosine varied considerably from one experiment to another. A summary of the results of the various modifications and the isolation yields is given in Table 5. Only one sample appears to have synthesised isodityrosine in a quantity to match the first incubation, and this sample contained too much tyrosine to enable GPC purification of the isodityrosine.

Variations in the concentration of isodityrosine between different batches of cell wall hydrolysate was suspected in the GPC experiments described in Section 3.3.2. The more comprehensive data on control cell wall samples, compiled in Chapter 4, confirm that there are indeed differing amounts of isodityrosine in different cell wall preparations. As an explanation of this observation, it is possible that the period of growth of shoots, for this work, was stopped at a critical stage in extensin synthesis and polymerisation, and that differences of less than a few hours' growing time could have a pronounced effect on the amount of isodityrosine present. Further, shoots at different stages of growth could have different capacities to synthesise isodityrosine. Thus, it is conceivable that relatively young shoots, which might contain less isodityrosine, could have a greater potential for synthesis of the dimer. In contrast, slightly older shoots, at a later stage of cell wall development, could have more isodityrosine present and a concomitant reduced capacity for further synthesis. It is likely that the capability for synthesising isodityrosine, as any other component, would vary over the growth period. There are a number of biochemical controls, such as synthesis and repression of enzymes, which could be in operation. Hence, if the point of harvest of shoots for cell wall preparation (which was controlled as far as was practically possible), was at a time when the plant's capacity to synthesise isodityrosine was changing, this could explain the different amounts of isodityrosine synthesis observed in the different incubations described in Section 3.4.2. In support of this hypothesis, it is now apparent, compared with the control data in Appendix C, that the concentration of isodityrosine in incubation 1 was low; these cell walls had a high capacity for isodityrosine synthesis.

This work has demonstrated that isodityrosine can be synthesised by simple incubation of cell walls with tyrosine. This novel system, which presumably involves endogenous wall enzymes, could provide a useful means of synthesising isodityrosine but the results show that there may be other factors which need to be controlled to ensure maximum yield.

It was beyond the scope of this study, but it would be interesting to investigate the effects of factors such as the precise growth stage and

synthesis capacity of cell walls, on the yield of isodityrosine. Cooper & Varner (1983) report that hydrogen peroxide is required for isodityrosine formation, although synthesis was achieved, in at least some of these experiments, without any addition. The possibility that a low concentration of this oxidising agent is required for optimum isodityrosine synthesis is worth testing. The inhibiting effects of any metabolites, such as ascorbate (Cooper & Varner, 1983), should also be considered.

4 INVESTIGATION INTO CHANGES IN CELL WALL COMPOSITION

4.1 Introduction

The practical approaches to this project, decided in the light of initial experiments, were outlined in Chapter 2. In order to study the behaviour of plant cell walls on processing, it was planned to adopt a combination of chemical analysis to investigate composition changes, and microscopy to look at structural changes. This chapter describes the study of changes in cell wall composition, and the parallel microscopy work is given in Chapter 5. The processes tested included different cooking and preservation treatments typically used for fruit and vegetables.

In view of the particular interest in extensin, compositional analysis concentrated on amino acid determinations to assess the effects of processing on this glycoprotein. Samples of cell walls were treated by a range of different food processes, and the amounts of key amino acids that were dissolved out of the cell walls and the amounts remaining in the wall residues were measured. This provided information on the proportion of amino acids which were destroyed or extracted by the different processes. The amino acids which were studied were hydroxyproline, in which extensin is unusually rich, and valine, tyrosine, histidine and lysine, which are the predominant components after hydroxyproline. In addition, the concentration of isodityrosine, the critical cross-linking unit which binds extensin in the wall was also assayed, using the automated method described in Chapter 3.

Further samples of cell walls were chemically treated by methods designed to extract pectin and glycoprotein fractions. In corresponding experiments, the amounts of the same key amino acids dissolved in these chemical fractions and the amounts left in the wall were determined. The patterns of amino acids extracted from the cell wall or destroyed by these chemical treatments are compared with those of the food processing tests.

In addition to detailed studies of amino acid composition, the carbohydrate content of the various pectin and glycoprotein wall extracts was monitored.

4.2 Changes in Amino Acid Composition Caused by Processing Treatments

4.2.1 Approach

Samples of isolated cell walls were treated with a variety of food processing conditions. Quantitative amino acid analysis was then performed to detect changes caused by the different processes. The processes tested were:

- acidified brine storage (as used for vegetables prior to pickling);
- sulphiting (as used for storing fruit for jam making);
- boiling in water for ten minutes;
- boiling in dilute sodium bicarbonate solution (as sometimes used in large-scale cooking of green vegetables to preserve the colour);
- jam making;
- deep frying in vegetable oil.

4.2.2 Sample preparation

Separate batches of cell walls were prepared for each processing test. These batches were subdivided into eight portions to provide triplicate samples each for processing and untreated controls, and duplicate portions for dry weight determination (Appendix A.2). The amount of cell walls in each subsample was prepared from approximately 9 g of shoots, and was equivalent to about 0.135 g dry weight. Unprocessed controls were prepared to allow for possible variations in amino acid composition between different preparation batches of cell walls.

The processing treatments are described in detail below. Each of the triplicate cell wall subsamples was treated quite separately. After processing, the cell wall mixture was centrifuged. The soluble fraction (supernatant) was then separated from the insoluble wall residue

(pellet). The soluble fraction contains material dissolved out of the wall during processing and is subsequently referred to as the extract. The soluble extracts, cell wall residues and unprocessed control samples were acid hydrolysed (Appendix A.5). The amino acid composition was then analysed (Appendix A.6).

4.2.3 Processing treatments

In this study the processing conditions used were deliberately selected to represent the more extreme levels likely to be used in practice.

The processing treatments were:

1. Acidified brine storage in 15% sodium chloride and 3% lactic acid for 2 months.
2. Sulphite storage in 3000 ppm sulphur dioxide solution for 4 weeks.
3. Boiling in water for 10 minutes.
4. Boiling in 1% sodium bicarbonate solution for 10 minutes.
5. Jam making, by boiling (for 10 minutes) to 68% total soluble solids with sucrose and pH 3 citrate buffer.
6. Deep frying in vegetable oil for 10 minutes.

Freezing and thawing was also tested but the amino acid analysis results from this process are not available.

Full experimental details of the treatments are given in Appendix A.14. Additional unprocessed controls for the cell walls made into jam were prepared to check for the effect of high sucrose concentrations on amino acid recoveries.

Each processed cell wall mix was separated into soluble extract and insoluble residue as described above. For obvious reasons there were no aqueous extracts resulting from deep frying. The extracts from

acidified brine storage could not be run on the amino acid analyser because of their high salt content.

4.2.4 Amino acid analysis

All the processed samples, except those sulphited, were analysed using an LKB amino acid analyser (Appendix A.6.1). However, this automatic method was unsatisfactory for hydroxyproline estimation because, despite efforts to improve separation, hydroxyproline was poorly resolved by this system. In addition, this imino acid is less efficiently detected than the other amino acids because it yields a yellow product with ninhydrin and consequently gives only a small absorbance at 570 nm. This wavelength is used to detect the blue complexes produced by the primary amino acids. Accordingly, a colorimetric assay for the determination of hydroxyproline was developed. This was adapted from the method of Drozd, Kucharz and Szyja (1976) for blood serum (Appendix A.15).

The complete amino acid profile of the sulphited samples was measured using a Pico Tag amino acid analysis system (Appendix A.6.2). It was discovered, unfortunately, that norleucine, the internal standard used for all the other samples, coeluted with isodityrosine under the chromatographic conditions essential to resolve other amino acids. It was, therefore, necessary to find an alternative internal standard. Taurine was found to elute in a blank portion of the chromatogram (see Fig.14) and was selected.

4.2.5 Calculation of results

The equivalent dry weight of the cell walls used for each processing test was determined from the dry weight of subsamples from the same cell wall preparation. This procedure is detailed in Appendix A.2. The concentrations of amino acids in the processed samples have been expressed as milligrams of amino acid per gram dry cell wall. These figures are tabulated for each process in Appendix C. To aid interpretation of the data, the weights of amino acids detected in the residues and soluble extracts have been expressed as a percentage of those present in the controls for that experiment, see Tables 6 and 7.

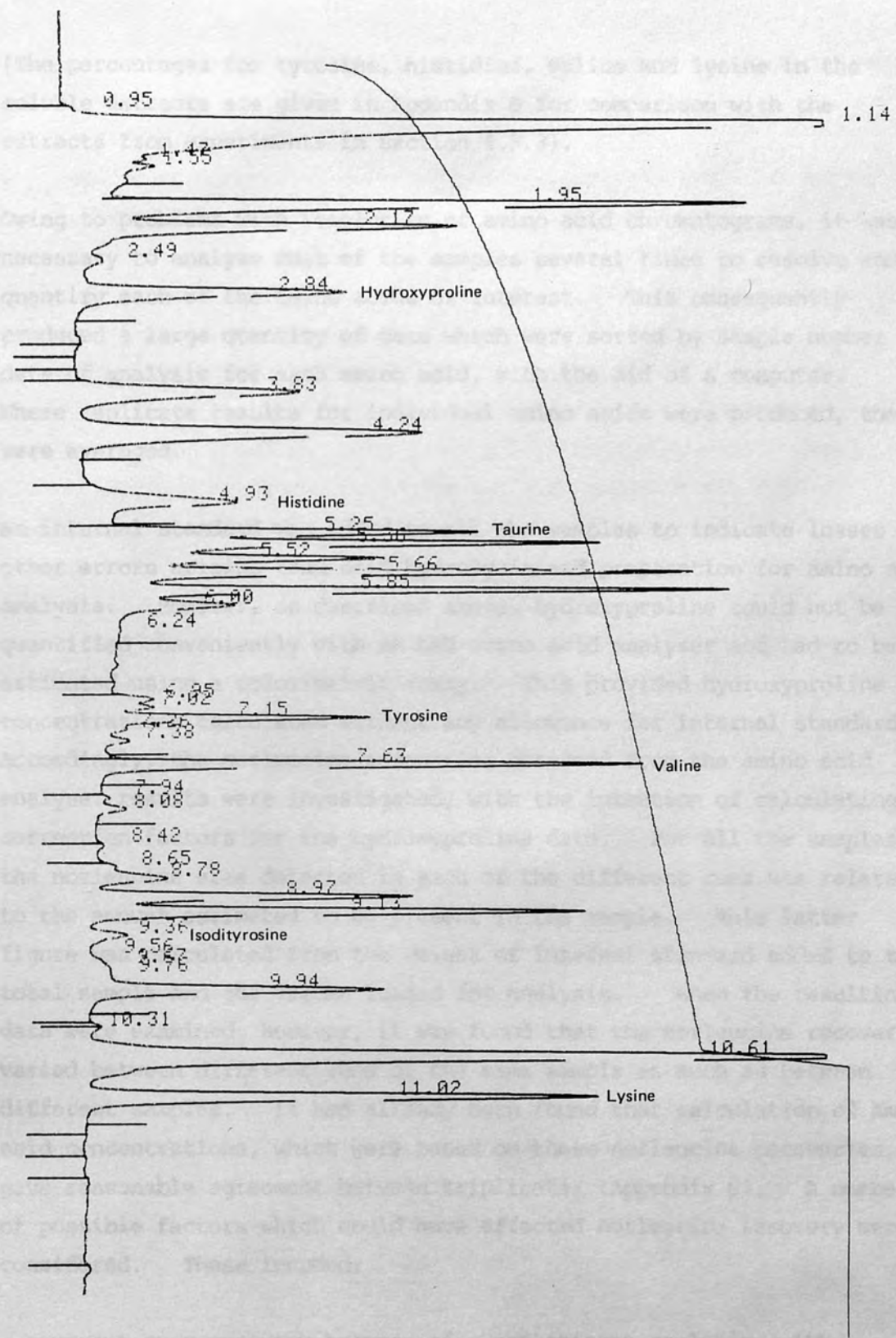


Fig. 14 Chromatogram of cell wall hydrolysate, Pico Tag System

(The percentages for tyrosine, histidine, valine and lysine in the soluble extracts are given in Appendix D for comparison with the extracts from experiments in Section 4.3.3).

Owing to problems with resolution of amino acid chromatograms, it was necessary to analyse most of the samples several times to resolve and quantify each of the amino acids of interest. This consequently produced a large quantity of data which were sorted by sample number and date of analysis for each amino acid, with the aid of a computer. Where replicate results for individual amino acids were produced, these were averaged.

An internal standard was added to all the samples to indicate losses or other errors arising from acid hydrolysis and preparation for amino acid analysis. However, as described above, hydroxyproline could not be quantified conveniently with an LKB amino acid analyser and had to be estimated using a colorimetric assay. This provided hydroxyproline concentrations calculated without any allowance for internal standard. Accordingly, the norleucine recoveries observed from the amino acid analyser results were investigated, with the intention of calculating correction factors for the hydroxyproline data. For all the samples, the norleucine area detected in each of the different runs was related to the amount estimated to be present in the sample. This latter figure was calculated from the weight of internal standard added to the total sample and the volume loaded for analysis. When the resulting data were examined, however, it was found that the norleucine recoveries varied between different runs of the same sample as much as between different samples. It had already been found that calculation of amino acid concentrations, which were based on these norleucine recoveries, gave reasonable agreement between triplicates (Appendix C). A number of possible factors which could have affected norleucine recovery were considered. These included:

- apparent over-recovery because of a contaminant coeluting with norleucine,

- apparent under-recovery because of loss of norleucine on processing, and
- erroneous norleucine concentration because sample prepared was too concentrated or too dilute.

It was finally concluded that the system for loading samples on to the LKB analyser was the main cause of variation. In this case hydroxyproline would not be affected, and the calculation of the concentrations of the other amino acids allows for the amount of internal standard loaded, thus eliminating this variation. Hence there was no merit in applying correction factors, based on norleucine recoveries, to the hydroxyproline results, and these data are therefore presented as measured. There is, nevertheless, likely to be some between-sample variation in hydroxyproline concentrations which has not been allowed for. This could have arisen from the rotary evaporating and dissolving stages of sample preparation.

4.2.6 Results

Full triplicate results for each of the processes are given in Appendix C.

The concentrations of amino acids in the residues, calculated as percentages of the controls are in Table 6.

The concentrations of hydroxyproline and isodityrosine in the extracts, calculated as percentages of the controls are in Table 7. The remaining data for the extracts are given in Appendix D, for comparison with various pectin and glycoprotein fractions.

Table 6 Amino acids remaining in insoluble cell wall residue after processing as a percentage of the amount in unprocessed walls

| Amino acid | Processing treatment | | | | | |
|----------------|----------------------|------------------|--------|-------------|-----|----------------|
| | Brined (ii) | Sulphited (i) | Boiled | Bicarbonate | Jam | Fried (iii) |
| Hydroxyproline | 74 | 71 | 96 | 66 | 47 | 101 |
| Isodityrosine | 72 | 46 | 84 | 35 | 44 | 75 |
| Valine | 60 | 69 | * | 40 | 50 | 39 |
| Tyrosine | 75 | 76 | 96 | 45 | 38 | 32 |
| Histidine | 46 | 52 | 99 | 33 | 42 | 29 |
| Lysine | 73 | 74 | 100 | 35 | 42 | 37 |

* not resolved

Notes

(i) Analysed using a Pico-Tag system. (All other samples analysed using an LKB amino acid analyser and separate hydroxyproline assay.)

(iii) Probably underestimated, see Section 4.2.7.

(iv) Analysed using a Pico-Tag system.

(All other samples analysed using an LKB amino acid analyser and separate hydroxyproline assay.)

Table 7 Amino acids extracted from the cell wall during processing as a percentage of the amount in unprocessed walls (i)

| Amino acid | Processing treatment | | | | | |
|----------------|----------------------|-------------------|--------|-------------|--------|---------------|
| | Brined (ii) | Sulphited (iv) | Boiled | Bicarbonate | Jam | Fried (ii) |
| Hydroxyproline | | 5 | 2 | 27 | 7(iii) | |
| Isodityrosine | | T | 0 | 11 | NC | |

T = trace detected

NC = not clear from chromatogram if present or not

Notes

- (i) Results for valine, tyrosine, histidine and lysine in Appendix D.
- (ii) No extracts from these processes, or extracts which could not be analysed.
- (iii) Probably underestimated, see Section 4.2.7.
- (iv) Analysed using a Pico-Tag system.
(All other samples analysed using an LKB amino acid analyser and separate hydroxyproline assay).

4.2.7 Discussion

This discussion of the amino acid data commences with a consideration of factors which may have affected specific results. The section continues with a general discussion of changes in cell wall amino acid composition caused by the different processes.

The experiments were designed so that the cell wall control samples were hydrolysed at a concentration of approximately 1.4 mg dry cell wall per ml of acid. This was to comply with the recommendation of Fry (1982) that hydrolysis should be carried out with less than 4 mg dry cell wall per ml. Protein-rich food samples are normally hydrolysed at a concentration of approximately 1 mg per ml of acid, according to Davies and Thomas (1973). If it is assumed that cell walls are less than 10% protein (see Table 1) it is clear that the resulting hydrolysates will contain much lower concentrations of amino acids than hydrolysates of protein-rich foods. Hydrolysis and subsequent preparation of low concentrations of amino acids would inevitably result in a higher than normal percentage error in estimation. This error would be likely to be greatest for the aqueous extracts which contain very low concentrations of amino acids. In particular, it was observed that the soluble fraction extracted from cell walls by boiling them in water contained only a small amount of amino acids. As might be expected, the weights of amino acids in these extracts appear to have been over-estimated because, when added to the amounts detected in the insoluble residue, the totals for tyrosine, histidine and lysine each exceed 100% of the unprocessed controls.

The extra controls prepared for the samples processed into jam provide a useful indication of the effects of high sugar concentration on amino acid determinations. The controls with added sugar, and the extracts from this process, contained large amounts of black humin precipitate after hydrolysis. This hampered subsequent preparation for amino acid analysis, and these samples were dark even after filtering. Each of these extracts and controls with added sugar was hydrolysed in a larger volume of acid than normal, because of the high sugar content and, as is now known, this may increase the error in amino acid estimation, as described above. Quantification of the amino acids, in these samples,

was difficult because the chromatograms were incompletely resolved and the control duplicates are in very poor agreement. The chromatograms of these controls contained a broad peak before histidine, and the peak interfered with isodityrosine quantification. There was also an extremely large peak at the start (around 400 s). These same two peaks were observed in the extracts, which contained most of the added sugar, but were not present in the residue samples. In view of these observations, it was concluded that the residue results were much more reliable and these alone should be used as a guide to the effects of jam processing.

The hydroxyproline contents of the control cell walls hydrolysed with sugar are significantly lower than those of standard controls. It is thought, however, that despite the preparation of appropriate blanks, the hydroxyproline determination of these samples is unreliable because the dark colour interferes with the assay. The extracts from cell walls prepared into jam were also dark and thus the hydroxyproline content is probably also underestimated along with the other amino acids.

The results in Table 6 suggest that the deep-fried cell walls have not lost any hydroxyproline, whereas approximately two-thirds of the other amino acids (except isodityrosine) have been extracted or destroyed. Whilst it may be a real observation, this result appears unlikely. It is possible that acid hydrolysis products of oil could also have interfered with the hydroxyproline assay, although no unusual effects were observed. Alternatively, the different behaviour of hydroxyproline from the other amino acids in this experiment could be related to an effect involving the internal standard. As explained in section 4.2.5 calculations of hydroxyproline concentrations, unlike the other amino acids, does not involve a factor for internal standard recovery.

