

TYROSINE CROSS-LINKS IN PLANT CELL WALL GLYCOPROTEIN

by

Karen Biggs.

Doctor of Philosophy
University of Edinburgh
1988.



ACKNOWLEDGEMENTS

I would like to express my thanks to the following:

Dr. Steve Fry, my supervisor, for his help, advice and encouragement;

Mrs. Janice Miller for practical advice and suggestions;

Dr. John Fry and staff at Leatherhead Food R.A. for assistance with amino acid analysis and ion chromatography; fellow workers in the original 'Cell Wall Group' for their friendship;

my parents for their help and support during many years of education

and Leatherhead Food R.A. and the Agricultural and Food Research Council (AFRC) for a studentship grant.

FOREWORD

Celery

Celery, raw,

Develops the jaw,

But celery, stewed,

Is more quietly chewed.

Ogden Nash.

To Richard.

CONTENTS

ABSTRACT	1
ABBREVIATIONS	2
INTRODUCTION	
Cell wall components	4
Classes of HRGP	5
Structure and properties of extensin	7
Interaction of extensin with other cell wall components	12
Possible roles of an extensin network	16
Cleavage of an extensin network	20
Aims of the project	21
MATERIALS	23
METHODS	
Cell suspension cultures	25
Cell wall isolation	25
Stains	26
Assays	27
Hydrolysis	27
Deglycosylation using TFMS acid	29
High voltage paper electrophoresis	29
Paper chromatography	30
Thin layer chromatography	30
Scintillation counting	31
Autoradiography	31
High performance liquid chromatography	31
Ion chromatography	34
Recrystallisation of sodium chlorite	34

Column chromatography	36
Preparation of [¹⁴ C]isodityrosine	37
Differences in counting efficiency between solubilised glycoprotein and cell wall pellets	39
RESULTS	
Analysis of suspension culture cell walls	
Introduction	40
Uptake and incorporation of [³ H]proline by cell suspension cultures	40
Variation in [³ H]proline incorporation during the growth cycle of <u>Capsicum</u> cultures	44
Conclusion	48
Optimisation of gel permeation chromatography system	
Introduction	49
Selection of gel permeation media and eluents	50
Column calibration	54
Role of molecular conformation in data interpretation	58
Conclusion	59
Cleavage reagents for isodityrosine	
Introduction	60
Solubilisation of glycoprotein from cell walls	60
Effects upon monomer extensin	63
Cleavage of isodityrosine	67
Purity of sodium chlorite	73
Conclusion	75
Investigation into conditions for specific cleavage of isodityrosine.	
Introduction	77
Acidified chlorite treatment of B.S.A.	78

Acidified chlorite treatment of lysozyme	84
Acidified chlorite treatment of monomer extensin in the presence of B.S.A.	87
Acidified chlorite treatment of isodityrosine in the presence of B.S.A.	89
Conclusion	93
Amino acid analysis of extensin	
Introduction	94
Purification and amino acid analysis of monomer extensin	94
Amino acid analysis of cell wall extensin	102
Conclusion	105
Isolation of extensin dimers	
Introduction	106
Salt elution of <u>Capsicum frutescens</u> f ₁ suspension culture cell walls	106
Effect of ascorbic acid pre-treatment	109
Salt elution of tomato and <u>Capsicum frutescens</u> f ₁ cultures	115
Effect of increasing radioactive precursor concentration	117
Solubilisation of glycoprotein fragments by use of acidified chlorite treatments	124
Continuous solubilisation of glycoprotein	125
Isolation of putative dimer	133
Conclusion	143
Release of trapped dimers	
Introduction	145
Driselase digestion of [<u>arabinosyl</u>]- ³ H]extensin	145
Driselase digestion of [<u>(hydroxy)prolyl</u>]- ³ H]extensin	149
Deglycosylation of extensin monomer	149

Driselase digestion in the presence of protease inhibitors	154
Conclusion	156
DISCUSSION	158
REFERENCES	167
Appendix 1: Composition of suspension culture medium for <u>Capsicum</u> <u>frutescens.</u>	180