The preceding paragraphs have included comments on specific results. This section continues with a discussion of general trends in compositional changes resulting from the different processes. Considering first the composition of cell wall residues, it is apparent that boiling in water and deep frying both result in most of the hydroxyproline remaining in the cell wall. These processes also showed

a limited degree of isodityrosine loss or breakdown. Possible reasons for the very high hydroxyproline content of deep-fried cell walls have already been discussed. In contrast, cell walls which had been boiled in bicarbonate or processed into jam had the least hydroxyproline left in the wall residue. These two processes, together with sulphiting, also resulted in the lowest isodityrosine concentrations. In general, the hydroxyproline and isodityrosine levels vary in parallel. There are also differences between processes in the concentrations of valine, tyrosine, histidine and lysine, which are the predominant extensin components after hydroxyproline, but which are not exclusively found in this glycoprotein. After boiling in water, most of these amino acids are still left in the cell wall. Much lower concentrations, however, are detected in the residues after boiling in bicarbonate, deep frying or processing into jam.

Where a reduced concentration of an amino acid, compared with controls, is found in the insoluble cell wall fraction after processing, the amino acid could have either been destroyed or dissolved. Table 7 gives the concentrations of the key extensin components found in the extracts. The composition of the extracts was determined after acid hydrolysis and thus this method does not distinguish between free amino acids and amino acids extracted in protein-bound form.

Thus, it was indicated above that the residues from sulphiting, boiling in bicarbonate and jam processing contained the least isodityrosine, and it is clear that there are different amounts of this component in the corresponding extracts. It has already been discussed that it is not possible to draw any valid conclusions from the jam extract results. However, comparison of the compositions of the bicarbonate and sulphite extracts is interesting. A significant amount of isodityrosine (11% of that in unprocessed controls) is dissolved out of the wall by boiling in bicarbonate, whereas only a trace is found in the sulphite extracts. The hydroxyproline contents of these extracts match the isodityrosine concentrations. Thus, it appears that bicarbonate treatment causes extensin to be extracted from the wall in a form that contains some isodityrosine. The extract and residue together account for most of the hydroxyproline, although about half the cell wall isodityrosine is

destroyed by this process. Examination of the results from sulphite treatment indicates, like the results from the bicarbonate test described above, that about half the cell wall isodityrosine is left in the residue. But, after sulphiting, the remaining isodityrosine is destroyed rather than detected in the extract. In addition, the total hydroxyproline in the extract and residue reveals that some of this imino acid is also destroyed by sulphiting. In complete contrast, boiling in water resulted in only small quantities of hydroxyproline, and no isodityrosine, being extracted. This process did, however, cause some isodityrosine breakdown.

4.3 Changes in Amino Acid Composition Caused by Chemical Extraction of Cell Wall Components

4.3.1 Background

O'Neill and Selvendran (1980) published methods for fractionating runner bean cell walls by extracting first pectin and then glycoprotein. In the present work these procedures were applied to mung bean cell walls (see Section 2.4) and the full fractionation method was compared with the glycoprotein extraction step alone. These early experiments indicated that the glycoprotein fraction contained more hydroxyproline if the pretreatment to remove pectin was omitted. Accordingly, it was planned to investigate in more detail the amino acid composition of the pectin extracts and the glycoprotein fractions prepared both with and without prior depectination. Experiments that were carried out to analyse these different extracts are described below. The previous section reports how the changes in marker amino acids for extensin, namely hydroxyproline, isodityrosine, tyrosine, valine, histidine and lysine were investigated after a variety of food processing treatments. The same amino acids were analysed after the chemical extractions to enable comparison of the pattern of amino acids extracted from the cell wall.

An experiment to compare the amino acid composition of the glycoprotein fraction with that of the whole cell wall is reported first. Although only providing data for hydroxyproline, this is a useful addition to results from the subsequent series of experiments.

4.3.2 Comparison of the composition of glycoprotein extract with whole cell walls

Cell walls were treated by the full fractionation procedure of O'Neill and Selvendran (1980) and the glycoprotein extracts were analysed compared with whole, untreated cell walls as controls.

A cell wall preparation was divided into duplicate subsamples (two each) for extraction, as control and for dry weight determination (Appendix A.2). The extraction samples were depectinated and treated with acidified chlorite by the method described in Appendix A.4 (Stages I and II).

It was intended to determine the amino acid composition of the glycoprotein extracts, cell wall residues and controls. However, at the time of this experiment, the LKB amino acid analyser was not working properly and only hydroxyproline results from the colorimetric assay (Appendix A.15) are available. These are given in Table 8.

An average of 37% of cell wall hydroxyproline was extracted with the glycoprotein fraction, and this result will be compared with later findings. It is clear that much of the wall hydroxyproline - 42% - was not accounted for by these two fractions. The remainder was presumably removed with the pectin extracts, although a proportion may be destroyed during the extraction process and subsequent hydrolysis. The composition of pectin extracts was analysed in the set of experiments described in the next section.

4.3.3 Amino acid composition of cell wall pectin and glycoprotein extracts

Approach

A number of experiments were carried out to compare the composition of the pectin extracts, glycoprotein extracts prepared with and without prior depectination and the corresponding residues. Untreated control cell walls were prepared for comparison, and further controls were prepared by heating cell walls under the same pH and temperature

Table 8 Hydroxyproline contents of glycoprotein extracts, cell wall residues and untreated controls (see Section 4.3.2)

| Sample | Hydroxyproline content mg/g dry cell wall, duplicates | | Mean hydroxyproline content expressed as % of control |
|--------------------------------|--|------|--|
| | 1 | 2 | |
| Control | 1.85 | 1.91 | - |
| Glycoprotein extract | 0.64 | 0.75 | 21 |
| Insoluble cell wall residue | 0.50 | 0.29 | 37 |
| Total extract plus residue | 1.14 | 1.04 | - |

Discussion

A number of observations which need to be taken into account when interpreting these results will be discussed first. The effects of chemical fractionation to extract pectin and glycoprotein on cell wall composition will then be reviewed.

The Pico-Tag system can be used quite satisfactorily to determine hydroxyproline in normal control cell wall hydrolysates. However, some of the other samples from these experiments contained additional components which eluted close to hydroxyproline and affected its quantification. Figure 15 shows a typical chromatogram from a cell wall residue and a number of components resulting from oxidized chlorite treatment, for example acetic acid, can be seen. Alpha-aminocaproic acid, which is an oxidation product of lysine, particularly interferes with hydroxyproline determination. As lysine

conditions used for glycoprotein extraction, to check for amino acids which might be dissolved. In this latter process cell walls were heated in pH 4.2 acetate buffer (Appendix A.14.8), this pH being that found for the mix of acetic acid and sodium chlorite used in acidified chlorite treatment. These extractions were performed in triplicate to provide data comparable to the processing results of Section 4.2.

One set of three cell wall samples was sequentially extracted for pectin, with water and ammonium oxalate, and then for glycoprotein (Appendix A.4). Each of the extracts was hydrolysed for amino acid analysis. Another set of samples was treated only with acidified chlorite to remove glycoprotein, without any depectination. The amino acid composition of all the fractions and cell wall controls, including hydroxyproline, was measured using a Pico-Tag System (Appendix A.6.2).

The means of the triplicate results for hydroxyproline and isodityrosine in the various fractions are given in Table 9. The amounts of amino acids are expressed as a percentage of the amount in untreated control cell walls. Additional compositional data for the extracts are given in Appendix D for comparison with the processing extracts.

Discussion

A number of observations which need to be taken into account when interpreting these results will be discussed first. The effects of chemical fractionation to extract pectin and glycoprotein on cell wall composition will then be reviewed.

The Pico-Tag system can be used quite satisfactorily to determine hydroxyproline in normal control cell wall hydrolysates. However, some of the other samples from these experiments contained additional components which eluted close to hydroxyproline and affected its quantification. Figure 15 shows a typical chromatogram from a cell wall residue and a number of components resulting from acidified chlorite treatment, for example acetic acid, can be seen. Alpha-amino adipic acid, which is an oxidation product of lysine, particularly interferes with hydroxyproline integration. As lysine

Table 9 Hydroxyproline and isodityrosine contents of various pectin, glycoprotein and buffer extracts and cell wall residues, as a percentage of the amount in unprocessed walls (see Section 4.3.3)

| | Hydroxyproline (i) | Isodityrosine |
|--|--------------------|---------------|
| Water-soluble pectin | 5 (ii) | 24 |
| Ammonium oxalate-soluble pectin | 5 | T |
| Glycoprotein extract (after depectination) | 0 | 0 |
| Cell wall residue (after all extractions) | 29 | 11 |
| Glycoprotein extract (no depectination) | 60 (iii) | T |
| Cell wall residue (after glycoprotein extraction only) | 62 (iii) | 14 |
| Extract from heating in pH 4.2 acetate buffer | 5 | 0 |
| Cell wall residue after heating in pH 4.2 acetate buffer | 93 | 80 |

T : Trace detected

Notes (see Discussion)

- (i) Hydroxyproline determination unreliable in acidified chlorite-treated samples
- (ii) Too low relative to isodityrosine
- (iii) Hydroxyproline overestimated in one or both of these samples.

becomes oxidized, the concentration of this amino acid would be expected to be reduced in all cell wall fractions. In addition, tyrosine and other aromatic amino acids are partially destroyed by this treatment. The authors have not concluded that the amino acid composition of the cell wall is not quantitative. The authors have also noted that the concentration of isodityrosine in the cell wall is higher than that of tyrosine. The authors also noted that the concentration of tyrosine in the cell wall is higher than that of histidine. The authors also noted that the concentration of taurine in the cell wall is higher than that of valine. The authors also noted that the concentration of lysine in the cell wall is higher than that of isodityrosine.

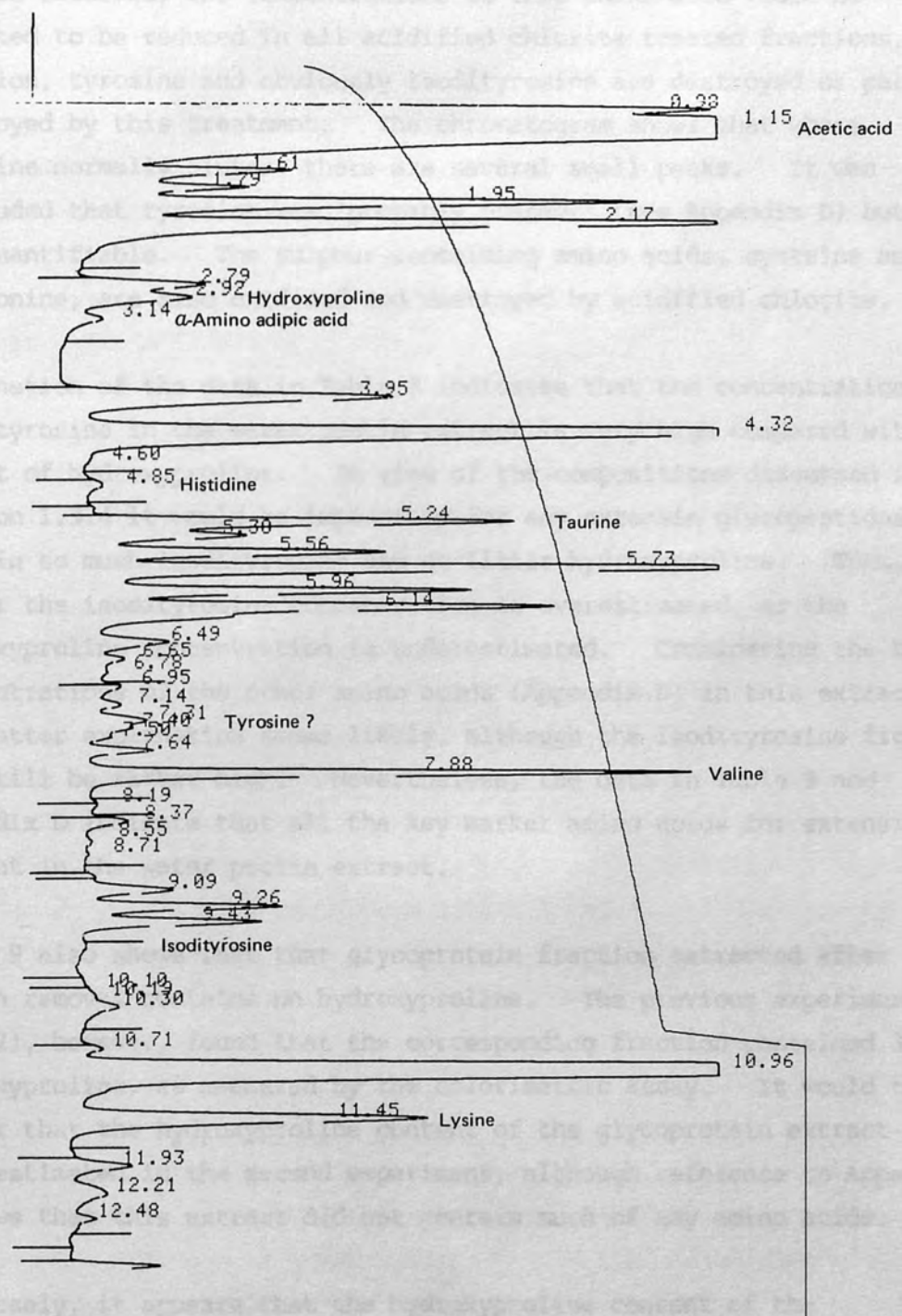


Fig. 15 Chromatogram of hydrolysate of cell wall residue remaining after pectin and glycoprotein extractions, Pico Tag System

becomes oxidised, the concentrations of this amino acid would be expected to be reduced in all acidified chlorite treated fractions. In addition, tyrosine and obviously isodityrosine are destroyed or partly destroyed by this treatment. The chromatogram shows that where tyrosine normally elutes, there are several small peaks. It was concluded that tyrosine was "probably present" (see Appendix D) but was not quantifiable. The sulphur-containing amino acids, cysteine and methionine, are also oxidised and destroyed by acidified chlorite.

Examination of the data in Table 9 indicates that the concentration of isodityrosine in the water pectin extract is very high compared with the amount of hydroxyproline. In view of the compositions discussed in Section 1.3.4 it would be impossible for any extensin glycopeptides to contain so much isodityrosine and so little hydroxyproline. Thus, either the isodityrosine concentration is overestimated, or the hydroxyproline concentration is underestimated. Considering the high concentrations of the other amino acids (Appendix D) in this extract, the latter explanation seems likely, although the isodityrosine figure may still be rather high. Nevertheless, the data in Table 9 and Appendix D indicate that all the key marker amino acids for extensin are present in the water pectin extract.

Table 9 also shows that that glycoprotein fraction extracted after pectin removal contains no hydroxyproline. The previous experiment (4.3.2), however, found that the corresponding fraction contained 37% hydroxyproline, as measured by the colorimetric assay. It would thus appear that the hydroxyproline content of the glycoprotein extract is underestimated in the second experiment, although reference to Appendix D shows that this extract did not contain much of any amino acids.

Conversely, it appears that the hydroxyproline content of the glycoprotein fraction extracted from cell walls which had not been depectinated, or alternatively the hydroxyproline content of the corresponding residue, is over-estimated as the sum of these two fractions exceeds 100% of controls. The possible causes of this inaccuracy have been discussed in the second paragraph of this Section.

These experiments, in which the amino acid compositions of all the different cell wall fractions were analysed, have shown that hydroxyproline is indeed extracted with pectin, as was earlier suspected. Further, isodityrosine and the amino acids which predominate in extensin are also extracted, a finding which indicates that whole molecules and possibly even cross-linked extensin oligomers may be rendered soluble. It appears that more extensin was extracted in the first, i.e. water stage, of pectin removal, than subsequent ammonium oxalate treatment.

Less of all the extensin marker amino acids were found in the glycoprotein fraction prepared after depectination, confirming the observation of experiments described in Section 2.4. However, as has already been discussed, it is not possible to compare directly the yields of hydroxyproline in the two different glycoprotein extracts.

The heating in acetate buffer test was included to see how specific the effect of chlorite was on extracting glycoprotein. It is clear from all these results that acidified chlorite extracts around half the cell wall hydroxyproline whereas pH 4.2 acetate buffer only dissolves 5%. This confirms that acidified chlorite effectively extracts glycoprotein, probably by breaking isodityrosine links, a reaction not caused by acid alone.

4.4 Effects of Chemical Extractions on Cell Wall Carbohydrate Compositions

Experiments described previously (Section 4.3) had indicated that glycoprotein was dissolved from the wall during pectin extraction. It was therefore decided to check whether pectin was correspondingly affected by glycoprotein extraction. Degradation of pectin, or other wall carbohydrate was estimated by assaying for presence of reducing sugars in different wall extracts. The assay was calibrated with galacturonic acid, the major monosaccharide constituent of pectin, as pectin is the wall carbohydrate fraction most likely to be extracted under the conditions of this experiment.

A cell wall preparation was divided into six portions, each equivalent to approximately 0.15 g dry weight, for separate duplicate extractions of pectin and glycoprotein (Appendix A.4) and control treatments. The different pectin fractions obtained from consecutive extraction stages, namely water, ammonium oxalate and water washes, were analysed separately. Controls were prepared by heating cell walls in water under the same conditions employed for glycoprotein extraction. All the soluble fractions were assayed for reducing sugars by the colorimetric method in Appendix A.16.

The results are given in Table 10.

This experiment indicated that no reducing sugars were present in the glycoprotein extracts. However, some sugars were detected in the control extracts and it was suspected that free reducing sugars were not found in the glycoprotein fractions because they had been destroyed by acidified chlorite.

A further check on the stability of free galacturonic acid solution to acidified chlorite was therefore carried out. Samples of galacturonic acid solution were treated by normal acidified chlorite conditions and compared with controls which were subjected to the same heat treatment. Analysis showed that no galacturonic acid was detected in the treated samples whereas about 90% of the initial concentration was found in the controls. This experiment confirmed that the free reducing sugar was destroyed by acidified chlorite.

It was concluded, from these experiments, that acidified chlorite treatment of cell walls does extract some pectin or other carbohydrate with the glycoprotein. However, under these conditions, galacturonic acid is unstable and it is thus not possible to estimate the quantity of sugars extracted. It is likely that glycoprotein extraction dissolves more than 20% of the amount of carbohydrate which is rendered soluble by the pectin extraction steps. This amount of carbohydrate was dissolved simply by the heating conditions used for glycoprotein extraction. In view of the observation that the uronic acid monomer is completely destroyed by acidified chlorite, it is reasonable to suggest that

Table 10 Reducing sugar contents of pectin and glycoprotein wall extracts

| Extract | μmol galacturonic acid in 2.05 ml extract | | total μmol galacturonic acid in extract (mean) |
|---------------------------------|--|-------|---|
| | 1 | 2 | |
| First water pectin extract | 0.505 | 0.480 | 3.6 |
| Second water pectin extract | 0.475 | 0.435 | 3.3 |
| Ammonium oxalate pectin extract | 0.765 | 0.760 | 5.6 |
| Pectin water washings | 0.510 | 0.510 | 3.7 |
| Glycoprotein extract | 0.000 | 0.000 | 0.0 |
| Control | 0.470 | 0.425 | 3.2 |

polygalacturonic acid, the major pectic polymer, is also unstable. If this were the case, a much greater proportion than the 20% observed would be dissolved by acidified chlorite treatment.

4.5 Discussion

The processing treatments tested in this study included different types of storage conditions and various cooking methods which are typically used for fruit and vegetables. The effects of these treatments on cell wall composition was assessed by measurement of the amino acid content of the wall fraction rendered soluble, and the residue remaining insoluble, after treatment. As the objective was to study extensin, the amino acids which predominate in this glycoprotein, namely hydroxyproline, as a specific marker; isodityrosine as the critical cross-linking dimer; and valine, tyrosine, histidine and lysine were assayed.

It was found that all the processes caused some degree of isodityrosine breakdown. Further, where it is possible to measure, it is apparent that these processes also resulted in hydroxyproline being extracted from the cell wall. The process which least affected extensin components was, as might be expected, boiling in water. Deep frying, boiling in bicarbonate and processing into jam all caused much greater changes to cell wall composition and generally reduced the concentrations of the key amino acids of extensin to less than half those of controls. Acidified brine storage and sulphiting were intermediate in effect, although the latter process destroyed about half the wall isodityrosine. Boiling in bicarbonate solution and processing into jam also markedly reduced the wall concentration of isodityrosine, and bicarbonate actually extracted isodityrosine, an observation which is discussed in more detail below.

As a comparison, the chemical fractionation experiments demonstrated that all the marker amino acids of extensin, including isodityrosine, were extracted with pectin. More of the extensin components were contained in the water pectin fraction than in the ammonium oxalate pectin extract. This is probably because water-soluble pectin is the

first fraction removed, although it may reflect the association of extensin with different types of pectin.

The soluble extracts resulting from both boiling in bicarbonate and water pectin fractionation contained more than 10% of the cell wall isodityrosine. This is an interesting observation as it was not thought that extensin could be so readily extracted from the wall. The presence of isodityrosine in the extract indicates that whole extensin molecules, or even cross-linked oligomers, may have been extracted.

The heating in pH 4.2 acetate buffer was included to check for the effects of pH and temperature of the acidified chlorite extraction conditions. Comparison of the composition of this extract with that of glycoprotein fractions clearly indicates that acidified chlorite is specifically required to extract extensin, presumably by breaking isodityrosine. The combined effects of heat and acid do not extract much extensin and the composition of the acid extract is similar to that resulting from heating in water. As might be expected, the pattern of amino acids in these two extracts is also similar to that of the water-soluble pectin fraction, although the latter contains more of all the amino acids because of the longer extraction time.

Some other similarities between the composition of various process extracts and chemically extracted fractions have been noted. The pattern of amino acids in the ammonium oxalate-soluble pectin is broadly similar to that in the bicarbonate extract, and this may be related to the pH conditions. The dilute solution of bicarbonate was pH 8, and the ammonium oxalate was 5.5, so that these two treatments were the least acid conditions used. There is also some similarity between the composition of the glycoprotein extracts and the soluble fraction resulting from sulphiting; both contain a predominance of valine.

All the experiments described in this chapter have demonstrated that the methods for fractionating cell walls are not specific. Amino acid analysis of pectin extracts has indicated that significant quantities of extensin are also rendered soluble. Furthermore, the extract contained isodityrosine, suggesting that the extensin is present as intact

molecules, and not just small fragments. Similarly, it appears that acidified chlorite extracts pectin or other carbohydrate in addition to glycoprotein. The simultaneous extraction of different components is not a surprising observation considering the complex, interwoven arrangement of wall polymers that was discussed in Section 1.3.

Microscopy study provided information on changes in the general conformation of the wall. The procedure used in this study for the chemical composition was discussed previously.

Previous studies of the structural changes which take place in plant tissues during food processing have usually concentrated on specific processes involving one type of vegetable. For example, Hogg and Jewell (1976) looked at the effects of blanching on carrots, and the softening of brins fermented cucumbers was studied by Heller, Simons and Triguero (1985). In the present study a number of different processes were tested on the type of plant tissue, namely, green bean seedlings, and this has enabled, for the first time, a comparison of the structural effects of a range of different processes. A comparison is not available between the effects of the different treatments on different plants. The procedure used in this study for the examination, preparation of samples and the methods used for the analysis were prepared. A comparison of the effects of different processes employed to produce structural changes in the wall of green bean seedlings to those of other types of vegetable is not available.

Further samples of cell walls were analysed using the methods described in this chapter to provide information on the changes in the wall structure caused by different processes. The effects of the different processes on the information to help in the interpretation of the data on the different processing tests.

The first section of this chapter describes the methods used for the analysis of the cell walls. The effects of the different processes on the wall structure and examined by electron microscopy. The effects of the different processes on the wall structure and examined by electron microscopy. The effects of the different processes on the wall structure and examined by electron microscopy.

5. INVESTIGATION INTO CHANGES IN CELL WALL STRUCTURE

5.1 Introduction

The effects of the various processing treatments described in Chapter 4 on cell wall structure were investigated by microscopy. This microscopy study provided information on changes in the physical conformation of the wall for comparison with modifications to the chemical composition assessed previously.

Previous studies of the structural changes which take place in plant tissues during food processing have usually concentrated on specific processes involving one type of vegetable. For example, Mirza and Jewell (1976) looked at the effects of blanching on carrots, and the softening of brine fermented cucumbers was examined by Walter, Fleming and Trigiano (1985). In the present study a number of different processes were tested on one type of plant tissue, namely mung bean seedlings, and this has enabled, for the first time, direct comparison of the structural effects of a range of different processes. Such comparison is not possible when the raw materials for the different treatments are different plants. To provide material for microscopical examination, pieces of whole beanshoots and isolated cell wall fragments were prepared. A combination of light and electron microscopy was employed to examine structural changes ranging from the arrangement of groups of whole cells to detailed conformation of the wall.

Further samples of cell walls were chemically extracted by different methods designed to remove specific components. These samples were examined by electron microscopy. The results provide additional information to help in the interpretation of micrographs from the food processing tests.

The first section of this chapter describes experiments with food processing treatments. Samples of isolated cell walls were processed and examined by electron microscopy and samples of whole tissues were similarly treated and examined by both light and electron microscopy. The effects of the different processes are compared in Section 5.2.3.

The investigation of cell wall structural changes caused by extraction of different components is described in 5.3. The chapter is concluded with a comparison of the effects of food processing and chemical extraction on cell wall structure.

5.2 Effects of processing treatments on cell wall structure

5.2.1 Experimental

The effects of food processing treatments were investigated by studying the structure of whole shoots and isolated cell walls. Beanshoots were grown and prepared as described in Appendix A.1. The method given in Appendix A.2 was used to prepare ground and washed cell walls. It is apparent from the micrographs (see e.g. Plates 21, 27, 28) that this grinding method results in small clusters of broken cells as well as individual wall fragments. These materials were treated by the range of processes used previously, namely:

- freezing and thawing
- acidified brine storage
- sulphiting
- boiling in water
- boiling in bicarbonate
- jam making
- deep frying

Details of these processing treatments are given in Appendix A.14. Untreated control material was also prepared.

The processed tissues were fixed, embedded and sectioned for microscopical examination. Details of these preparation methods are given in Appendix A.17. Portions of processed shoots were prepared by each of two procedures, namely periodic acid thiocarbohydrazide-osmium (PATCH-osmium) and glutaraldehyde-osmium. The cell wall samples were treated only with PATCH-osmium.

The glutaraldehyde-osmium treated shoots were sectioned and further stained for examination by light microscopy. Tissues from both treatments were sectioned for electron microscopy and at this stage the glutaraldehyde fixed samples were post-stained with uranyl acetate. PATCH-osmium specifically stains 1,2 glycol groups (Jewell & Saxton, 1970) and is used to demonstrate changes in cell wall polysaccharides. Glutaraldehyde-osmium is a general fixative which gives good preservation of cell contents, and samples from this fixing method were post-stained with uranyl acetate to visualise the protein and carbohydrate of these structures.

5.2.2 Results

Whole shoots

A diagram indicating the different cell types in beanshoot tissue is given in Plate 2. This transverse section shows two bands of small, and therefore relatively strong cells: the epidermis which forms a protective outer layer and the cambium and thickened xylem cells of the vascular tissue. The remaining large, thin-walled cells which make up most of the structure are parenchyma. It was discussed in Section 2.1 that beanshoots were used for this study because their structure is representative of a wide range of plant foods. The edible parts of plants are mostly composed of parenchyma tissue.

Plates 3a, 4 and 5 show control beanshoot tissue. The cell walls are thin (of the order of $0.5 \mu\text{m}$ wide). The cells contain a large central vacuole and the cell contents are arranged around the inside edge of the wall. Adjacent cells appear closely associated, which is consistent with tight binding of the cell walls. The intercellular spaces are therefore small in size and are triangular in cross-section. The texture of the shoots was assessed during cutting of samples in preparation for microscopy. The control tissue had, as expected, a firm texture.

Freezing and thawing tissue softens the texture by causing mechanical damage which can be seen in Plates 3b, 6 and 7. Layers of cells have

been torn apart along the middle lamella, the pectin-rich cementing layer, forming irregular gaps between cells. The cell contents have been completely disrupted and dispersed.

Storage in acidified brine affects the structure of the cell wall as shown in Plates 3c, 8 and 9. The wall is swollen and there is some splitting into layers both within the wall and along the middle lamella. The air spaces have become enlarged along the junction between cells. The contents are plasmolysed (i.e. shrunk away from the cell wall) and partly precipitated. Some of the cellular organelle membranes have broken down and formed vesicles. The resulting tissue was firmer than that from other processes, but softer than that of the control.

Plates 3d, 10 and 11 show beanshoots which have been stored in sulphite solution. This treatment causes the most extreme cell wall swelling observed; the walls are of the order of 6 μm wide, compared with 0.5 μm for controls. Sulphiting also causes the formation of very fine layers within the wall. The electron micrographs show that the layering is more pronounced on the middle lamella side of the cell walls. There is some splitting of the middle lamellae but the air spaces remain relatively compact. At the edges of these spaces there appear to be regions which act as constraints to cell separation. This process causes the tissue to become very soft. As observed in brined tissue, the cell contents are plasmolysed.

Boiling in water effects only little damage to the structure and some softening in texture. Plates 3e, 12 and 13 show how the cell wall is slightly swollen and the air spaces are starting to extend as the middle lamella dissolves. The cell contents are precipitated within the cell because boiling denatures cytoplasmic proteins but is not severe enough to cause further disruption.

In contrast, boiling in bicarbonate solution causes severe damage to the tissue and results in a very soft texture. This cooking treatment causes an unusual separation of the cell wall, which can be seen in Plates 3f, 14 and 15. The wall itself has parted along the middle lamella and has also, in some places, split into coarse fibres. This

has caused the existing intercellular spaces to enlarge irregularly, and caused new spaces to open up between cells. The cell contents have been disrupted.

Plates 3g, 16 and 17 show beanshoots which have been made into jam, and acquired a gelled texture. The cell wall is swollen but with rather less layering than results from other processes such as sulphiting or boiling in bicarbonate. Some of the air spaces have elongated along the middle lamella, while other spaces have greatly enlarged in a similar way to those, described below, in deep fried tissue.

Deep frying beanshoots causes major changes to the structure, as seen in Plates 3h, 18 and 19. The cell walls are swollen but dense, and the whole shoot has dehydrated and shrivelled to a crisp texture. The outside layers of cells are compressed to an extent that the contents are completely flattened. In this area, the intercellular air spaces are very expanded and rounded in shape. The centre tissues show elongated spaces and swollen separating cell walls, although there is less compression in this area.

Cell Walls

Plates 20 - 28 show cell walls which were isolated from whole tissues prior to treating with the same range of processes described above.

The detailed structure of unprocessed control cell walls can be seen in Plates 20 and 21. The walls are evenly stained throughout, whereas after some processes the middle lamella is damaged and stain is able to permeate causing the appearance of a dark line. The mechanical weakening of cell walls after freezing and thawing is clearly visible in Plate 22. In addition to the folded conformation of the wall, the middle lamella is damaged and thus stained.

It is not possible to discern much information about wall ultrastructure from the micrographs of acidified brine stored isolated cell walls. For example, Plate 23 shows a cell wall fragment remaining after such treatment. It is apparent, however, that brining results in much more

extensive damage to separate cell walls than it does when they are constrained within cellular tissue.

The effects of sulphiting can be seen in Plate 24. Whilst the cell wall is swollen and layered, the extent of damage is not as severe as observed in sulphited whole tissues (Plates 10 and 11). An explanation for this finding is discussed later (Section 5.2.3).

The cell wall structures left after boiling in water or in bicarbonate solution are shown in Plates 25 and 26, respectively. Water treatment extracts material from the middle lamella and leads to separation in this region. The same effect is observed after boiling in bicarbonate but, in addition, layers are formed within the wall.

Processing isolated walls into jam causes marked structural changes, shown in Plate 27. The wall is swollen but homogeneous and entire rather than layered as in other processes. The intercellular spaces are enlarged although there is no splitting along the middle lamella.

The air spaces are also very expanded in deep fried cell walls (Plate 28). The wall itself, however, remains thin and compact.

5.2.3 Discussion

Sections of all the processed whole tissues were viewed in a light microscope and an electron microscope and compared with control, unprocessed material. The various treatments affected both structure and texture of beanshoots to different degrees. In all cases the cell contents were disrupted or precipitated to an extent that the texture of the processed tissue would depend on cell wall strength and integrity (Saxton & Jewell, 1969). In fresh, unprocessed tissue the turgor pressure from the cell contents also contributes to the texture. Changes in cell wall structure that were observed included swelling, layering, splitting at the middle lamella and expansion of intercellular spaces. In general, as might be expected, the treatment of cell walls isolated from surrounding cells caused more pronounced changes in structure than when whole tissues were processed.

The outermost tissues of the whole beanshoot were affected to the greatest extent because they were more exposed to the processing conditions. Within the central area, the stronger vascular cells were more resistant than the thin walled inner parenchyma tissue (see Plate 2). During preparation of shoots which had been sulphited or boiled in bicarbonate, it was noticed that the tissue as a whole was held together by the vascular fibres which remained much stronger than the parenchyma cells. This is similar to the finding of Jewell, Rantsios and Scholey (1973) that the vascular and epidermal tissues of strawberries retain their structural integrity after jam making.

Micrographs of control tissue show that the cell walls are thin and adjacent cells are closely associated and hence the intercellular spaces are small. In general, heat treatment causes a swelling of the cell wall, expansion of intercellular spaces and separation of adjacent cells due to loss of pectin binding in the middle lamella.

Freezing and thawing causes layers of cells to be torn apart along the middle lamella. The slow freezing employed in this investigation would be likely to cause the formation of large ice crystals, which is consistent with the observed disrupted cell organelles and enlarged intercellular spaces. Fast freezing methods would be expected to result in smaller ice crystals, which would minimise disruption.

Storing whole beanshoots in sulphite solution causes the cell wall to become very swollen and laminated. The degree of layering is particularly extreme compared with other processes. Comparison of Plate 24 with Plates 10 and 11 appears to indicate that sulphite treatment of isolated cell walls results in less swelling and layering than similar processing of whole tissues, a somewhat paradoxical observation. However, during preparation of the isolated cell walls for microscopy it was found that the material was severely damaged and there were problems in locating suitable samples for sectioning. It is likely that the only fragments which remained available for examination were strengthened, lignified walls. In support of this explanation, the wall shown in Plate 24 shows a similar degree of layering to that observed in vascular areas of whole tissue.

Sulphiting and jam making both cause pronounced wall swelling although some areas near the air spaces remain as constrictions (see Plates 11 and 17). The changes in the cell wall after sulphiting are not, however, uniform. The region of the wall closest to the cell is relatively compact whereas the cell walls after jam making are expanded evenly across the width of the wall and middle lamella. The pattern of delamination observed after sulphiting (i.e. progressively less from the middle lamella side of the wall to the cell side) may be accounted for by the diffusion of sulphite through the network of intercellular spaces and middle lamellae. Hence the cell side of the wall would be attacked last. Alternatively, the varying proportions of structural components across the width of the wall may provide an explanation and this is discussed in Section 5.4.

In a similar way to sulphiting, acidified brine storage of isolated cell walls appears to cause complete disintegration except for lignified walls. The effects of this process can be seen more clearly in the micrographs of whole tissue. Brining causes cell walls to swell and layer and causes the middle lamella to split. The changes are more extreme towards the outside tissue, but this storage treatment does not cause the same degree of softening as sulphiting.

In contrast to the acid processes described above, bicarbonate solution is alkaline. Boiling with this additive produces a very soft tissue. Adjacent cells are only weakly bound together and outer tissues are easily washed away. Bicarbonate causes the wall to part along the middle lamella but it also causes some splitting of the wall into coarse fibre-like layers which have a hairy appearance. A possible mechanism for this unusual layering, based on the effects of bicarbonate on specific wall constituents is discussed later (5.4).

Short cooking of plant tissue in water does not cause much damage to the structure. The softening that is observed is mainly accounted for by the loss of turgor pressure from the cell contents. The wall swelling and middle lamella separation is clearly more pronounced in the isolated walls (Plate 25) than in the cooked whole tissues (Plates 12 and 13).

Deep frying in oil, however, is clearly a much more severe treatment. The application of extreme heat and a non-aqueous solvent combine to form a different structure from the other processes, and a crisp texture. The shoots as a whole become shrivelled and dehydrated but there are marked differences between the inner and outer tissues. The outermost cells lose moisture and form a dense, compact layer with the contents precipitated between swollen cell walls. A striking feature of this compressed tissue is the gross expansion of some intercellular spaces presumably caused by gases becoming trapped and expanding rapidly with the sudden increase in temperature. In contrast the inner parenchyma cells are not compressed. A similar expansion of the air spaces which remained in clusters of isolated cell walls was also observed (Plate 28). These wall samples show clearly that material is not dissolved as in other processes, and the walls remain compact and dense.

Processing tissues into jam causes the wall to swell and form a homogeneous gel structure. These changes are particularly pronounced in the isolated cell walls, and the effectiveness of this process in extracting wall pectin (discussed in Section 5.4) is demonstrated by the way in which the cell wall jam was firmly set.

Shorter lengths of shoots were used for jam processing as it was found that larger pieces became shrivelled and dehydrated rather than undergoing softening and pectin extraction. This observation is similar to the industrial problem encountered when trying to make jam with too large pieces of fruit, such as whole strawberries. The fruit tends to toughen, and it floats because it has not absorbed sufficient sugar and is consequently less dense than the bulk of the jam. The larger pieces of shoot that shrank and hardened could be seen to be similar in structure to the deep fried samples. The outer layers of cells were dehydrated and compressed, preventing diffusion of syrup into the centre of the tissue.

5.3 Effects of chemical extractions on cell wall structure

5.3.1 Experimental

Samples of isolated cell walls were chemically extracted by a variety of methods to dissolve particular components and combinations of components. Water-soluble and ammonium oxalate-soluble pectin fractions were removed by the method which is intended as a pretreatment for glycoprotein extraction (Appendix A.4 Stage 1). Glycoprotein was extracted (Stage 2) both as a single treatment, and combined with depectination. In other experiments general deglycosylation of cell walls was carried out using anhydrous hydrogen fluoride treatment, by the method of Van Holst and Varner (1984). (Experimental details are given in Appendix A.18). Hydrogen fluoride treatment was employed with the objective of removing as much carbohydrate as possible from the wall to leave a structure containing mostly protein.

The treated cell wall samples were fixed and stained with PATCH-osmium and sectioned as described for processed cell walls (Section 5.2.1 and Appendix A.17). Untreated controls were prepared for comparison.

5.3.2 Results and discussion

The effects of chemical extraction of different components on cell wall structure was investigated by electron microscopy. The electron micrographs are described, and the different extractions are compared and discussed below. These results will be related to those from the food processing tests in the general discussion at the end of this Chapter.

The structure of untreated control cell walls (Plate 29) is compact and adjacent walls are tightly bound. Compared with this, extraction of water-soluble pectin (Plate 30) causes slight wall swelling and separation.

The pectin appears to be dissolved out of the middle lamella first, which results in splitting along this layer. Plates 31 and 32 show the

effects of removing the ammonium oxalate soluble pectin fraction in addition to the water-soluble pectin. The wall is very swollen and layered and generally much more damaged. Adjacent walls have split along the middle lamella in places, although constrictions are present in other areas. The middle lamella side of the wall is most swollen and finely layered, while the cell side is relatively compact.

Sequential extraction of glycoprotein after pectin causes further swelling, particularly in the interior of the wall. Thus Plates 33 and 34 show that the middle lamella area is swollen and the cell side is generally still entire, but the wall in between these two regions is more layered. In some places (see Plate 34) even the cell side of the wall is starting to disintegrate. The characteristic constrictions, noted above, are, however, still present. The combined effect of pectin and glycoprotein removal causes the wall to adopt a fine stripey appearance.