ABSTRACT

Plant cell walls contain the hydroxyproline-rich glycoprotein called extensin. Present knowledge of the structure and properties of extensin are reviewed and its possible roles within the primary cell wall are discussed. It is suggested that extensin forms a structurally important network cross-linked by the phenolic amino acid isodityrosine and a demonstration of the existence of such intermolecular links is the main aim of the work detailed here. The approach that has been taken was to use cell walls where the extensin contains radioactive hydroxyproline or arabinose residues and by specifically cleaving isodityrosine bonds to demonstrate the production of smaller extensin fragments. A potential specific cleavage reagent was a solution of warm, acidified sodium chlorite but this was found to cleave peptide bonds in salt-soluble extensin molecules. From a study of acidified chlorite treatment of a protein, bovine serum albumin (B.S.A.), it was shown that the ratio of B.S.A. (amino acyl residues):sodium chlorite was important in determining peptidyl cleavage; at a ratio of 0.75:1 (mol/mol), or higher, peptidyl cleavage was not detected. Furthermore, it was shown that non-radioactive B.S.A. could be used to protect peptide bonds in [³H]extensin against peptidyl cleavage during acidified chlorite treatments whilst not preventing the cleavage of isodityrosine; therefore this reagent could be used specifically. Acidified chlorite treatment of radioactive cell walls in the presence of B.S.A. solubilised intact monomer extensin and it is concluded that extensin is held in the cell wall by cross-links susceptible to acidified sodium chlorite treatment, probably intermolecular isodityrosine.

ABBREVIATIONS

aq.	aqueous
B.S.A.	Bovine serum albumin
2,4-D	2,4-dichlorophenoxyacetic acid
DCPIP	2,6-dichlorophenolindophenol
Glu	Glucose
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
His	Histidine
HPLC	High pressure liquid chromatography
HRGP	Hydroxyproline-rich glycoprotein
HVPE	High voltage paper electrophoresis
Hyp	Hydroxyproline
Idt	Isodityrosine
Lys	Lysine
Mes	2[N-morpholino]ethanesulphonic acid
M _r	Molecular weight (relative molecular mass)
PAGE	Polyacrylamide gel electrophoresis
PAW	Phenol/acetic acid/water 2:1:1 (w/v/v)
pI	isoelectric point
PITC	Phenylisothiocyanate
PMSF	Phenylmethanesulphonyl fluoride
POPOP	1,4-di-2(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
Pro	Proline
R.I.	refractive index
rpm	revolutions per minute
Ser	Serine

Taps	<u>N</u> -tris[hydroxymethyl]methyl-3-amino-propanesulphonic acid
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TFMS	Trifluoromethanesulphonic acid
Thr	Threonine
TLC	Thin layer chromatography
Tris-HCl	Tris[hydroxymethyl]aminomethane hydrochloride
Tyr	Tyrosine
u.v.	ultraviolet
Val	Valine
V_e	sample elution volume
V_0	void volume
V_t	included volume
v/v	volume / volume
w/v	weight/ volume

INTRODUCTION

The walls of growing plant cells, primary cell walls, are of fundamental importance to the plant, both at the cellular level where they determine cell size and shape, and at higher levels of organisation where they are involved in plant growth, disease resistance and defining the properties of foodstuffs. In order to understand how these functions of cell walls are effected, it is first necessary to have an understanding of the structure and interaction of the components of the cell wall. Many studies have been made on the polysaccharide components of the primary cell wall but, until recently, little attention had been paid to the protein part which can comprise up to 10% of the wall dry weight. In this chapter brief mention will be made of the various components of the primary cell wall; then the classes of hydroxyproline-rich glycoprotein (HRGP) will be described and one group of HRGPs, extensin, will be discussed in detail. Possible roles of extensin in the cell wall will be outlined. The ways in which extensin has been suggested to interact with other cell wall components will be described and this will lead to an outline of the aims of this particular study.

Cell wall components

The principal components of the primary cell walls, their constituent molecules and relative abundance as % of the wall dry weight are given below. Information is taken from McNeil et al. (1984) and from Fry (1985). Cellulose constitutes about 20-30% of the wall dry weight and is composed of β -4-linked glucose units, joined to form unbranched chains that are organised into microfibrils. Hemi-

celluloses include xyloglucan, predominant in dicots as 20-30%, and xylans, predominant in monocots as 20-40% of the wall dry weight. Xyloglucan consists of a β -4-linked glucose backbone with short side-chains of xylose, galactose, fucose and arabinose residues attached, whilst xylans consist of a backbone of β -4-linked xylose residues. Pectins include: homogalacturonan, composed predominantly of α -4-linked galacturonic acid residues and found mainly in dicots (approx. 10%); rhamnogalacturonan I (10-20% in dicots), composed of alternating 4-linked galacturonic acid and 2-linked rhamnose residues and bearing oligomeric side-chains of arabinose and galactose residues; and rhamnogalacturonan II (2-5% in dicots), whose structure has not yet been fully elucidated, although it is known to contain galacturonic acid, rhamnose, fucose and arabinose residues, arranged in repeating heptasaccharides. Pectins are acidic molecules bearing a negative charge at physiological pH values.