Glycoprotein extraction alone, Plates 35 and 36, can be seen to cause much less effect. In some places the middle lamella has separated, presumably because this pectin layer is dissolved by the heat and acid conditions of glycoprotein extraction. The wall itself is swollen and slightly layered. In contrast to all the other extractions, this layering occurs evenly across the whole wall. Thus, in this case, the cell side is not more compact, and at higher magnification (Plate 36) fragments can be seen disintegrating from this area of the wall. As the cell wall contains a higher proportion of pectin than glycoprotein it is to be expected that pectin extraction will cause more damage than glycoprotein removal. The importance of pectin and glycoprotein in controlling the structure of different areas of the wall will be discussed later (Section 5.4).

Plate 37 shows the very severe damage caused by partial extraction of carbohydrates by anhydrous hydrogen fluoride. This non-specific treatment will render the wall polysaccharides soluble, including cellulose, hemicellulose and pectin. Complete hydrogen fluoride digestion should leave a wall skeleton of protein, stripped of carbohydrate side-chains, together with lignin if present. However, as

these samples were successfully stained by PATCH-osmium, which is specific for 1,2-glycol groups of sugars, it is clear that carbohydrate degradation was not exhaustive. It is thought that the presence of traces of moisture in the cell walls quenched the hydrogen fluoride and prevented complete reaction. Nevertheless, during preparation of the hydrogen fluoride treated samples for microscopy, it was difficult to find many sections which contained discernible wall pieces. The fragment shown in Plate 37 is one of the few remaining and it therefore represents the least damaged end of the range of effects. More completely extracted walls would be barely visible.

5.4 General Discussion

The effects of a range of food processing treatments and chemical extractions of individual wall components on cell wall structure have been studied using microscopy techniques. Food processing treatments were investigated for their effects on whole tissues and for specific changes to the cell wall. The results from experiments with whole tissues were discussed in detail in Section 5.2.3. The purpose of this general discussion is to compare the effects of the food processes with those of the chemical extractions. This section will therefore concentrate on the results of tests carried out on isolated cell walls.

Throughout this discussion attempts will be made to relate observed changes in wall structure to effects on individual wall polymers. The different wall components are not distributed evenly across the width of the cell wall; this concept will be explained with reference to Plates 20 and 22. The cell wall, which forms a boundary between a pair of adjacent cells, actually comprises two halves, one from each cell, which are cemented together by the middle lamella. The whole unit forms a continuous layer (Plate 20) when intact, but in Plate 22 the middle lamella can be seen as a dark line in the centre. During formation of new cell walls the middle lamella is laid down first, and a primary cell wall is deposited on this. At a later stage of cell development the secondary wall layer, which contains lignin, is added. Thus consecutive layers are deposited on the cell side of the wall. The tissues used for this study were processed prior to the deposition of

much secondary wall. Hence this layer will not be considered in the following discussion.

The relative proportions of the wall components change from the middle lamella to the area of wall nearest the cell (Northcote, 1963). The middle lamella itself contains mainly pectin and the concentration of pectin decreases towards the cell side of the wall. The hemicellulose fraction is similarly distributed across the wall, although this component is not present in the middle lamella. In contrast, the concentration of cellulose is highest at the cell side of the wall. Glycoprotein is thought to be more evenly distributed throughout the width of the wall.

Sulphite treatment was found to cause swelling and layering of the cell wall. These effects were most extreme at the middle lamella side of the wall, whereas the cell side remained relatively compact. It is possible that these position-related structure changes may be accounted for by sulphite acting on particular wall components. The areas of wall richest in sulphite-labile components would thus be expected to be most damaged.

The acid conditions in sulphite solution will degrade pectins, while cellulose and hemicelluloses are resistant. It is also likely that sulphite solution will hydrolyse extensin side chains because the strength of acid is similar to 12 mM oxalic, which is employed to remove the arabinosides (O'Neill & Selvendran, 1980). Although oxalic acid treatment is carried out at a higher temperature than sulphiting, the prolonged exposure of cell walls in the latter system could result in glycoprotein side chain hydrolysis. As these carbohydrate groups are important in protecting the structure of the glycoprotein molecule (Section 1.2.5), their removal could weaken the wall by making the glycoprotein network less rigid.

The observation that sulphite-treated cell walls are most swollen and layered at the middle lamella side is consistent with this reagent degrading pectins. The magnitude of wall damage after sulphiting, which is extreme compared with other processes, could well be accounted

for by an additional effect on extensin side chains. Comparison of the structure of sulphited cell walls (see particularly Plates 10 and 11) with that of walls from which pectin and glycoprotein have been extracted (Plates 33 and 34) supports these suggestions. The extent of swelling, formation of fine layers and the retention of a compact area at the cell side of the wall are notably similar in both cases.

It is likely that the cell wall changes resulting from brining are also accounted for by acid-breakdown of pectin. Middle lamella swelling and splitting has occurred (Plates 8 and 9) but to a lesser extent than after sulphiting. In agreement with this explanation, the type of changes in cell wall structure found after acidified brining are similar to those found after pectin is extracted (Plates 30, 31 and 32). It should be noted that the extent of changes in these two groups of plates cannot be compared directly because Plates 8 and 9 are whole shoots whereas the rest are isolated cell walls. It has been mentioned earlier that acidified brine storage of isolated cell walls results in much more extensive damage than when the walls are constrained within cellular tissue. The prolonged storage of tissues in acidified brine is probably the reason that this treatment caused more damage than observed after pectin extraction.

Pectin is the wall component also most affected by boiling in water. Plates 25 and 30 both show a typical feature of extraction of water-soluble pectin, i.e. splitting along the middle lamella. It would appear that the higher temperature, shorter time conditions of cooking in water are more effective at dissolving pectin than the prolonged but more gentle fractionation treatment.

The samples which were used for making jam demonstrate a completely different effect on pectin compared with the processes described above. The combination of heat and acid partly degrades and dissolves the pectin so that, on cooling in the presence of sugar, the whole structure forms a homogeneous gel. These gelled cell walls (Plate 27) are swollen across the width of the wall and the middle lamella is also expanded but is intact and shows no signs of separating.

Sulphiting, brining and jam making all involve acidic conditions, and the effects of these processes on wall structure can be explained by changes in the pectin component. In the specific case of sulphiting it appears that glycoprotein may also be degraded, and the combined effects on these two components could explain why this process caused the most severe damage observed. In contrast, bicarbonate solution is alkaline, and boiling tissues in this solution causes changes in wall structure which are unlike any other process. Middle lamella splitting is observed in bicarbonate treated cell walls, but there is also splitting into unusual coarse layers within the wall (Plate 26). Part of these changes will be accounted for by pectin breakdown as this polymer is known to be degraded by heating in both acid and alkaline conditions. Hydrolysis of pectin will cause middle lamella splitting and general weakening of the wall. The formation of the distinct layers may be attributable to breakdown of the hemicellulose fraction of the wall. Hemicelluloses include a number of different polysaccharides which are alkali-labile to differing extents.

Chemical extraction of glycoprotein results in even swelling and layering across the wall, as would be expected from the distribution of this component. Glycoprotein extraction is accompanied by solution of some pectin (4.4) and this explains the middle lamella splitting observed.

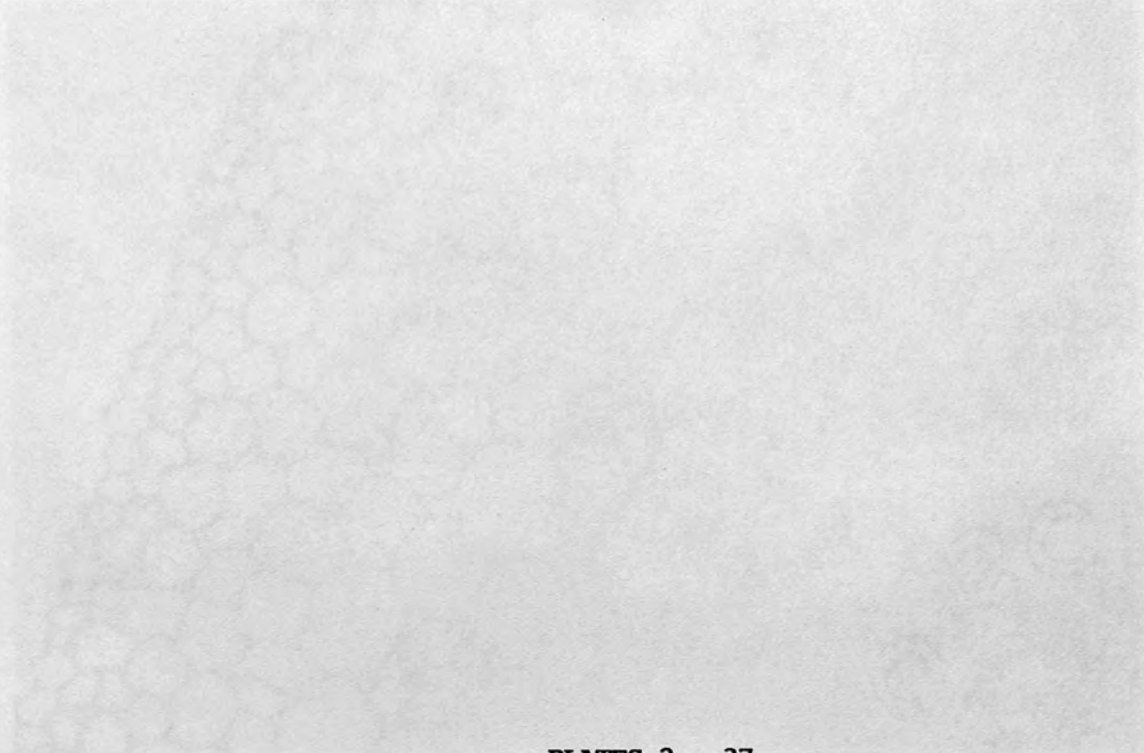
The extent of changes after glycoprotein extraction (Plate 35) can be contrasted with the changes after hydrogen fluoride treatment to remove carbohydrate and leave protein (Plate 37). The latter process causes much more severe swelling and disintegration. The relative effects of these two extractions is consistent with the wall comprising mostly carbohydrate and less than 10% protein.

Finally, two processes, namely freezing and deep frying, cause changes in wall structure which are related more to physical damage than chemical modification or extraction of different constituents. In the case of freezing the damage is caused by ice crystals, whereas in deep frying the heat-induced expansion of entrapped air causes separation of adjacent cell walls from the intercellular spaces. Although material

is not dissolved from isolated cell walls by deep frying, Plate 19 shows that the inner tissues of whole shoots are affected differently. It appears that moisture at the centre of the tissue is prevented from escaping by the dense outer layers. The high temperature of deep frying probably damages pectin, which is then dissolved in this moisture, resulting in cell wall swelling and middle lamella splitting.

PLATES 2 - 37

Plate 2. Light micrograph of cranial nerve section in longitudinal section showing the nerve fibers.



PLATES 2 - 37

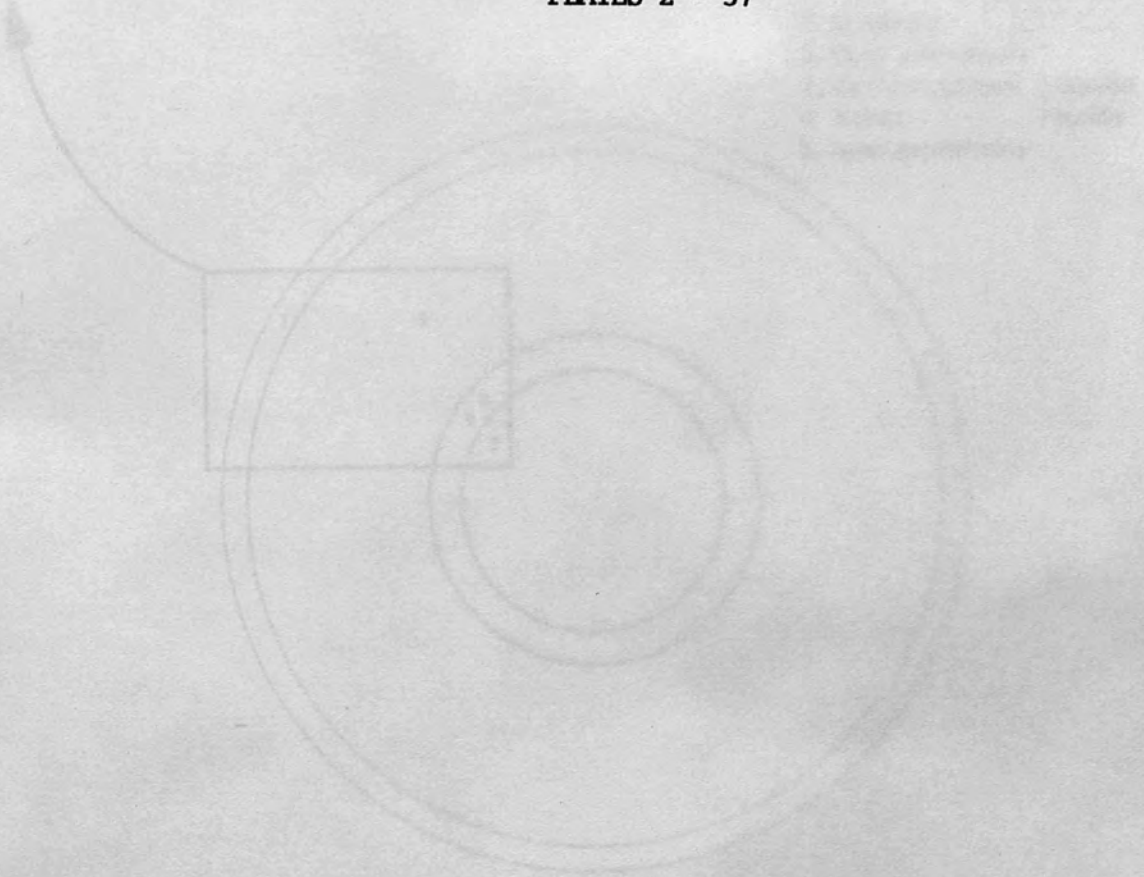
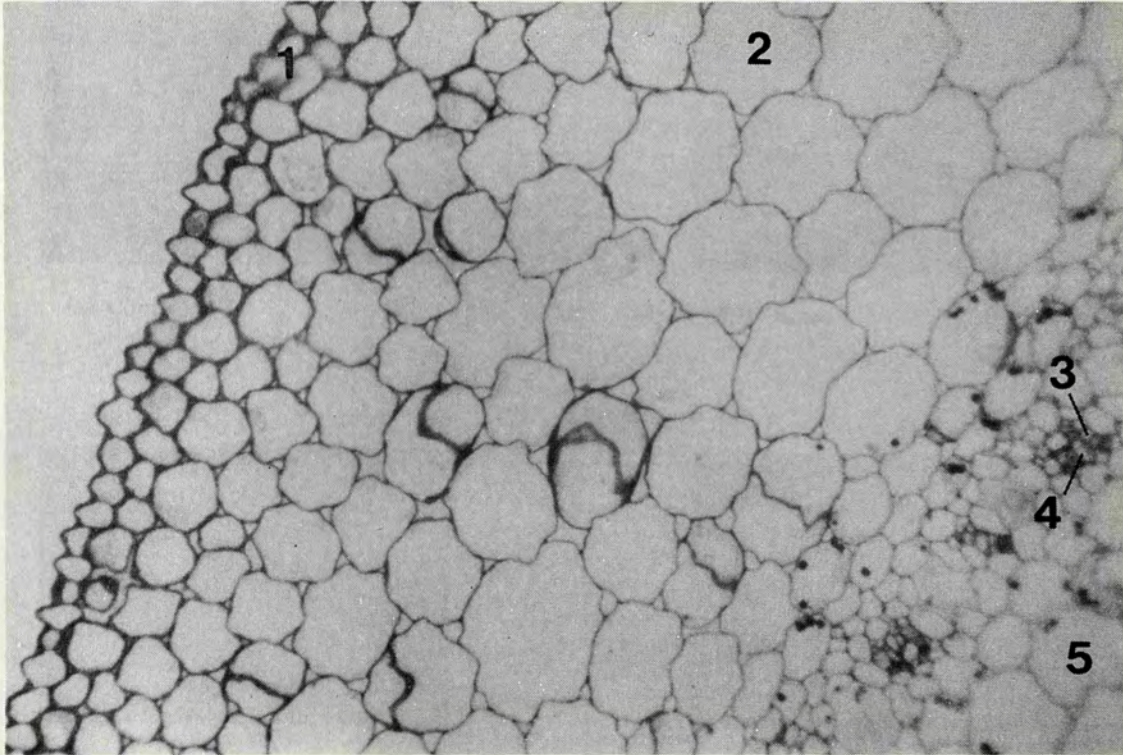


Plate 2. Light micrograph of transverse section of beanshoot to show different cell types
x 200



- 1. Epidermis
- 2. Outer parenchyma
- 3. Cambium/phloem } vascular
- 4. Xylem } bundle
- 5. Inner parenchyma

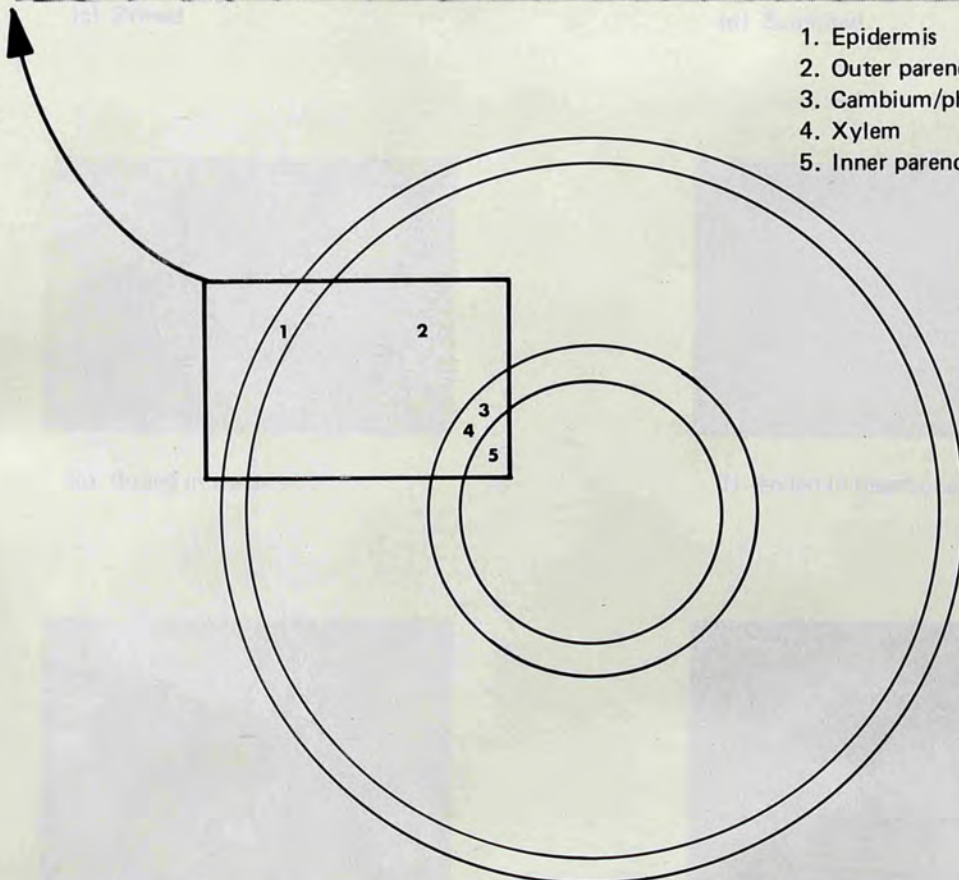
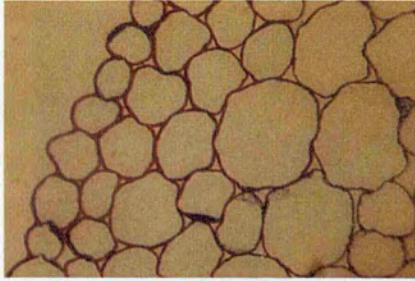
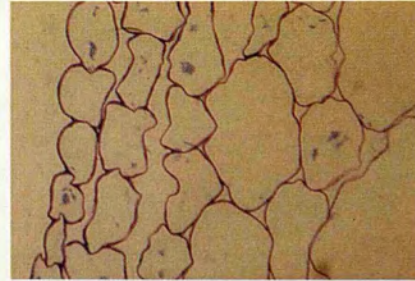


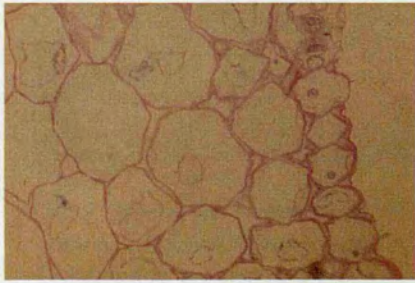
Plate 3. Light micrographs of processed beanshoots, stained with methylene blue, azure II and basic fuchsin x 300



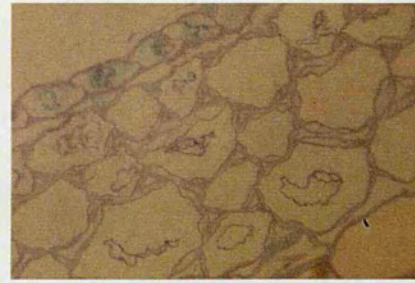
(a) Control



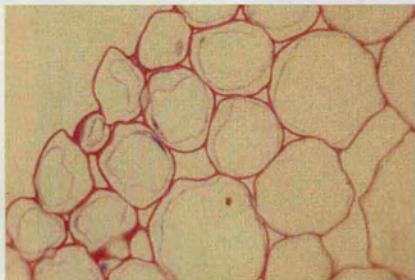
(b) Frozen



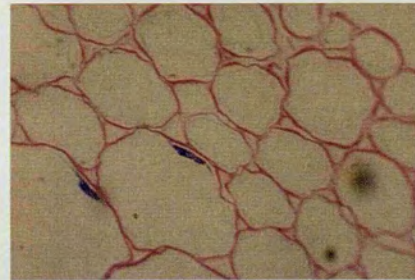
(c) Brined



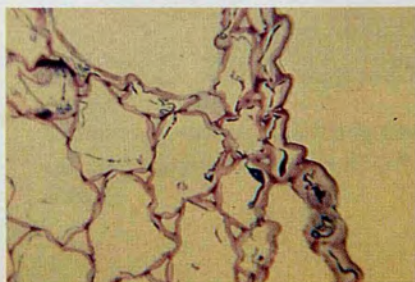
(d) Sulphited



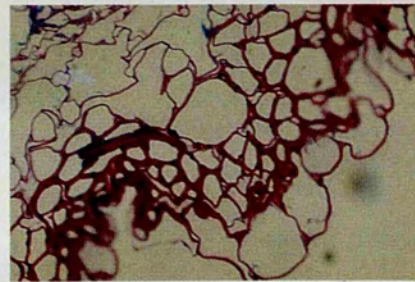
(e) Boiled in water



(f) Boiled in bicarbonate solution



(g) Jam-preserved



(h) Deep-fried

Transmission electron micrographs of processed beanshoots



Plate 4. Control
Outer layer of tissue showing epidermal and outer parenchyma cells with thin cell walls. Cell contents are arranged around inside of wall. x 2000



Plate 5. Control
Outer parenchyma cells demonstrating close binding of adjacent cell walls and small size of intercellular spaces. x 5000



Plate 6. Frozen
Cambium-xylem area depicting disruption of cell contents and separation of cell walls. x 2000



Plate 7. Frozen
Inner parenchyma cell wall showing splitting along middle lamella from air spaces. x 5000

Transmission electron micrographs of processed beanshoots

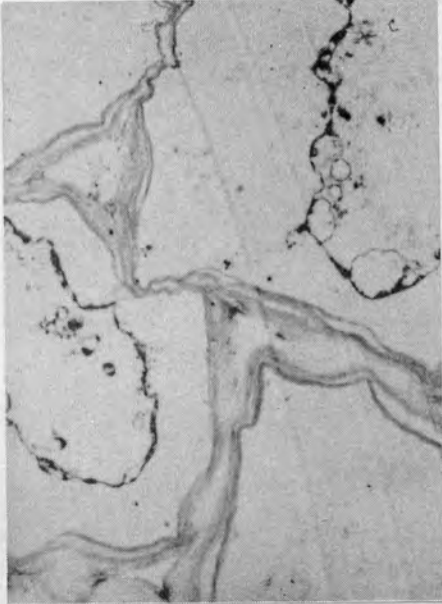


Plate 8. Brined
Outer parenchyma cells showing swollen, separating cell walls and plasmolysed cell contents. x 2000



Plate 9. Brined
Outer parenchyma region depicting, more clearly, the cell wall layering and separation. x 5000

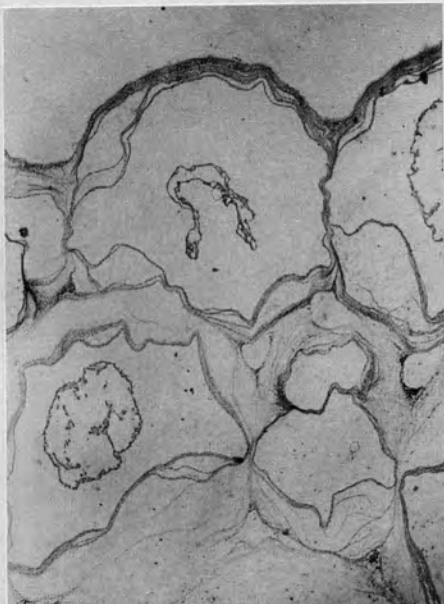


Plate 10. Sulphited
Epidermal and outer parenchyma cells demonstrating extremely swollen cell walls and plasmolysed contents. x 2000

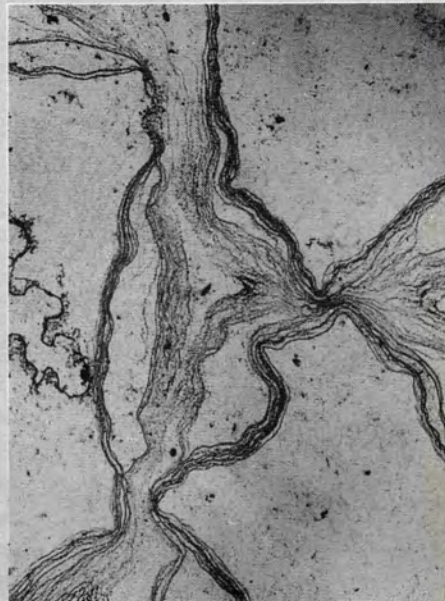


Plate 11. Sulphited
Outer parenchyma cells showing, in more detail, the laminated structure caused by separation occurring within the wall. x 3000

Transmission electron micrographs of processed beanshoots

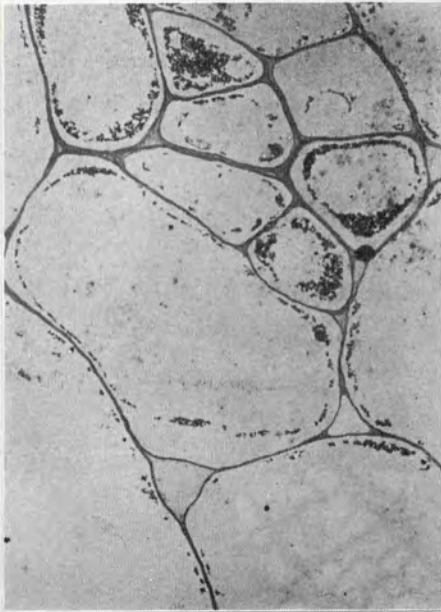


Plate 12. Boiled in water
Cambium-inner parenchyma tissues demonstrating precipitated contents and a little damage and swelling of cell walls, especially around the edges of the air spaces. x 2500

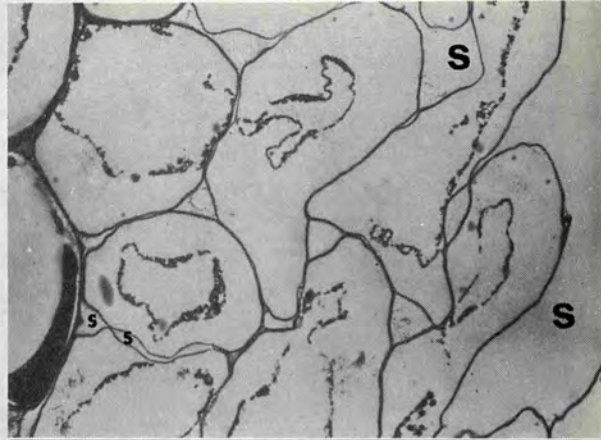


Plate 14. Boiled in bicarbonate solution
Inner parenchyma cells showing marked splitting in some areas of middle lamella. The cell contents are disrupted. x 2500

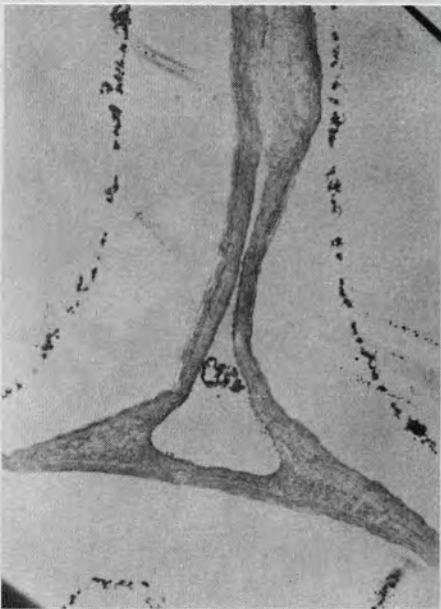


Plate 13. Boiled in water
Outer parenchyma cells which have been affected by processing to a greater extent than inner cells shown in Plate 12. The cell walls are swollen and the intercellular spaces are enlarging, causing cells to part along the middle lamella. x 5000

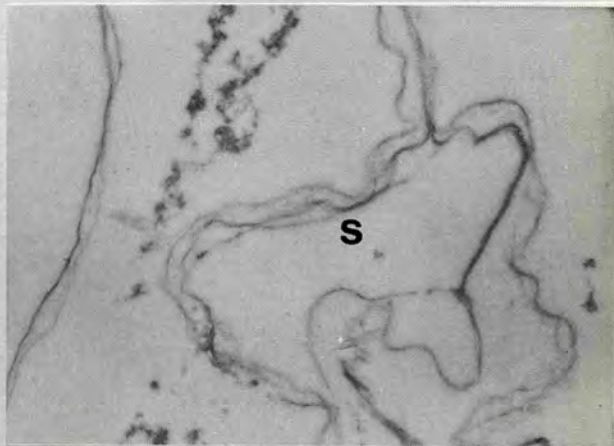


Plate 15. Boiled in bicarbonate solution
Inner parenchyma cells illustrating, more clearly, the layering of the cell wall into discrete fibre-like strands and the formation of enlarged intercellular spaces. x 5000

S = space (intercellular)

Transmission electron micrographs of processed beanshoots

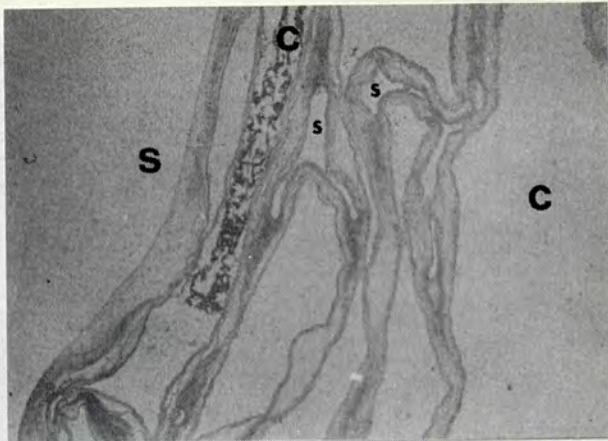


Plate 16. Jam-preserved
Outer parenchyma cells which have been flattened together. The cell walls are swollen and some of the intercellular spaces are elongating as adjacent cells part along the middle lamella. Some of the air spaces are greatly enlarged, for example the one on the left side of the Plate. x 2500

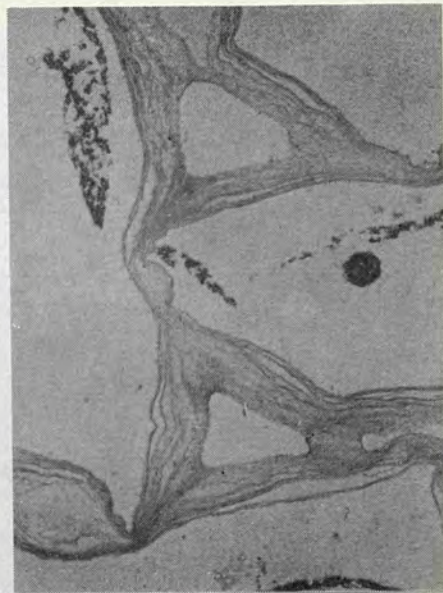


Plate 17. Jam-preserved
Outer parenchyma cells demonstrating the swelling of the walls and precipitated cell contents. x 2500

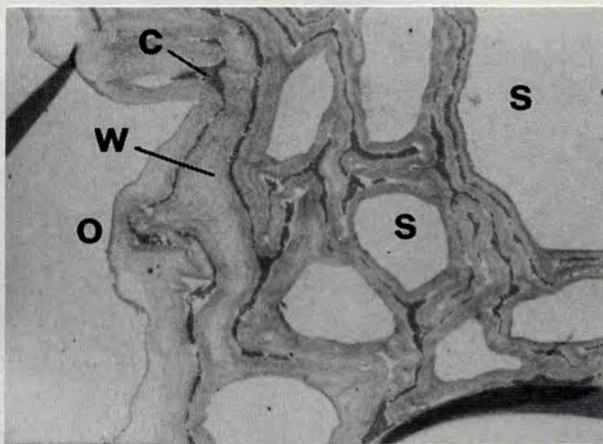


Plate 18. Deep-fried
Epidermis and outer parenchyma region showing layers of cells compressed together and swollen cell walls. It should be noted that the dark areas are flattened, precipitated cell contents and the white areas are greatly enlarged intercellular spaces. x 2500

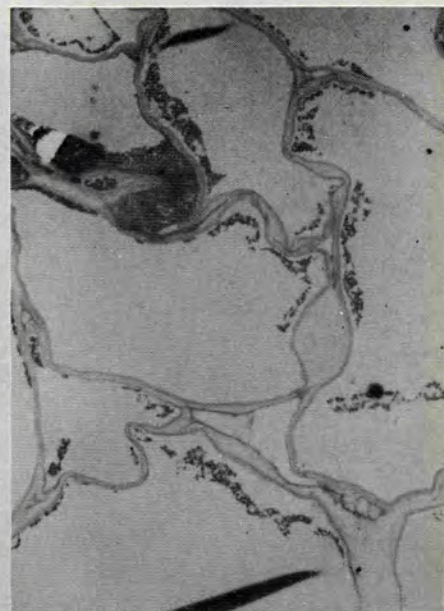


Plate 19. Deep-fried
Inner parenchyma cells, which have been less affected than those in Plate 18, showing swollen, separating cell walls. x 2500

S = space (intercellular)

O = outside edge

C = contents

W = wall

Transmission electron micrographs of processed cell walls



Plate 20. Control
Wall evenly stained. Middle
lamella undamaged. x 25 000

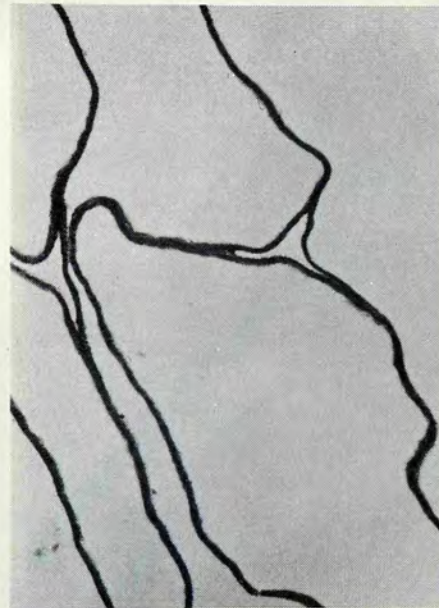


Plate 21. Control
Cluster of thin walls. Note
small size of space at inter-
cellular junction. x 5000



Plate 22. Frozen
Wall is a collapsed folded
structure. The middle lamella,
visible as a dark line, has
partially weakened and allowed
stain to penetrate. x 25 000



Plate 23. Brined
Severely damaged wall
fragment. x 5000

Transmission electron micrographs of processed cell walls

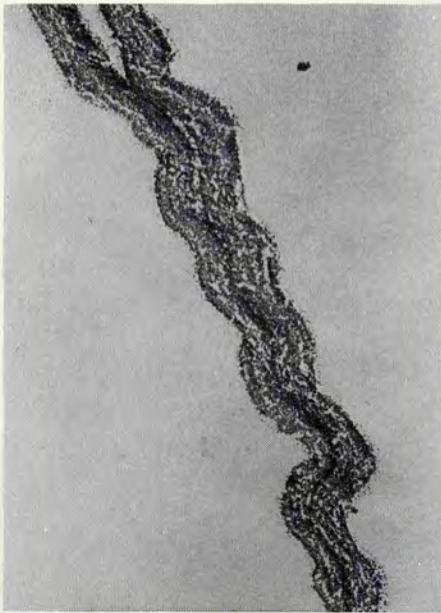


Plate 24. Sulphited
Wall very swollen and disintegrating, causing speckled appearance. Middle lamella starting to split. x 10 000



Plate 25. Boiled in water
Middle lamella split and wall adopts a fuzzy appearance as material is dissolved. x 50 000



Plate 26. Boiled in bicarbonate solution
Swollen, layered structure which has split at the middle lamella and within the wall. Individual fuzzy layers appear to be disintegrating further. x 10 000

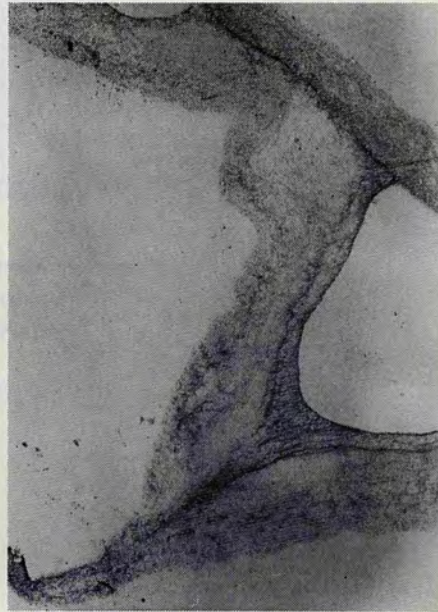


Plate 27. Jam-preserved
Group of extremely swollen cell walls showing homogeneous structure. Middle lamellae are intact and edges of intercellular spaces are rounded. x 5000