Proteins in the plant cell wall also have carbohydrate moieties associated with them and are therefore termed glycoproteins; they constitute up to about 10% of the wall dry weight. The two main groups of wall glycoprotein are extensin and arabinogalactan proteins; extensin is present as 1-10% in dicots and 0.1-2% in monocots. Both groups contain hydroxyproline in their polypeptide backbones and are described as HRGPs.

Classes of HRGP

Although extensin, arabinogalactan proteins and potato lectin can all be grouped together as HRGPs, there are significant differences between them and it is therefore important to distinguish between them. A brief outline of the characteristic properties of

extensin will be given here, for the purpose of allowing a comparison with arabinogalactan protein and potato lectin; however, a more detailed account of extensin's structure will follow later.

Extensin is covalently bound in the cell wall and is composed of approximately 50% carbohydrate and 50% protein. The carbohydrate is present as short, unbranched side-chains of arabinose and galactose units and the protein backbone is highly basic and rich in the amino acids hydroxyproline, serine, lysine, valine, histidine and tyrosine (Stuart & Varner, 1980).

Arabinogalactan proteins are water soluble and are secreted into the medium of suspension cultured cells. Although they are HRGPs, arabinogalactan proteins are structurally quite different from extensin; arabinogalactan proteins contain about 90% carbohydrate and 10% protein. The carbohydrate in arabinogalactan protein molecules exists as long, branched side-chains incorporating arabinose, galactose and glucuronic acid residues. The protein component is rich in both hydroxyproline and serine, as in extensin, but it also contains alanine and glycine; these amino acids are not abundant in extensin. The overall charge of arabinogalactan proteins is acidic, whilst extensin is a strongly basic molecule. Some arabinogalactan proteins are capable of binding reversibly to Yariv's artificial antigen, whilst no such reaction has been reported for extensin. For a review of arabinogalactan proteins see Clarke et al. (1979).

Potato lectin, purified by Allen & Neuberger (1973), binds reversibly to N-acetylglucosamine oligomers and can therefore be purified by affinity chromatography (Owens & Northcote, 1980). The purified lectin is water soluble, composed of approximately 47% arabinose and 3% galactose, and 50% protein including 11%

hydroxyproline. Cystine is also present and this amino acid is absent from extensin. There are, however, some similarities between potato lectin and extensin in that both components contain serine residues with single galactose residues attached and hydroxyproline residues with oligoarabinosides attached (Murray & Northcote, 1977). It has been suggested that potato lectin could belong to a class of soluble glycoproteins that includes extensin precursors (Allen et al., 1978).

Structure and properties of extensin

The first report of an integral cell wall protein was made by Lamport & Northcote (1960) and they showed that the wall contained most of the cell's hydroxyproline residues. The protein was also shown to have carbohydrate groups associated with it (Lamport, 1967), and Lamport (1965) proposed the name 'extensin' for this HRGP, in the belief that it was a structural component of the wall involved in cell extension; however, no evidence that extensin is essential for cell extension growth has yet been provided. Extensin is a structural component of the primary cell wall; this is perhaps reflected by the difficulty encountered in extracting it from the cell wall. Covalently bound extensin is insoluble in many of the usual cell wall extraction solvents such as detergents (SDS), salt solutions, dilute acids and alkalis, phenol:acetic acid:water and anhydrous liquid hydrogen fluoride (Mort & Lamport, 1977). This insolubility has obviously hindered study of extensin's structure but the isolation of extensin precursors, which are soluble in salt solutions, has enabled rapid progress to be made in recent years. The systems used to study extensin fall into two groups, (i) cell suspension cultures, such as tomato (Smith et al., 1984) and (ii) aerated carrot root discs (Stuart

& Varner, 1980); both systems produce enhanced levels of HRGPs compared with natural plant tissues. Extensin molecules produced by these systems share fundamental properties, although there are some differences in the amino acid sequences of the polypeptide backbone, as will be described later; consequently extensin is currently viewed as a family of related molecules.

Alkaline hydrolysis of tomato cell walls resulted in the isolation of hydroxyproline-oligoarabinosides (Lampport, 1967) and further work, involving enzymic degradation, demonstrated that galactose was an additional sugar component of the glycoprotein (Lampport, 1969). Acid hydrolysis of cell walls to remove oligoarabinosides, followed by trypsin digestion, revealed that the galactose units were attached to serine residues (Lampport et al., 1973). From these findings a structure was proposed for a portion of an extensin glycopeptide, serine-(hydroxyproline)₄, (see Fig. 1) which has since been shown to be common to all extensins. Akiyama & Kato (1976, 1977) elucidated the nature of the bonds in hydroxyproline-oligoarabinosides produced by alkaline hydrolysis of tobacco cell walls; in the predominant hydroxyproline-tetra-arabinoside it was shown that the arabinose unit farthest from the polypeptide backbone was α -linked whilst the remaining three residues were β -linked (Akiyama et al., 1980).

As previously mentioned, extensin is approximately 50% protein. The principal amino acids are hydroxyproline (approx. 50 mol % of the amino acid residues), serine (13 mol %), tyrosine (10 mol %), lysine, histidine, valine and threonine; cysteine, methionine and phenylalanine are absent from extensin (Stuart & Varner, 1980). These values are for salt-soluble extensin from carrot cell walls. From

their work, Stuart & Varner (1980) also estimated that extensin 'monomer' (a single extensin precursor molecule) has a relative molecular mass (M_r) of 86-90 kDa; it is difficult to obtain an accurate value for the M_r of extensin because there are no standard glycoprotein molecular weight markers with which to compare it, but this value is in agreement with other estimations of M_r (see Results-Optimisation of gel permeation chromatography system). Extensin is a highly basic molecule with a strong positive charge at physiological pH values; this is due to the abundance of lysine and histidine residues. An isoelectric point for extensin has not been determined experimentally but a value of 10-12 has been predicted (Cooper et al., 1984).

The principal sugars of extensin are arabinose (96 mol % of sugar residues) and galactose (4 mol %) (van Holst & Varner, 1984); these are attached to hydroxyproline and serine respectively, and are thought to be involved in maintaining extensin molecules in a rigid, rod conformation. Measurements made by van Holst & Varner (1984), using circular dichroism techniques to study secondary structure, ascertained that the polypeptide backbone of extensin molecules is held in a polyproline II conformation. Deglycosylation of extensin caused much of this conformation of the peptide backbone to be lost. The effects of deglycosylation have also been studied by Stafstrom & Staehelin (1986b), who visualised extensin molecules under the electron microscope. Glycosylated molecules appeared to exist in a highly elongated form whilst deglycosylated molecules were difficult to resolve.

Amino acid sequencing of extensin peptides has shown that repeat amino acid sequences other than serine-(hydroxyproline)₄ occur.

Identification of repeat sequences was carried out using classical amino acid sequencing techniques by Smith et al. (1986). They found the following repeats in two salt-soluble extensin precursors (P1 and P2) from tomato suspension culture cells:

-Ser-Hyp-Hyp-Hyp-Hyp- -Tyr-Lys-Tyr-Lys- -Thr-Hyp-Val-
-Val-Lys-Pro-Tyr-His-Pro-.

From these sequences they suggested that extensin exists as a polymer of rigid domains [glycosylated serine-(hydroxyproline)₄ regions] interspaced with more flexible, non-glycosylated sequences. Antibodies have been raised against glycosylated and deglycosylated forms of precursors P1 and P2 (Kieliszewski & Lamport, 1986). The results of cross reactivity of these antibodies with each type of antigen implied that the nonglycosylated domains of the intact precursors differ from one another, and therefore confirmed that precursors P1 and P2 are different (Smith et al., 1986). It has been suggested that the extensin molecule is arranged so that the basic lysine and histidine residues would be exposed for interaction with acidic pectins and the tyrosine residues would be available as potential sites for cross-link formation (Cooper & Varner, 1984; Smith et al., 1986). This representation of an extensin molecule is important when considering the roles of extensin within the cell wall.

An alternative approach to sequencing the amino acids in extensin was used by Chen & Varner; they cloned the gene for carrot extensin and then predicted the amino acid sequence from this (Chen & Varner, 1985a, 1985b). The number of post-translational modifications found in extensin (for example, hydroxylation of proline residues) make an accurate prediction difficult but, nevertheless, considerable similarity between the two methods is observed. Repeat sequences from

the cloned extensin gene include:

-Ser-Hyp-Hyp-Hyp-Hyp- -Thr-Pro-Val-Tyr-Lys-Tyr-Lys-
-His-His-Tyr-Lys-Tyr-Lys-.

All Pro residues occurring in quadruplets are arbitrarily shown as Hyp; other Pro residues may also be modified to Hyp. There was an estimated total of 274 amino acids in this carrot extensin, with hydroxyproline present as 42 mol % of amino acid residues. Since Chen & Varner's amino acid sequencing of carrot extensin by gene cloning techniques, the amino acid sequences of other extensin molecules have been determined, including that for tobacco extensin (Memelink, 1988), and these also show remarkable sequence repetition. However, differences exist between the sequences in carrot and tobacco extensin.