Transmission electron micrograph of processed cell walls

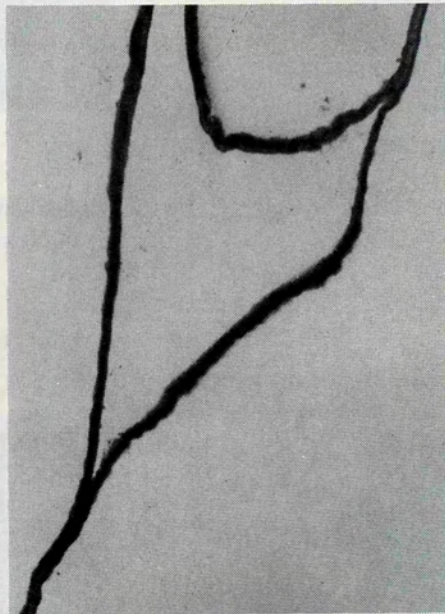


Plate 28. Deep-fried
Cluster of cell walls showing
grossly enlarged intercellular
space (compare with Plate
21). Walls dense and
undamaged. x 5000

Transmission electron micrographs of chemically extracted cell walls



Plate 29. Untreated control

Thin cell walls with intercellular junction compact. x 5000



Plate 30. After extraction of water-soluble pectin fraction

Walls showing some splitting along middle lamellae x 5000

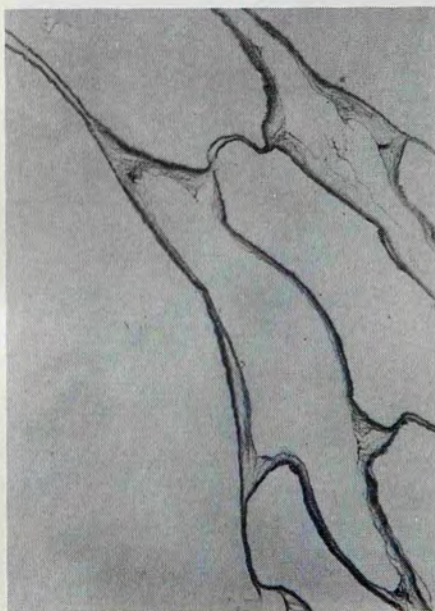


Plate 31. After complete pectin extraction

Cluster of cell walls showing middle lamellae splitting and disintegration across wall from middle lamella side. Constrictions present in some places. x 2000

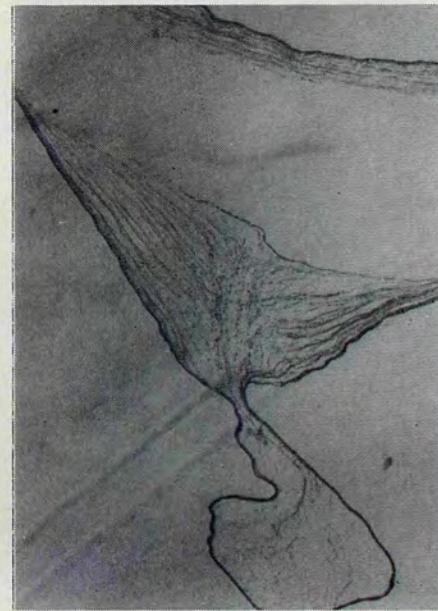


Plate 32. After complete pectin extraction

Cell side of wall is relatively compact whereas middle lamella side is very swollen and finely layered. x 5000

Transmission electron micrographs of chemically extracted cell walls

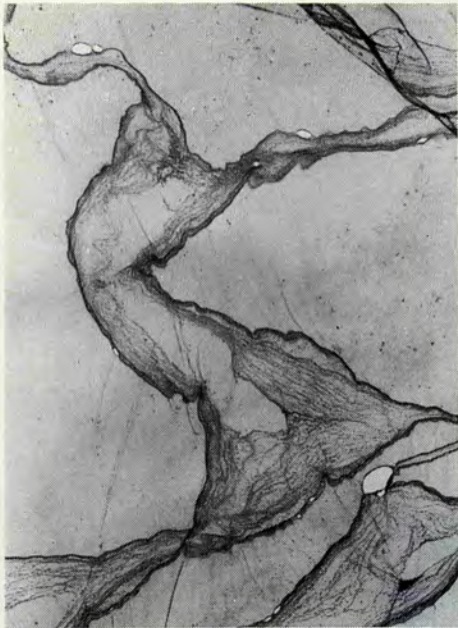


Plate 33. After depectination and glyco-
protein extraction

Walls are extensively swollen
although some areas are con-
stricted. Cell side of wall remains
compact and rest of wall forms
very fine layers. x 2000



Plate 34. After depectination and glyco-
protein extraction

Fine layers clearly visible
within wall. Cell side of wall
also starting to disintegrate
(area top left). x 10 000

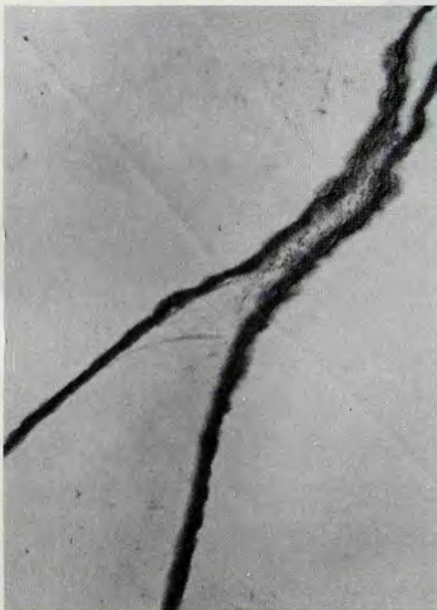


Plate 35. After glycoprotein extraction only

Middle lamella split and cell walls
slightly swollen. Wall appears stripey
in places and is disintegrating evenly
across its width. x 5000

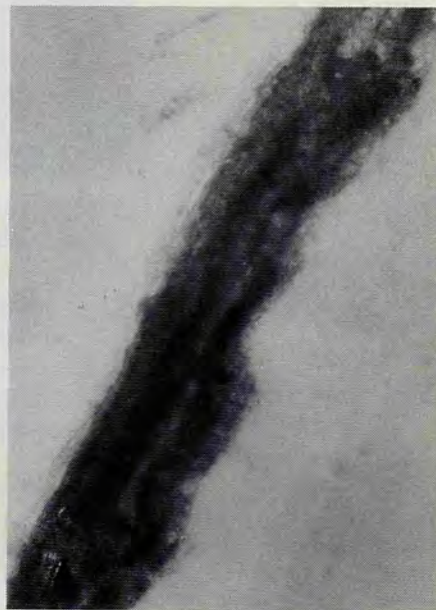


Plate 36. After glycoprotein extraction only

Area of wall not split at middle
lamella. Wall appears thin in
patches as material dissolved,
and fragments seen disintegrating
from cell side of wall. x 30 000

Transmission electron micrograph of chemically extracted cell walls

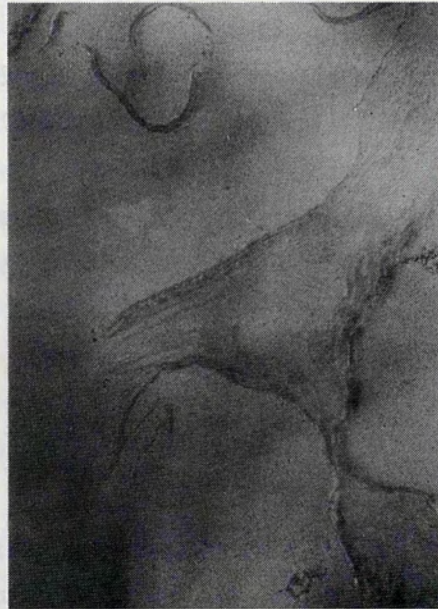


Plate 37. After extraction of carbohydrate using hydrogen fluoride

Very damaged, swollen fragment. It appears that cell side of wall may be most intact, and is seen splitting away from rest of wall (top right)
x 2500

DISCUSSION

Initial experiments in this project were aimed at extracting extensin from the cell wall in order to study changes in its composition caused by food processing. Various isolation procedures were employed and these are discussed in detail in Section 2.6. The major conclusion from these experiments was that it was not feasible to extract extensin from the wall in an intact form that contained isodityrosine cross-links. In addition to this experimental evidence, new information from the literature had indicated that extensin was more interwoven with other wall components than was originally imagined. As a specific example, close association of the glycoprotein with pectin was observed throughout subsequent work.

In view of the evidence that extensin is an integral component of the cell wall, it was decided to investigate the effects of processing on extensin as a part of the wall as a whole. Amino acid analysis was used to monitor changes in wall composition as a result of processing treatments. The characteristic extensin components, namely hydroxyproline and isodityrosine, were measured to identify changes in this specific wall polymer. At the same time, microscopical techniques were used to study the effects of the processing treatments on cell wall structure.

Prior to investigating changes in amino acid composition of cell walls it was necessary to develop an automated assay for isodityrosine. The peak corresponding to this dimer was identified in chromatograms of cell wall hydrolysates, and the evidence which supports this identification is summarised in Section 3.2.6. Attempts were then made to obtain enough isodityrosine to calibrate the amino acid analysis method. As one approach, GPC was used to isolate naturally-occurring isodityrosine from cell wall hydrolysates. During the course of these isolation experiments it was noticed that the concentration of isodityrosine appeared to vary between different batches of cell wall hydrolysates. The more extensive amino acid data compiled for unprocessed control cell

walls (reported in Chapter 4) confirmed that the isodityrosine concentration really did vary. A possible explanation for this finding is discussed below.

Synthesis of isodityrosine was also attempted, and the different systems employed are described in Section 3.4. A novel method which involved simple incubation of cell walls with tyrosine was identified as an effective way of synthesising isodityrosine. Incubation of a batch of cell walls in this way resulted in a six-fold increase in the concentration of isodityrosine. The dimer formation is presumably catalysed by endogenous wall peroxidase enzymes. This incubation method was repeated, with various minor modifications, in attempts to increase the yield of isodityrosine. However, it was found that the amounts of isodityrosine synthesised varied quite markedly between different experiments. An hypothesis which might explain these differences in synthesis was discussed in Section 3.5, and it is apparent that it may also explain the variations in cell wall isodityrosine content reported above. The basis of this hypothesis is that younger shoots may contain less isodityrosine but could have a greater capacity for synthesis than more mature shoots. If the plant material used for these studies was harvested at a critical stage of extensin polymerisation it is possible that the amounts of isodityrosine and the activity of enzymes involved in its synthesis could vary between different batches. An investigation into the effect of precise growth stage of shoots on enzyme activity and isodityrosine concentration is suggested under Future Plans. Effects of other conditions on isodityrosine synthesis by this cell wall incubation method may also be worthy of study, as the possibility remains that the successful syntheses were caused by some adventitious contamination.

Throughout this work several observations have indicated that the cell wall pectin fraction and the glycoprotein fraction may be coextracted. During various extractions using the acidified chlorite method (Sections 2.4 and 4.3) the amount of extensin rendered soluble was compared with and without use of the pretreatment to remove pectin. These experiments clearly demonstrated that more extensin was extracted from

the cell wall if the depectination treatment was omitted. Analysis of the pectin extracts confirmed that they contained hydroxyproline. Furthermore, from the data in Table 8 it can be inferred that a similar quantity of hydroxyproline may be extracted with pectin as is removed specifically as the glycoprotein fraction. As complementary evidence to amino acid analysis, results from measurement of carbohydrate composition of the various wall fractions (Section 4.4) also support the idea that pectin and glycoprotein extractions may not be very specific. The results of these experiments indicate that glycoprotein extraction dissolves more than 20% of the amount of wall carbohydrate which is rendered soluble by the pectin extraction stages. This carbohydrate was measured as galacturonic acid and is likely to be mostly pectin.

A close association and parallel extraction of pectin and extensin are not surprising considering the current ideas of wall structure shown in Fig.5 and discussed in detail in Section 1.3.4. The basis of this wall model is a woven cellulose structure and a separate but entangled extensin network, surrounded by an amorphous matrix of pectin and hemicellulose.

It is possible that the extensin component in the wall actually consists of fractions with different degrees of solubility. There could be a readily extracted portion which would dissolve during pectin solution; and it is feasible that more extensive cross-linking could cause another fraction to be less easily dissolved from the wall. It is interesting that the water-soluble pectin fraction contains significant quantities of the amino acids found in extensin, including isodityrosine, whereas much smaller amounts are present in the ammonium oxalate pectin fraction. As discussed in Section 4.5, this may reflect an association of extensin with a specific type of pectin polymer.

The data on changes in cell wall composition and the results from the microscopy studies have been fully discussed in Sections 4.5 and 5.4, respectively. Findings from these two different aspects of the processing study will be linked and discussed below.

From the range of food processing treatments and chemical extractions to which cell walls were subjected in these tests, water pectin extraction and boiling in bicarbonate both caused isodityrosine to be dissolved out of the wall. It can be inferred from these observations that large fragments of extensin molecules, or even cross-linked oligomers, may have been rendered soluble. The extract resulting from boiling in bicarbonate contained, in addition to one-tenth of the cell wall isodityrosine, one-third of the wall hydroxyproline. This process caused an unusual separation of the wall into coarse fibres, as seen microscopically. It has previously been suggested that this may be explained by a combined effect of bicarbonate on the pectin and hemicellulose fractions of the wall. It is now clear from the amino acid data that this process also damages glycoprotein. It is possible that the treatment attacks extensin via alkali-labile amino acids such as serine. Such hydrolysis could result in formation of small glycosylated peptides, perhaps denuded of galactosyl residues, originally attached to the serine groups.

In addition to causing some isodityrosine to be extracted, bicarbonate treatment also destroys about half the isodityrosine content of the wall. Sulphiting similarly causes hydrolysis of isodityrosine and also loss of some hydroxyproline. It has earlier been proposed that the magnitude of the structural changes after sulphiting, namely extreme swelling and formation of fine layers, results because these conditions degrade both pectin and extensin. In particular, it was mentioned that sulphite could cause hydrolysis of extensin's carbohydrate side-chains and hence reduce the rigidity of this polymer. In support of the suggestion that both pectin and glycoprotein may be affected, the micrographs of sulphited cell walls show similar features to those of walls from which these two wall components have been chemically extracted.

It is likely that the structural modifications observed after acidified brine storage and jam making are mainly attributable to changes in pectin. Pectin is certainly dissolved and extracted from the wall by the heat and acid of the jam process, and sets to form a gel on cooling. The wall content of hydroxyproline and isodityrosine is

reduced to one half of the level of untreated controls and hence, presumably, extensin is extracted in parallel with pectin. Brining causes changes in wall amino acid composition similar to, but less markedly than, those caused by jam making. The accompanying structural changes after acidified brine storage are swelling and layering of the wall in a corresponding way to that observed after pectin extraction. This finding supports the suggestion that commercial brining degrades mainly pectin.

The small reduction in the wall content of extensin's characteristic amino acids after boiling in water is, as expected, accompanied by very little physical damage to the wall. Deep frying, however, causes more outstanding decreases in the marker compounds. These amino acids have no aqueous phase available into which to be extracted and thus it appears that they are degraded by the extreme heat of frying.

Thus it may be concluded that most food processing treatments affect the pectin component of the wall because this polymer is easily hydrolysed by acid or alkaline conditions. As the wall consists of 30-40% pectin (Albersheim, 1976) the consequent structural changes, such as splitting of the middle lamella and swelling of the wall, will be clearly noticeable. However, in addition to effects on pectin, some of the processes tested, notably boiling in bicarbonate, sulphiting and jam making, also resulted in damage or extraction of extensin. It has further been suggested that the unusual layering observed after boiling in bicarbonate may be caused by this treatment dissolving the hemicellulose fraction. All of the processes tested, including boiling in water or heating in pH 4 acetate buffer, resulted in breakdown of some of the isodityrosine. It has been clearly confirmed, however, that acidified chlorite is specifically required to split isodityrosine links in order to extract glycoprotein, and acid conditions alone are not sufficient.

This work has shown that glycoprotein extraction causes concomitant solution of cell wall carbohydrate. In a corresponding way, and of particular interest, it was found that the water-soluble pectin fraction

contains all the predominant extensin amino acids including isodityrosine. The boiling in bicarbonate test also produced a similar result. These findings suggest that at least part of the wall extensin, possibly a less extensively cross-linked fraction, is more readily extracted than was originally thought.

to explain the presence of isodityrosine in the wall extensin fraction synthesized during the growth of the cell wall. It was suggested that isodityrosine is formed from dityrosine by a decarboxylation reaction during the synthesis of the cell wall.

It would be interesting to know whether the presence of isodityrosine in the cell walls is due to the presence of isodityrosine in the cell wall or whether the activity of the enzyme isodityrosinase is high in the cell wall. Isodityrosinase is a member of the tyrosinase family of enzymes and is a hydroxylase enzyme.

2. Isodityrosine in Cell Walls

Cell walls have been shown to contain isodityrosine in the form of isodityrosine with tyrosine in the form of isodityrosine. Isodityrosine is also found in other cell wall components such as chitin and cellulose. The synthesis of isodityrosine in the cell wall is described in Section 2.1. The presence of isodityrosine in the cell wall is due to the presence of isodityrosine in the cell wall.

With the objective of determining the role of isodityrosine in the cell wall system, several studies have been carried out. The results of these investigations are described in Section 2.2.

3. Isolation of Isodityrosine

The application of the method of isolation of isodityrosine from the cell wall hydrolysates and the results of the isolation of isodityrosine from the cell wall are described in Section 3.1. The isolation of isodityrosine from the cell wall is based on ion exchange chromatography.

FUTURE PLANS

1. Effect of wall growth stage on isodityrosine content

An hypothesis was proposed in Section 3.5 and extended in the Discussion to explain the variations in isodityrosine content and isodityrosine synthesising ability observed between different batches of cell walls. It was suggested that relatively young cell walls may contain less isodityrosine but have a greater potential for synthesis than more mature walls.

It would be interesting to investigate at which growth stage seedling cell walls have the most active isodityrosine synthesising capacity. The activity of peroxidase enzymes could be monitored, and the isodityrosine content measured in relation to dry weight and hydroxyproline content.

2. Isodityrosine synthesis

Cell walls can be used to synthesise isodityrosine by simple incubation with tyrosine (3.4.2). However, this work has shown that there may be other factors which need to be controlled for optimum isodityrosine synthesis. Examples of these factors have been discussed in detail in Section 3.5, and include cell wall growth stage (as 1. above), addition of hydrogen peroxide and the inhibitory effect of ascorbate.

With the objective of synthesising isodityrosine using this cell wall system, possible effects of these and other factors would be worth investigating.

3. Isolation of isodityrosine

The application of GPC to isolate isodityrosine from cell wall hydrolysates and some of the difficulties encountered with this method are described in Section 3.3.2. A different chromatographic technique based on ion exchange was briefly tried as an alternative. Initial

tests with the method of Epstein and Lamport (1984) indicated that further development would be required because of the limited experimental detail available on suitable elution gradients. It is suggested that this ion exchange method might be more convenient with a different buffer system from pyridine acetate, such as ammonium acetate. There is also an alternative ion exchange procedure, reported by Cooper and Varner (1983) which may be more promising.

4. Effect of processing on extensin's carbohydrate side chains

The peptide backbone of extensin is glycosylated by short arabinose side chains which stabilise the helical conformation of the molecule. Van Holst and Varner (1984) found that removing the carbohydrate side chains caused the ordered secondary structure to be lost. Thus, any processing treatments which remove or possibly shorten these critical arabinosides would change the structure of extensin. Such a change could consequently reduce the strength of extensin as discussed in Section 1.2.5.

In order to investigate the effects of processing on extensin carbohydrate side chains a method to extract the glycoprotein, or at least glycopeptides, from the wall with these groups still intact is required. The ideal approach would be to employ a beta-arabinofuranosidase but no mention of this enzyme has been found in the literature. Only enzymes with pyranosidase specificity and enzymes active with alpha-linked sugars are described.

Papain is reported to be used in the cherry processing industry to soften fruit prior to syruping (Blakemore, 1967). The effect of this enzyme is specifically attributed to its proteolytic activity. Papain preferentially hydrolyses peptide bonds with the basic amino acids arginine, lysine and phenylalanine (Bergmeyer, 1974). These amino acids constitute about 10% (mol per 100 mol) of the cell wall glycoprotein (O'Neill & Selvendran, 1980). It was therefore assumed that there would be sufficient such sites to split the glycoprotein into glycopeptides, and this method was tried as a means of extraction.