Recent advances in the study of extensin's structure have involved visualisation of salt-soluble precursor molecules from carrot root cell walls under the electron microscope (Stafstrom & Staehelin, 1986a; Heckman et al., 1988). From such a study the length of an extensin monomer (extensin-1) molecule has been estimated as 80 nm (Stafstrom & Staehelin, 1986a). Electron microscopy has also revealed that extensin molecules appear to be kinked, the possible significance of which will be discussed later. Salt-soluble oligomers of extensin have also been identified. Stafstrom & Staehelin (1987) have reported the presence of a second extensin (extensin-2) in carrot cell walls. This is about half the size of extensin-1, 40 nm, and shows many properties similar to extensin-1; however they did not determine whether this molecule was covalently cross-linked in the cell wall. Stafstrom & Staehelin (1988) have used electron microscopy to examine the distribution of labelled anti-extensin antibodies in carrot cell

walls; they observed that extensin was distributed uniformly throughout the wall but was apparently present at reduced levels in the middle lamella.

As mentioned earlier, monocots contain low levels of HRGP but recently a HRGP has been isolated from a cell suspension culture of the graminaceous monocot, Zea mays (Kieliszewski & Lamport, 1987). It was shown that this material was also rich in threonine (therefore named THRGP) and it had a characteristic amino acid profile; like extensin it was highly basic. Cross-reactivity of THRGP with antibodies raised against tomato extensins P1 and P2 was significant and it was concluded that this THRGP was 'extensin-like'. A HRGP has also been isolated from maize pericarp cell walls by Hood et al., (1988) and there are similarities in amino acid composition between this and THRGP.

Having so far described the structure and main properties of extensin molecules, the next issue to be addressed is: how are extensin molecules held in the cell wall?

Interaction of extensin with other cell wall components

Originally it was proposed that covalent bonds with cell wall polysaccharides would hold the extensin molecules in the cell wall (Lamport, 1965) with the link mediated through the galactose and arabinose side-chains of extensin. This idea was extended by Albersheim's group who described the primary cell wall as a single macromolecule of covalently connected components (Keegstra et al., 1973), and proposed that extensin was linked into this macromolecule by glycosidic bonds between galactose and serine residues. Modifications of this model were made throughout the 1970's.

(Albersheim, 1975,1978) but no conclusive evidence was obtained for attachment between extensin and wall polysaccharides. Preston (1979) argued against the idea of a single macromolecule wall because it did not allow for the observed swelling and shrinking properties of primary walls.

Mort & Lamport (1977) applied an anhydrous liquid hydrogen fluoride treatment to cell walls; this dissolved the wall polysaccharides but left the peptide bonds intact. Following such a treatment, they observed that an insoluble protein residue remained and they also noted the presence of an unknown phenolic component. It was concluded that the residue must contain a non-polysaccharide cross-link, possibly involving phenolic material (Lamport, 1980). Isolation of a new phenolic amino acid from cell wall hydrolysates (Fry, 1982) supported this suggestion, especially as the amino acid, called isodityrosine, was derived from tyrosine and therefore fitted Lamport's earlier discovery of an 'unknown tyrosine derivative' in extensin (Lamport, 1977). The structure of isodityrosine, an oxidatively coupled dimer of tyrosine units linked by a diphenyl ether bridge, is shown in Fig. 2. Isodityrosine was found in cell wall hydrolysates of calli from a variety of plant species and was consistently present in amounts proportional to the hydroxyproline content (Fry, 1982); it was also estimated that there would be about 9 half-isodityrosine residues per extensin molecule, which would be sufficient to form cross-links between cell wall glycoprotein molecules.

The structural animal protein, resilin, has been shown to contain tyrosine cross-links, present as dityrosine and trityrosine (Andersen, 1964; Neville, 1967) and these are believed to allow

formation of a macromolecular network as an elastic skeletal component in insects.

As discussed earlier, tyrosine residues are present in extensin's polypeptide backbone and these could be involved in the formation of intramolecular (within an extensin molecule) and intermolecular (between extensin molecules) isodityrosine cross-links. The former type of isodityrosine bridge has been detected in a tryptide of extensin; -Ser-[Hyp]₄-Val- $\frac{1}{2}$ Idt-(Tyr or Lys)- $\frac{1}{2}$ Idt-.

Extensin tryptides are short polypeptide sequences formed by trypsin cleavage of deglycosylated extensin (Epstein & Lampert, 1984). Determination of the formula, M_r and net charge of the tryptide showed that the bridge was intramolecular. It has been suggested that these intramolecular links may be responsible for the kinks observed in extensin monomer molecules viewed under the electron microscope (Stafstrom & Staehelin, 1986a). The amino acid sequence -Tyr-Lys-Tyr-Lys- is a prime candidate for the site of formation of these intramolecular links. However, it is intermolecular isodityrosine cross-links that would be important to the formation of a cross-linked glycoprotein network within the cell wall and the existing evidence for these links will be discussed in a separate section. Lampert (1986) has proposed that the amino acid sequence

-Val-Lys-Pro-Tyr-His-Pro-

is a potential domain for the intermolecular cross-link.

Coupling of tyrosine residues to form isodityrosine is suggested to be catalysed by the action of the enzyme peroxidase (Fry, 1986b; Biggs & Fry, 1987). Peroxidases are located within the primary cell wall and they are known to catalyse reactions involving phenolic groups by the generation of free radicals from the phenols; these free

radicals can then easily undergo coupling to form dimers. Incubation of tyrosine with peroxidase and hydrogen peroxide has been shown to result in production of dimers (Fry, 1987); at the reaction pH optimum of 9.5 the product is dityrosine, but at lower pH values (3-5) some isodityrosine is formed. It has been proposed that, in the cell wall, acidic pectins may be important in regulating the formation of isodityrosine (Fry, 1987); basic extensin molecules could interact with acidic pectins by ionic binding.

Everdeen et al. (1988) claim to have identified an extensin peroxidase which was present in the salt leachate of young tomato suspension culture cells, and they showed that this preparation could cross-link salt-soluble extensin molecules in the presence of hydrogen peroxide. Cross-linking was detected as a decrease in extensin monomer pool size with an accompanying increase in extensin oligomers as resolved by FPLC gel permeation chromatography. A role for peroxidase in insolubilisation of extensin within the cell wall has also been reported by Fry (1982) and Cooper & Varner (1984), who showed that peroxidase inhibitors, such as L-ascorbate and dithiothreitol, prevented insolubilisation of extensin. Cooper & Varner speculated that a wall bound peroxidase-ascorbate oxidase system could control the rate of phenolic cross-linking in the cell wall.

Possible roles of an extensin network

The existence of an independent, cross-linked extensin network in plant cell walls has several important implications, both structural and physiological.

Extensin can be considered as an important structural

component of the plant cell wall because of (i) the repetition of amino acid sequences within its polypeptide backbone, which implies a structural rather than an enzymic function (Lampport, 1985), (ii) the presence of short carbohydrate side-chains that fold back to help maintain a polyproline II conformation which is responsible for maintaining extensin molecules in a rigid rod-like conformation (Stafstrom & Staehelin, 1986b) and (iii) its marked insolubility in wall extraction solvents. Parallels have been drawn between extensin and the animal glycoprotein, collagen, which is also a structural molecule (Ashford & Neuberger, 1980; Robinson et al., 1985), and between extensin and the cell walls of some algae. In Chlamydomonas, the cell wall is composed entirely of HRGPs and work by Roberts et al., (1972) has shown that several layers exist; an insoluble inner wall layer, and an outer wall layer that consists of two glycoproteins assembled into a crystalline lattice (Catt et al., 1976). Isodityrosine has not been isolated in any of these layers. A review of the work on HRGP walls in Chlamydomonas reinhardtii is given in Roberts et al., (1985) and it is suggested that the model of algal HRGPs is a useful one for understanding the structural role of extensin in higher plants. Deposition of extensin has been shown to be important to cell morphology (Cooper, 1984) because protoplasts treated with 3,4 dehydroproline, a specific inhibitor of prolyl hydroxylase, did not form proper cell walls. Cooper concluded that HRGP is important for the correct assembly of wall polymers.

Lampport & Epstein (1983) proposed a new model for the cell wall, referred to as the 'warp-weft' hypothesis. This model consists of interlinking cellulose microfibrils and extensin network, that are not covalently linked, but which weave through one another. These

networks would be surrounded by a pectin-hemicellulose gel. It is suggested that cellulose microfibrils, forming the warp, run parallel to the plane of the cell wall whilst the extensin precursors are inserted perpendicular to this plane and link to form a weft. As yet, the only evidence for transmural insertion of extensin precursors into the cell wall (Lampert, 1986) comes from the rapid elution of these molecules from the cell wall during leaching in salt solutions (Smith et al., 1984; Heckman et al., 1988). Lampert (1985) suggests that this model is attractive because 'concatenation of polymeric systems by a glycoprotein network would mechanically couple the load-bearing polymers and hence distribute stress throughout the cellulose microfibrillar network.'