Cell walls were incubated with papain (Sigma Type IV) in 50 mM acetate buffer pH 4 containing 0.2 M sodium chloride. The conditions for enzyme activation had been previously assessed with the substrate benzoyl-arginine ethylester. However, it was found that papain extracted a large proportion of carbohydrate but only about 5% of the wall hydroxyproline. It was concluded that the enzyme preparation used contained more carbohydrate splitting activity than protease action and was therefore not suitable for extracting glycopeptides from the cell wall.

It is suggested that an alternative source of this enzyme, or a preparation from which protease activity has been purified, may be effective at extracting extensin glycopeptides. The possibility remains, however, that proteolytic papain may not be able to gain access to the extensin backbone. Prior to trypsin digestion of cell walls, the arabinosides are normally removed by acid hydrolysis (Lampert, Katona & Roerig, 1973), to enable enzyme to reach the polypeptide.

As an alternative, the acidified chlorite method of O'Neill and Selvendran (1980) remains a suitable means of extracting extensin glycopeptides from the wall. An enzymic method was attempted, in the present study, in preference to this chemical treatment because of the observed breakdown of pectin (discussed in Section 4.4). Nevertheless, acidified chlorite is reported to cause little, if any, hydrolysis of extensin arabinosides.

A further alternative which might enable an investigation of extensin carbohydrate side chains is a method described by Tang and Williams (1984) for collagen. This procedure involves nitrosation followed by GLC or HPLC.

As an extension of this present study, an investigation into the effects of the range of food processes on extensin carbohydrate side chains would be most interesting. As indicated above, papain, acidified chlorite or a nitrosation method could possibly be used to release extensin glycopeptides. These glycopeptides could then be treated by the various processes and the arabinoside profile compared, possibly using HPLC or TLC, with untreated controls.

Routine Experimental Procedures

Unless otherwise stated, all materials were obtained from Sigma and all % are m/V. Addresses of suppliers are given at the end of the Appendix.

1. Growth of seedlings

Mung bean seeds were kindly supplied by Masterfoods B.V., Netherlands. Seeds were soaked overnight, at room temperature, and grown on sterilised sponges in sealed containers. Each unit contained 15 g (dry weight) of seeds and 200 ml water. The containers were placed in an incubator at 28^o-30^oC for four days. The shoots, final length 10-15 cm, were trimmed to remove hook and root, and then used for cell wall preparation. For processing the whole shoot tissue for microscopical examination, the seedlings were cut into 5 cm-long pieces.

2. Cell wall preparation

All equipment and reagents (except sodium dodecyl sulphate solution) were cooled on ice. The trimmed shoots, in portions of approximately 15 g, were ground thoroughly in a pestle and mortar with 2-3 ml 0.1M pH6 phosphate buffer containing 0.6M sucrose. The homogenate was transferred to 50-ml glass centrifuge tubes with approximately 30 ml of the same buffer containing 0.3M sucrose. It was found that the addition of approximately 1 ml of 0.5% sodium dodecyl sulphate (SDS) solution (BDH) at this stage collapsed the foam formed during grinding and enabled the cell wall fragments to be spun into a pellet. The tubes were spun at about 2000 r.p.m. in a bench centrifuge and the supernatants were discarded. The pellets were washed four times with 30 ml 0.5% SDS followed by four washes with distilled water.

For some experiments the shoots (up to 100 g) were homogenised in a Kenwood liquidiser. Sufficient 0.6M sucrose buffer was added to cover the blades (approximately 75 ml), and the tissues were liquidised at maximum speed for two periods of one minute. After the first minute of

liquidising, the material on the sides of the blender were washed down to the blades with approximately 20 ml buffer. The effectiveness of the procedure was checked by light microscopy and found to be effective at breaking the tissue into very fine fragments. The liquidised homogenate was then spun and washed as described above.

For quantitative portioning of the cell wall preparation the pellets were combined and made into a dilute suspension with water. While the suspension was being stirred, aliquots were taken using a 5-ml Gilson automatic pipette, and placed in tared containers and weighed. Duplicate portions were also accurately weighed into evaporating dishes. These were dried on a steam bath and then in a vacuum oven at 70°C to constant weight. A vacuum oven was used as cell wall samples char at normal drying oven temperatures. These weights were used to calculate the dry weight per gram of suspension, enabling conversion of the portions taken to equivalent weight of dry cell wall.

3. Enzyme digestion of cell walls

Cell walls, prepared from about 30-g shoots, were incubated in a total volume of 100 ml with the following:

- 0.239g cellulase (Type I 1.34 U/mg)
- 0.08g polygalacturonase (Pectinase 6.6 U/mg, 15 mg/ml in 40% glycerol)
- 0.02mg pectinesterase (Tomato, 166 U/mg 1.25 ml of 1.6 mg/100 ml stock)
- 0.005g thimerosal, preservative
- 0.1M citrate-phosphate buffer pH7, containing 0.1M sodium chloride

The incubation was carried out in a shaker bath at 30°C for 48 hours. After 24 hours the pH was adjusted to 5, with 0.1M citric acid, to provide suitable pH conditions for each of the enzymes for part of the time (pH optima varied between 4 and 7.5). The incubation mix was filtered and the soluble extract dialysed.

4. Sodium chlorite/acetic acid procedure for extracting glycoprotein from cell walls

All extraction steps were performed on cell walls prepared from about 30-g shoots, in a total volume of 100 ml.

Stage 1

Preliminary pectin removal was carried out by heating cell walls in distilled water adjusted to pH 4 (with dilute hydrochloric acid), at 80°C for two hours. In all extractions, except those for microscopical examination, this step was carried out in a shaker bath. The cell wall suspension was centrifuged and the pectin extract removed from the cell wall pellet. This process was repeated. The cell walls were then treated with 0.5% ammonium oxalate (BDH) at pH 5.5, under the same conditions. After removing the ammonium oxalate soluble pectin fraction the cell wall pellet was washed once with water.

Stage 2

The depectinated cell wall sample was made up to 90 ml in a wide-necked conical flask. This flask was arranged in a glycerol bath on a thermostatically controlled stirrer hot plate. A slow stream of nitrogen was bubbled through the cell wall suspension, and the temperature was maintained at 70°C. Five ml of 2.4% $\frac{V}{V}$ acetic acid (BDH) and 5 ml of 7.5% sodium chlorite (BDH) were added and the reaction was allowed to proceed for 15 minutes, when a further 5 ml of each of the reagents were added. After a total of 30 minutes the hotplate was turned off, and the flask was allowed to cool, with nitrogen still flushing. The suspension was centrifuged to separate the glycoprotein extract from the cell wall residue. The pellet was washed once with water, and the washings pooled with the extract.

5. Acid hydrolysis

Samples were hydrolysed into free amino acids by refluxing with 100 ml 6N hydrochloric acid (Fisons) at 110°C for 20 hours. Any samples which

were dilute solutions were rotary evaporated prior to addition of acid. An internal standard was added before hydrolysis.

Cell walls were hydrolysed at a concentration of less than 4 mg per ml (Fry, 1982); an average of 1.35 mg dry cell walls per ml acid was used for the processed samples. For samples analysed on an LKB, norleucine was used as an internal standard. This was added at a concentration of 0.1 mg in 100 ml for controls and residues and 0.01 mg for soluble extracts. Taurine, 0.14 - 0.34 mg per 100 ml, was used as an internal standard for samples analysed on a Pico-Tag, because norleucine coeluted with isodityrosine.

The extracts and the controls prepared with added sugar, from the jam processing tests, were hydrolysed in a total of 600 ml acid because of the high concentration of sugar.

6. Amino acid analysis

6.1 Using an LKB amino acid analyser

Most of the amino acid analysis for this study was carried out using an LKB 4400 analyser linked to a Spectra Physics SP4000 computing integrator. All of the determinations of isodityrosine for Chapter 3, and the analysis of processed samples, except sulphited, were carried out with this system.

The processing samples were prepared by rotary evaporating the total hydrolysate and dissolving the residue in 3 ml 0.2N sodium citrate buffer at pH 2.2 (loading buffer). Amino acid separation was on an Ultropac 8 (LKB) column of cation exchange resin. Elution was by a sodium citrate buffer system with increasing pH, temperature and ionic strength during the run (see Table 11). Detection was by post-column derivatisation with ninhydrin.

The analysis method was calibrated using 30 μ l of the following mixture:

1.0 ml 0.656 mg/ml hydroxyproline*
1.5 ml 0.288 mg/ml proline*
0.5 ml 0.41 mg/ml norlucine*
0.5 ml protein hydrolysate calibration mix (LKB)
0.5 ml loading buffer

* 100-ml stock solutions prepared in loading buffer.

The concentrations of amino acids were calculated using the following formulae:

(AA : Amino Acid ; IS : Internal Standard)

(i) Calibration run

$$KF_{AA} = \frac{\text{concentration}_{AA} \times \text{area}_{IS}}{\text{area}_{AA} \times \text{concentration}_{IS}}$$

(ii) Sample run

$$\text{concentration}_{AA} = \frac{\text{IS amount}}{\text{sample amount}} \times \frac{KF_{AA} \times \text{area}_{AA}}{\text{area}_{IS}}$$

Isodityrosine concentrations were calculated manually because in some samples this component was present in small concentrations and peak integration was unreliable. The area of the isodityrosine peak was calculated from the product of height and width at half height. The amount of isodityrosine was then calculated using a response factor for tyrosine, also calculated in the same way. Peak width was taken into account in isodityrosine determination because the peak shape was very broad compared with the standard amino acids.

A typical chromatogram from the LKB amino acid analyser is given in Fig.9.

Table 11. Programme used for separation of cell wall hydrolysates on an LKB amino acid analyser

| <u>Step No.</u> | <u>Buffer</u> | <u>Time (mins)</u> | <u>Temp °C</u> |
|-----------------|---------------|--------------------|----------------|
| 1 | C | 2.0 | 45 |
| 2 | C | 10.0 | 62 |
| 3 | B | 23.0 | 62 |
| 4 | B | 2.0 | 83 |
| 5 | E | 23.0 | 83 |
| 6 | F | 2.5 | 83 |
| 7 | A | 8.0 | 83 |
| 8 | A | 3.0 | 45 |
| 9 | A | 20.0 | 45 |
| 10 | A | 1.0 | 45 |

Buffers:

- A: 0.2N sodium citrate pH 3.00
- B: 0.2N sodium citrate pH 4.25
- C: 0.2N sodium citrate pH 3.42
- E: 1.2N sodium citrate pH 6.45
- F: 0.4N sodium hydroxide

6.2 Using a Pico-Tag system

The sulphite-processed samples and those analysed for experiments described in Section 4.3.3. were analysed using a Waters Pico-Tag amino acid analysis system. This system involves pre-column derivatisation with phenylisothiocyanate and separation by reverse-phase HPLC on a Nova-Pak C18 column. Because the amino acid composition of these samples was fairly low, it was necessary to dry 80-200 µl of acid hydrolysates in up to four portions, for derivatisation.

Derivatisation was carried out by adding 20 µl ethanol 95% : water : triethylamine : phenylisothiocyanate (7 : 1 : 1 : 1) (Pierce & Warriner UK Ltd). The reagent and sample were mixed and allowed to stand at room temperature for twenty minutes, and the reagent was removed under

vacuum. The derivatised samples were then dissolved in 40 μ l diluent (5 mM sodium phosphate buffer pH 7.4 : acetonitrile (Fisons) (95 : 5)) and 25 μ l of the resulting solution was injected for analysis. Gradient elution at 38°C was employed, with a two eluent system providing an increasing concentration of acetonitrile (see Table 12). The phenylthiocarbamyl amino acid derivatives were detected by U.V. absorbance at 254 nm.

The analysis method was calibrated using protein hydrolysate calibration mix (Pierce & Warriner UK Ltd) mixed with taurine and hydroxyproline solutions, which were each prepared in 0.1M hydrochloric acid. The final calibration solution contained 2 μ mol/ml of each amino acid except cystine which was present at 1 μ mol/ml. This standard solution, 5 μ l, was derivatised, made up in 200 μ l diluent and 10 μ l was injected.

The concentrations of amino acids were calculated using the following formulae:

(AA : Amino Acid ; IS : Internal Standard)

(i) Calibration run

$$RF_{AA} = \frac{\text{concentration}_{AA}}{\text{area}_{AA}}$$

(ii) Sample run

$$\text{concentration}_{AA} = \frac{\text{area}_{AA} \times RF_{AA}}{\text{area}_{IS} \times RF_{IS}} \times \frac{\text{IS amount} \times \text{scale factor}}{\text{sample amount}}$$

A typical chromatogram from the Pico-Tag system is given in Fig.14.

Table 12. Programme used for separation of cell wall hydrolysates on a Pico Tag system

| Time (i) (min) | Flow rate (ml/min) | % A ⁽ⁱⁱ⁾ | % B ⁽ⁱⁱⁱ⁾ | Curve profile ^(iv) |
|-------------------|------------------------|---------------------|----------------------|-------------------------------|
| Initial | 1.0 | 100 | 0 | |
| 10.00 | 1.0 | 54 | 46 | 5 |
| 10.50 | 1.0 | 0 | 100 | 6 |
| 11.50 | 1.0 | 0 | 100 | 6 |
| 12.00 | 1.5 | 0 | 100 | 6 |
| 17.00 | 1.5 | 0 | 100 | 6 |
| 17.50 | 1.5 | 100 | 0 | 6 |
| 25.00 | 1.5 | 100 | 0 | 6 |
| 25.50 | 1.0 | 100 | 0 | 6 |
| 36.00 | 1.0 | 0 | 100 | 10 |
| 85.00 | 0.0 | 0 | 100 | 6 |

(i) 13 min run time, 12 min equilibration and regeneration time (total analysis 25 min)

(ii) A: 940 ml 0.14 M sodium acetate pH 6.1, containing 0.05% triethylamine mixed with 60 ml acetonitrile

(iii) B: 60% acetonitrile and 40% water (by vol)

(iv) 5: slightly convex
6: linear
10: strongly concave

7. Isodityrosine synthesis involving ferricyanide oxidation of tyrosine

A 4-g aliquot of tyrosine was dissolved in 100 ml 17% aqueous ammonia (BDH) and 6.67 ml 0.75M potassium ferricyanide (Fisons) was added, with stirring. The reaction was allowed to proceed for three hours at room temperature.

For GPC a 20-ml aliquot of the synthesis mix was concentrated and made up in 8 ml column buffer. This was filtered through Whatman GF/A filters to remove precipitated tyrosine. The sample was cleaned by running through mini-columns (see method 11), prior to loading on the GPC column.

8. TLC purification of isodityrosine synthesis mix

A 10-ml portion of the crude synthesis mix resulting from ferricyanide oxidation of tyrosine was rotary evaporated to dryness. The residue was suspended in 5 ml 17% aqueous ammonia (BDH), and applied to silica gel TLC plates, (0.25 mm precoated, Camlab). Reference standards of tyrosine and synthetic dityrosine were used for comparison. The plates were developed in propan-1-ol:25% ammonia in water (7:3) (Fry, 1982). The plates were viewed under U.V. light to distinguish dityrosine (which fluoresces) and isodityrosine (which does not). Strips were cut from the plates and stained with Folin and Ciocalteu's phenol reagent (0.5N), and developed in ammonia vapour. Isodityrosine, dityrosine and tyrosine were identified by comparison with R_fs reported by Fry (1982). The bands corresponding to the position of isodityrosine on the unstained plates were cut out and eluted with three portions of 2M ammonia.

9. Isolation of isodityrosine by adaptation of assay procedure

This procedure was adapted from the methods of Fry (1982, 1984), and employed high-voltage paper electrophoresis. Electrophoresis was carried out on an LKB Multiphor unit at 1500V for 75 minutes, in formic acid (Fisons) (90%):glacial acetic acid (Fisons):water (1:4:45). Visualisation was as described for TLC in method 8. After electrophoresis the paper was dried and cut just beyond the band of isodityrosine, at right-angles to the direction of migration. The anode end of the paper, containing isodityrosine, was then subjected to descending chromatography in the opposite direction to electrophoresis, in propan-2-ol (Fisons) :17% aqueous ammonia (4:1) for 1.5 hours.

10. Synthesis of dityrosine

An aliquot of 1 g tyrosine was dissolved in 910 ml water. To this was added 5000U of Type II Horseradish Peroxidase dissolved in 40 ml water, and 50 ml 0.1% hydrogen peroxide solution (to give a final concentration of 0.005% peroxide). The pH was adjusted to 9.2 by the addition of sodium hydroxide. The solution was incubated at 37°C for 24 hours, with occasional shaking. After incubation, a 500-ml aliquot was taken and concentrated, by rotary evaporating, to approximately 10 ml. This concentrated solution was cooled on ice and 200 ml cold 95% ethanol (James Burrough) were added. The resulting suspension was filtered twice through Whatman GF/A filters to remove the precipitate.

For amino acid analysis, the filtrate was evaporated to dryness and dissolved in 3 ml loading buffer containing 0.15 mg norleucine.

11. Gel permeation chromatography

GPC was carried out on a 1.6 x 100 cm column of Sephadex G-10 (Pharmacia) eluted with 0.007M citrate buffer, pH 2.2, with an ionic strength of 0.025. (This is amino acid analyser loading buffer diluted 30 times). A total of 130 50-drop fractions, approximate volume 2.2 ml (except experiments in Sections 3.3.2 and 3.4.1, in which 65 fractions of 100 drops) were collected at a flow rate of approximately 30 ml per hour. The eluate was monitored at 280 nm.

A preliminary clean-up stage was introduced for cell wall hydrolysates to avoid contaminating the main column. Samples were shaken with three 25-ml portions of ethyl acetate to extract phenolic acids and polyphenols whilst leaving amino acids in aqueous solution. The samples were then applied to mini-columns (0.5 x 10 cm) ("Econocolumns", Bio Rad) also packed with Sephadex G-10. At least 2.5 column volumes of buffer (as above) were used to elute components not adsorbed to the resin. This eluate was concentrated by rotary evaporating, and a solution equivalent to approximately 0.5 g dry cell wall per ml was loaded on to the main column. The mini-columns were cleaned with excess 8M urea between samples.

12. Incubation of cell walls with peroxidase
(Section 3.4.2)

Cell walls, prepared from 10-g shoots, were incubated with 4000 U horseradish peroxidase (as used in dityrosine synthesis, method 10), and 40 ml 0.1% hydrogen peroxide (to give a final concentration of 0.04%). The incubation was in 0.1M phosphate buffer, pH7, at room temperature for 24 hours.

13. Incubation of cell walls with tyrosine
(Incubation 1 Table 5)

Cell walls prepared from 10-g shoots were incubated with 0.02 g tyrosine in 100 ml of 0.1M phosphate buffer at pH7. The system was left at room temperature for 24 hours. The suspension was then centrifuged and the cell wall pellet was acid hydrolysed (method 5) to provide free isodityrosine. In one experiment the supernatant was checked and found not to contain any isodityrosine.

14. Processing treatments

Samples of cell walls were treated quantitatively for amino acid analysis, and both whole shoot pieces and isolated cell walls were treated for microscopical examination.

14.1 Freezing

Tissue was submerged in water, and frozen (slowly) by placing in a -18°C cold store. After several weeks' storage the samples were allowed to thaw at room temperature.

14.2 Acidified brine storage

Pieces of shoot, or cell wall suspension were added to a brine solution to give a final concentration of 15% sodium chloride and 3% (V/V) lactic acid. The samples were stored for two months and shaken occasionally. Prior to acid hydrolysis and amino acid analysis, the cell wall samples were washed four times with water to remove salt.

14.3 Sulphiting

Tissue was stored in sulphurous acid (Fisons), diluted to give a final concentration of 3000 ppm sulphur dioxide solution, in a tightly sealed container. Samples were kept for three weeks at room temperature and shaken occasionally.