The 'warp-weft' hypothesis can also be used to consider the role of the cell wall in extension growth. Primary walls of young cells are viewed as having relatively few cross-links, which would facilitate cell extension by allowing cellulose microfibrils to slip through the extensin weft. As more cross-links are formed and cellulose microfibrils become fixed, leading to formation of a cell wall with a high tensile strength, the cell growth rate decreases. Experiments with excised pea epicotyls (Sadava & Chrispeels, 1973; Sadava et al., 1973) led to the suggestion that extensin accumulation was related to the cessation of cell elongation. Cassab et al., (1985) reported a developmental regulation of hydroxyproline accumulation in soybean seed coats and Hood et al., (1988) made similar findings in a study on maize pericarp cell walls. They showed that in maize, peptidyl hydroxyproline accumulates throughout development and that extractability of HRGP decreased late in development. However, it is more probable that a reduction in wall

extensibility is related to the amount of hydroxyproline bound in the wall than to the amount secreted into it (Wilson & Fry, 1986).

Cell walls of woven networks could have a role in the plant's defence against infection because a tightly cross-linked network will provide a good physical barrier against invading pathogens. Various workers (Esquerré-Tugayé et al., 1979; Hammerschmidt et al., 1984) have reported significant accumulations of HRGP in the cell walls of plants subjected to fungal infection, although a defence mechanism that works by providing a barrier to pathogen entry would need to be successful at an early stage of infection. It is possible that detection of fungal elicitors by the plant cells could induce production of increased levels of HRGP (Showalter et al., 1985). Recent work by Conrad et al., (1978) has been aimed at developing an antibody technique to quantitate the amounts of extensin precursors that accumulate in response to stress, such as infection. For a more detailed account of the evidence for extensin's roles in extension growth and plant defence mechanisms see the review by Wilson & Fry (1986).

Another issue to be considered when describing the roles of extensin is that of the possible localisation of extensin within certain types of tissue in the plant. As mentioned earlier, the systems used to study extensin are plant cell suspension cultures and carrot root discs; it is not possible, from the results of experiments performed on these systems, to draw any conclusions about the localisation of extensin. Cassab & Varner (1988) suggest that extensin levels are tissue specific; in soybean plants extensin content was found to be predominant in layers of the seed coat and tissues of the root nodules (Cassab et al., 1985). Extensin has also

been detected in the cell walls of sclerenchyma tissue; its presence here could again be related to a function of mechanical strength (Cassab & Varner, 1988). It is possible to envisage that the walls of epidermal cells would contain elevated levels of extensin because the epidermal tissue exerts a strong influence over the swelling properties of plant tissue. This can be easily demonstrated by removing the epidermal layers from a length of rhubarb petiole placed in water; the petiole increases in length as the tissue swells (von Sachs, 1865).

The properties of plant cell walls are also important to studies in food science because it is the cell walls that determine the characteristics of plant foodstuffs after processing and preservative treatments. Processes such as pickling and jam-making modify wall structure (Wilson, 1987) and so an understanding of the cross-linked extensin network, and the susceptibility of isodityrosine bonds to cleavage during food processing and storage treatments, could help to explain the textural modifications observed.

Cleavage of an extensin network

At present only indirect evidence exists for the presence of intermolecular isodityrosine cross-links in extensin. Studies on the isolated carrot root disc system (Cooper & Varner, 1983, 1984) showed that radioactively labelled soluble extensin molecules were secreted from the cytoplasm and slowly became insolubilised in the cell walls. This process was accompanied by isodityrosine formation but Cooper & Varner did not prove that the isodityrosine formed was responsible for the cross-linking of extensin.

Glycoprotein has been solubilised from bean cell walls by

treatment with solutions of acidified sodium chlorite (Selvendran, 1975; Selvendran et al., 1975). Hydroxyproline-rich polymers were extracted from the walls and it was suggested that solubilisation occurred as a result of cleavage of phenolic cross-links (O'Neill & Selvendran, 1980). Solutions of warm, acidified sodium chlorite have been extensively used in delignification processes in the pulp and paper industry; sodium chlorite acts by splitting the ether linkages between phenolic lignin subunits (Yang & Goring, 1978; Dence et al., 1962). In addition to an ability to solubilise wall glycoprotein, acidified chlorite has been shown to cleave pure isodityrosine, initially to tyrosine and then causing degradation of the tyrosine (Fry, 1982).

Aims of the project

From the evidence outlined, the hypothesis that extensin is cross-linked via phenolic bonds is an attractive one but the true identity of the intermolecular bridge remains elusive. The aim of this project is to test for the presence of tyrosine cross-links in vivo. The approach taken to study this was to use cell walls where the extensin contains radioactive hydroxyproline or arabinose residues and by specifically cleaving isodityrosine bonds to demonstrate the production of smaller extensin fragments. It had been suggested that a solution of warm, acidified sodium chlorite could act as a specific cleavage reagent for phenolic isodityrosine bonds. The recovery of some extensin fragments as dimers would be evidence for intermolecular links. Purified dimers could then be further treated with acidified sodium chlorite to demonstrate conversion to monomers with concomitant loss of isodityrosine. In order to achieve this aim the experiments

carried out had to answer the following questions:

What is a suitable source of extensin?