The samples for amino acid analysis were separated into soluble extract and insoluble cell wall residue. The residue was then washed once with water and the washings combined with the extract for analysis.

14.4 Boiling in water

Tissue was boiled in distilled water for 10 minutes and allowed to cool.

14.5 Boiling in bicarbonate

As 14.4. using a 1% solution of sodium hydrogen carbonate (Fisons). The samples for amino acid analysis were washed as described for sulphiting.

14.6 Jam making

Three grams of shoots (cut into 2 mm pieces) were mixed with 16 g sucrose, 3 ml 1M sodium citrate buffer pH 3 and 7 ml distilled water. Alternatively, a portion of cell wall preparation, equivalent to 0.114 g (average) dry cell walls was used, with the water omitted, to give the same final concentration. This mix was stirred and heated, in a glycerol bath at 120°C, to boiling and then boiled for 10 minutes to a final total soluble solids of 68%. The jam preparation was allowed to cool, and it was observed to set.

The samples for amino acid analysis were heated with addition of about 5 ml water to melt the gel. The soluble extract was separated from the cell wall by centrifuging, and the residue was washed four times with water. The washings were combined with the extracts for analysis. Additional controls were prepared for amino acid analysis, as follows:

duplicate portions of cell walls were hydrolysed with the addition of 14 g sucrose. It was calculated that an addition of 14 g would give a sugar concentration approximating to that in the combined extracts and water washes.

14.7 Deep frying

Vegetable oil was heated to 140° - 160°C on a thermostatic hot plate. The shoots were added and deep fried for 10 minutes to a brown colour, and then allowed to cool. The cell wall samples were first filtered under vacuum on Whatman 54 paper, to remove as much water as possible. To enable quantitative transfer, the cell wall sample and filter paper were added to the oil and treated as above.

The samples for amino acid analysis were washed with three 70-ml portions of hexane to remove oil.

14.8 Heating in acid

Cell wall samples were heated in acid for amino acid analysis, to investigate the effects of the heat and pH conditions employed in acidified chlorite glycoprotein extraction. The pH of cell walls with added acetic acid and sodium chlorite reagents (in the concentrations given in method 4) was found to be 4.2. Samples of cell walls were heated in 0.1 M pH 4.2 acetate buffer at 70°C for 30 minutes. The residue was washed once with water and the washings were combined with the extract for analysis.

15. Hydroxyproline determination

This assay was adapted from the method of Drozd, Kucharz and Szyja (1976) for the determination of hydroxyproline in blood serum. The oxidation step with chloramine-T was modified according to Klis and Eeltink (1979).

Samples had been prepared in pH 2.2 buffer for loading on an LKB amino acid analyser. They were adjusted to pH 6 with 0.1M sodium hydroxide and buffered with acetate-citrate buffer, pH 6, containing propan-2-ol

(Fisons) (Drozd, Kucharz & Szyja, 1976). In this operation, control and residue samples were diluted twenty times, and extracts were diluted ten times. The samples, (3 ml) were decolorized by shaking with 50 mg activated charcoal (40-50 μm) for three minutes, and filtered through GF/A (Whatman) and 0.22 μm millipore.

The assay procedure was as follows: duplicate 1 ml samples were mixed with 0.4 ml buffer (as above) and 0.2 ml 10% chloramine-T (BDH), and heated to 30°C for ten minutes. Then 0.24 ml 60% (V/V) perchloric acid (Fisons) and 1 ml dimethylaminobenzaldehyde colour reagent (5% in propan-2-ol) were added, followed by heating to 60°C for twenty minutes. Samples were cooled and the absorbance at 560 nm measured immediately.

Standard curves were prepared using a stock of 100 $\mu\text{g}/\text{ml}$ hydroxyproline in loading buffer. This was neutralised and diluted to give a final range of 1-5 $\mu\text{g}/\text{ml}$. Reagent blanks were prepared with loading buffer instead of standard. Sample blanks were prepared for the jam processed tests (which were very dark because of the high sugar concentration), using propan-2-ol instead of the colour reagent.

Hydroxyproline concentrations were calculated from A_{560} using a Genstat program to determine the equation of the calibration line.

Hydroxyproline results are shown rounded up, although all the calculations were done to three significant figures. It was found for a typical hydroxyproline assay, that the coefficient of variation (95%) was 1.7%.

16. Assay for reducing sugars

The colour reagent used in this assay was dinitrosalicylic acid, made up as a 1% solution with 30% potassium sodium tartrate (Bergmeyer, 1974). The samples, 2.05 ml, were incubated with 2 ml colour reagent in a boiling water bath for five minutes, cooled, and the absorbance at 546 nm was measured. Blanks were prepared using water instead of sample. A standard curve was prepared using a stock of 10 $\mu\text{mol}/\text{ml}$ galacturonic acid diluted to give a final range of 0.1 - 1.5 $\mu\text{mol}/\text{ml}$.

17. Preparation of samples for microscopy

Unless otherwise stated, all microscopy reagents were obtained from Taab Laboratories Limited.

The shoots and cell walls which had been treated by some of the food processing conditions required preliminary preparation as follows. The shoots which had been treated by sulphiting, acidified brine storage and jam making were rinsed with water prior to treating with microscopy reagents. The deep fried shoots were washed with the solvent CNP-30 to remove excess oil and then rinsed with absolute ethanol.

The shoots were cut into 2-mm lengths for further preparation. Samples of cell walls and shoots were treated by the PATCH-osmium procedure and further samples of shoots were treated by glutaraldehyde-osmium.

(a) PATCH-osmium

Samples were placed in 1% periodic acid for 2 hours; rinsed in water and transferred to 1% thiocarbonylhydrazide in 5% acetic acid at 60°C for 2 hours; rinsed in water and fixed in 1% osmium tetroxide for 2 hours.

(b) Glutaraldehyde-osmium

Samples were fixed in 2.5% glutaraldehyde in cacodylate buffer for 2 hours; rinsed in water and fixed in 1% osmium tetroxide for 2 hours.

All the samples were then rinsed in water and dehydrated by soaking in a series of increasing strength ethanol solutions ending with absolute ethanol. The tissues were infiltrated and embedded in L.R. white resin (London Resin Company).

Thin transverse sections (2 μm) of the glutaraldehyde-osmium treated shoots were cut for light microscopy. These were stained in methylene blue, azure II (Gurr) and basic fuchsin (Gurr), and viewed in a compound light microscope. This differential staining technique usually makes the cell wall appear red and the cell contents appear blue.

Ultra-thin transverse sections (less than 0.1 μm) of tissues from both preparation procedures were cut for electron microscopy. At this stage the glutaraldehyde fixed samples were post-stained in 7% uranyl acetate in methanol and viewed in a JEOL 1200 EX electron microscope.

18. Hydrogen fluoride deglycosylation of cell walls

Cell walls were dehydrated by soaking in a series of increasing strength alcohol solutions (70%, 80%, 90%) ending with two changes of absolute ethanol. The samples were left for one hour at each stage. The cell walls were then soaked in two changes of absolute methanol, the solvent used by Van Holst and Varner (1984) for hydrogen fluoride treatment.

Portions of the alcohol-dehydrated walls, equivalent to a dry weight of approximately 1 mg, were transferred to small plastic snap-top tubes. 180 μl hydrogen-fluoride-pyridine (Aldrich) was added to each tube. The deglycosylation treatment was left to proceed for 90 minutes at room temperature. At the end of this period ice cold water (1 ml per tube) was added to quench the reaction. The remaining wall fragments were dialysed against 0.1M sodium chloride solution overnight, followed by distilled water for two days.

Addresses of suppliers

Aldrich Chemical Co Ltd, Gillingham, Dorset, SP8 4JL

BDH Chemicals Ltd, Poole, Dorset, BH12 4NN

Bio-Rad Laboratories Ltd, Watford, Herts, WD1 8RP

Camlab Ltd, Cambridge, CB4 1TH

Fisons Scientific Apparatus, Loughborough, Leics

Gurr, BDH Chemicals Ltd

James Burrough, London, SE11 5DF

LKB Instruments Ltd, Croydon, Surrey CR2 8YD

London Resin Co, Woking, Surrey, QU21 1AE

Pharmacia Ltd, Milton Keynes, Bucks, MK9 3HP

Pierce & Warriner, Chester, CH1 4EF

Sigma Chemical Co Ltd, Poole, Dorset, BH17 7NH

Taab Laboratories Ltd, Aldermaston, Berks, RG7 4QW

Waters Associates Ltd, Millipore (UK) Ltd, Harrow, Middx, HA1 2YH

Whatman Lab Sales Ltd, Maidstone, Kent, ME15 9XN

Retention time
(minutes)

| | |
|------|----------------------|
| 272 | Phenol |
| 281 | 2-Nitrophenol |
| 389 | 4-Nitrophenol |
| 478 | 2,4-Dinitrophenol |
| 686 | 2,6-Dinitrophenol |
| 750 | 2,4,6-Trinitrophenol |
| 908 | Phenylamine |
| 1434 | Phenylacetone |
| 1463 | Phenylacetylene |
| 1640 | Phenylacetylene |
| 3023 | Phenylacetylene |
| 3095 | Phenylacetylene |
| 3122 | Phenylacetylene |
| 3191 | Phenylacetylene |
| 3281 | Phenylacetylene |
| 3425 | Phenylacetylene |

Notes:

- (1) All of these compounds are distilled from the mixture and will all hydrolyse characteristically.
- (2) Peaks at 389 minutes and 478 minutes correspond to the components in all well hydrolysed.

APPENDIX B

Table of typical retention times of unusual amino acids and related compounds analysed using an LKB (see 3.2.1.) (i)

| <u>Retention time</u> (seconds) | <u>Compound</u> |
|------------------------------------|---|
| 272 | Phospho-L-serine |
| 281 | Cysteic acid, homocysteic acid |
| 380 | Taurine (distinct from 389 and 440 (ii)) |
| 476 | Urea (distinct from 440 (ii)) |
| 686 | Methionine sulphoxide (just before aspartic acid) |
| 750 | Methionine sulphone (just after aspartic acid) |
| 908 | Carboxymethyl-cysteine |
| 1424 | Homoserine (coelutes with glutamic acid) |
| 1463 | Sarcosine (methyl-glycine) |
| 1640 | alpha-aminoadipic acid |
| 3023 | 3-chloro-tyrosine |
| 3095 | Pipecolic acid (2-piperidinecarboxylic acid) |
| 3122 | Glycyl-tyrosine |
| 3191 | 3-amino-tyrosine |
| 3251 | Djenkolic acid (5,5'-methylenebiscysteine) |
| 3425 | Anserine nitrate (N-beta-alanyl-1-methyl histidine) |

Notes:

- (i) All of these compounds are distinct from any unknown peaks in the cell wall hydrolysate chromatogram (Fig.8).
- (ii) Peaks at 389 seconds and 440 seconds correspond to unidentified components in cell wall hydrolysates.

Tables of Amino Acid Compositions of Soluble Extracts, Insoluble Residues and Controls from Processing Treatments (see Section 4.2). Results expressed as mg per g dry cell wall.

Key

* : missing data (sample lost, or amino acid not quantifiable because peak not resolved)

T : trace detected (but not quantifiable)

NC : not clear from chromatogram if component present or not.

Notes

1. Although presented rounded up, all concentrations, and calculations for Tables 6 and 7 (in Chapter 4) were determined to 3 significant figures.
2. The sulphited samples were analysed by a Pico Tag system. All other processed samples were analysed using an LKB and a hydroxyproline colorimetric assay.

1. Acidified Brine Stored

| | | <u>Residue</u> | <u>Control</u> |
|----------------|---|----------------|----------------|
| Hydroxyproline | A | 1.22 | 1.18 |
| | B | 0.90 | 1.53 |
| | C | 1.05 | 1.59 |
| Isodityrosine | A | 0.18 | 0.20 |
| | B | 0.15 | 0.18 |
| | C | 0.13 | 0.26 |
| Valine | A | 1.34 | 1.96 |
| | B | 1.24 | 2.29 |
| | C | 1.17 | 2.00 |
| Tyrosine | A | * | 1.46 |
| | B | 1.15 | 1.48 |
| | C | 1.06 | 1.46 |
| Histidine | A | 0.85 | 1.61 |
| | B | 0.84 | 2.00 |
| | C | 0.73 | 1.64 |
| Lysine | A | 1.67 | 2.11 |
| | B | 1.61 | 2.23 |
| | C | 1.45 | 2.14 |

2. Sulphited

| | | <u>Residue</u> | <u>Extract</u> | <u>Control</u> |
|----------------|---|----------------|----------------|----------------|
| Hydroxyproline | A | 1.74 | 0.12 | 2.24 |
| | B | 1.71 | * | 2.10 |
| | C | 1.51 | 0.12 | 2.62 |
| Isodityrosine | A | 0.14 | T | 0.28 |
| | B | 0.15 | * | 0.31 |
| | C | 0.08 | T | 0.22 |
| Valine | A | 1.86 | 0.61 | 2.29 |
| | B | 1.82 | * | 2.65 |
| | C | 1.69 | 0.55 | 2.84 |
| Tyrosine | A | 1.64 | 0.10 | 1.91 |
| | B | 1.60 | * | 2.07 |
| | C | 1.43 | 0.14 | 2.20 |
| Histidine | A | 1.40 | 0.33 | 1.97 |
| | B | 0.99 | * | 2.15 |
| | C | 0.90 | 0.19 | 2.26 |
| Lysine | A | 2.03 | 0.17 | 1.71 |
| | B | 1.55 | * | 2.32 |
| | C | 1.26 | 0.11 | 2.54 |

3. Boiled in water *hicultivata*

| | | <u>Residue</u> | <u>Extract</u> | <u>Control</u> |
|----------------|---|----------------|----------------|----------------|
| Hydroxyproline | A | 1.24 | 0.02 | 1.19 |
| | B | 0.99 | 0.02 | 1.13 |
| | C | * | 0.02 | * |
| Isodityrosine | A | 0.25 | 0.00 | 0.26 |
| | B | 0.20 | 0.00 | 0.25 |
| | C | 0.21 | 0.00 | 0.27 |
| Valine | A | * | * | 1.95 |
| | B | * | * | 2.05 |
| | C | * | * | 1.95 |
| Tyrosine | A | 1.09 | 0.13 | 1.10 |
| | B | 0.98 | 0.14 | 1.15 |
| | C | 1.13 | 0.10 | 1.08 |
| Histidine | A | 1.75 | 0.29 | 1.57 |
| | B | 1.61 | 0.36 | 1.64 |
| | C | 1.36 | 0.31 | 1.56 |
| Lysine | A | 2.76 | 0.33 | 2.66 |
| | B | 2.71 | 0.38 | 2.78 |
| | C | 2.45 | 0.36 | 2.48 |

4. Boiled in sodium bicarbonate

| | | <u>Residue</u> | <u>Extract</u> | <u>Control</u> |
|----------------|---|----------------|----------------|----------------|
| Hydroxyproline | A | 0.66 | 0.30 | * |
| | B | 0.60 | 0.29 | 1.15 |
| | C | 0.92 | 0.32 | 1.06 |
| Isodityrosine | A | * | 0.04 | * |
| | B | 0.09 | 0.03 | 0.30 |
| | C | 0.13 | 0.03 | * |
| Valine | A | 0.99 | 0.54 | * |
| | B | 0.71 | 0.53 | 2.05 |
| | C | 0.99 | 0.62 | 2.41 |
| Tyrosine | A | 0.77 | 0.30 | * |
| | B | 0.60 | 0.25 | 1.64 |
| | C | 0.79 | 0.31 | 1.57 |
| Histidine | A | 0.77 | 0.74 | * |
| | B | 0.55 | 0.66 | 2.00 |
| | C | 0.94 | 0.88 | 2.60 |
| Lysine | A | 1.18 | 0.90 | * |
| | B | 0.85 | 0.77 | 3.02 |
| | C | 1.51 | 1.05 | 3.79 |

* Results unreliable, see Section 5.2.1

5. Jam processed

| | | <u>Residue</u> | <u>Extract</u> ⁺ | <u>Control</u> | <u>Control + Sugar</u> ⁺ |
|----------------|---|----------------|-----------------------------|----------------|-------------------------------------|
| Hydroxyproline | A | 0.57 | 0.11 | 1.74 | 0.21 |
| | B | 0.73 | 0.02 | 1.37 | 0.19 |
| | C | 0.92 | 0.09 | 1.59 | |
| Isodityrosine | A | T | NC | 0.39 | NC |
| | B | T | NC | * | NC |
| | C | 0.17 | NC | * | |
| Valine | A | 0.92 | 0.12 | 1.96 | 0.13 |
| | B | 0.87 | 0.13 | 1.67 | 0.89 |
| | C | 1.06 | 0.40 | 2.02 | |
| Tyrosine | A | 0.57 | 0.14 | 1.65 | 2.72 |
| | B | 0.55 | 0.04 | 1.54 | 0.53 |
| | C | 0.69 | 0.16 | 1.47 | |
| Histidine | A | 0.75 | 1.19 | 2.00 | 1.85 |
| | B | 0.76 | 0.25 | 1.85 | 0.61 |
| | C | 1.11 | 1.70 | 2.38 | |
| Lysine | A | 1.46 | 1.29 | * | 2.64 |
| | B | 1.44 | 0.56 | 3.50 | 1.66 |
| | C | 1.95 | 1.68 | 4.30 | |

⁺ Results unreliable, see Section 4.2.7

6. Deep fried

| | | <u>Residue</u> | <u>Control</u> |
|----------------|---|-------------------|----------------|
| Hydroxyproline | A | * | 1.16 |
| | B | 1.41 ⁺ | 1.34 |
| | C | 1.13 | * |
| Isodityrosine | A | * | 0.22 |
| | B | 0.20 | 0.21 |
| | C | 0.12 | * |
| Valine | A | * | 2.90 |
| | B | 1.30 | 3.00 |
| | C | 1.02 | * |
| Tyrosine | A | * | 1.65 |
| | B | 0.38 | 1.75 |
| | C | 0.69 | * |
| Histidine | A | * | 3.34 |
| | B | 1.00 | 2.76 |
| | C | 0.77 | * |
| Lysine | A | * | 5.35 |
| | B | 1.94 | 4.33 |
| | C | 1.67 | * |

⁺ may be overestimated, see Section 4.2.7

Notes

- (i) Analyzed on a Pico Tag
- (ii) Analyzed on an LRP
- (iii) Jan extract results available from Section 4.2.7
- (iv) Histidine probably overestimated
- (v) Several peaks close to position of tyrosine (see Section 4.3.1)

Amino Acids Extracted from the Cell Wall by Different Processing and Chemical Treatments, as a Percentage of the Amount in Unprocessed Controls
(see Sections 4.2 and 4.3)

| Treatment | Amino Acid | | | |
|---|------------|----------|-----------|--------|
| | Valine | Tyrosine | Histidine | Lysine |
| Sulphited (i) | 22 | 6 | 12 | 6 |
| Boiled (ii) | * | 11 | 20 | 14 |
| Bicarbonate (ii) | 25 | 18 | 33 | 27 |
| Jam (ii) | 11 | 10 | 70 (iii) | 40 |
| Acid (ii) | 18 | 5 | 20 | 23 |
| Water-soluble pectin (i) | 56 | 41 | 86 | 34 |
| Ammonium oxalate - soluble pectin (i) | 13 | 4 | 37 | 18 |
| Glycoprotein fraction after depectination (i) | 1 | 0 | 0 | 1 |
| Glycoprotein fraction without depectination (i) | 22 | PP (iv) | 12 | 1 |

* : missing data (peak not resolved)

PP : probably present

Notes

(i) Analysed on a Pico Tag

(ii) Analysed on an LKB

(iii) Jam extract results unreliable (see Section 4.2.7)
histidine probably overestimated

(iv) Several peaks close to position of tyrosine (see Section 4.3.3)

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