Does acidified sodium chlorite solubilise glycoprotein by specifically cleaving phenolic cross-links, or are other bonds also broken?

What is the mechanism of isodityrosine cleavage by acidified sodium chlorite?

How can the solubilised glycoprotein fragments be separated into different sized molecules?

Is it possible to leach salt-soluble oligomers from suspension culture cell walls and, after purification of these molecules, identify intermolecular isodityrosine cross-links in them?

MATERIALS

Chemicals

All chemicals used were obtained from either BDH Chemicals Ltd. or Sigma Chemical Co. and were of AnalaR purity where available.

All water used was singly distilled in a glass still, unless otherwise stated.

Radiochemicals

Radiochemicals were obtained from Amersham International plc. The radiochemicals used were: L-[5-³H]proline (specific activity 555 GBq/mmol); D-[U-¹⁴C]glucose (specific activity 10 GBq/mmol); L-[1-³H]arabinose (specific activity 92 GBq/mmol); L-[U-¹⁴C]tyrosine (specific activity 19 GBq/mmol.)

Plant Material

Cell suspension cultures of Capsicum frutescens were a generous gift from Robert Hall and Sue Holland, Botany Dept. Plants of Capsicum frutescens (Mill) had been grown from seed (McNair, Edinburgh) and suspension cultures were obtained from stem callus.

Equipment

Chromatography paper was from Whatman and aluminium backed TLC plates were from Merck. TLC plates were monitored on a Quickscan R&D densitometer, supplied by Helena Laboratories.

HVPE was performed on a flatbed, water-cooled system from Shandon, with a high voltage power pack from Savant Instruments, Inc.

A Speed-Vac centrifugal evaporator from Savant Instruments,

Inc. was used to dry samples under vacuum.

Amino acid analysis was performed on a Pico-Tag HPLC system from Waters. The gel permeation HPLC system used was from Gilson/Scotlab. Ion chromatography of inorganic ions used a Dionex Series 2000i ion chromatograph.

Radioactive samples were counted on an Intertechnique SL-3000 liquid scintillation counter from Kontron Instruments.

Bench-top centrifugation was carried out in a Centaur 2 centrifuge from M.S.E. Spectrophotometry was performed on an SP8-100 ultraviolet spectrophotometer from Pye-Unicam.

Cell walls were sonicated using a Soniprep 150 from M.S.E.

X-ray film used for autoradiographs was Agfa-Gevaert CURIX RP1 (30 cm x 40 cm).

Autoradiographs were developed in an automatic developer, Gevamic 60 from Agfa-Gevaert.

METHODS

Cell Suspension Cultures

Cell suspension cultures of Capsicum frutescens were grown in Schenk & Hildebrandt medium (Schenk & Hildebrandt, 1972) supplemented with 0.5 mg/ml 2,4-D, 0.1 mg/ml kinetin and 30 g/l sucrose (see Appendix 1 for composition of medium). Cultures were agitated on an orbital shaker (diameter of orbit = 1.3 cm) at 90 rev/min and at 25°C. The cells were subcultured fortnightly; 4-5 g fresh weight of cells were subcultured into 50 ml fresh medium in a 250 ml flask sealed with a cotton wool bung and aluminium foil.

Cell Wall Isolation

Cells were filtered on muslin, frozen and thawed, and 1 g cells suspended in 5-10 mls of a solution containing 20 mM-Hepes and 10 mM-ascorbic acid, adjusted to pH 7 with sodium hydroxide. Cells were ground with a pestle and mortar to break up any large lumps and then sonicated three times for periods of two minutes each. The sonicator probe had a diameter = 1 cm and an amplitude of oscillation = 18 µm; the suspension was cooled on ice between sonications. A small proportion of the suspension was examined under a light microscope to check that all cells had been ruptured. The suspension was centrifuged at 2000 rpm for 5 min to pellet the cell walls, the pellet suspended in approx. 5 ml phenol/acetic acid/water (PAW) (2:1:1 w/v/v) and stirred for 4-6 h at 20°C in a fume cupboard. After further centrifugation to pellet the cell walls, the PAW supernatant was removed and the presence of extracted proteins/glycoproteins tested for by adding 0.2 ml of 10% (w/v) aq. ammonium formate and 25

ml acetone to the 5 ml PAW supernatant. The solution was shaken and allowed to stand at 0°C for 1 h; at the end of this time any protein present appeared as a white precipitate. The PAW treatment was repeated several times until no further protein precipitates formed. The cell wall pellet was washed several times to remove all traces of phenol and the walls were lyophilised.

Stains

The marker lanes of paper chromatograms and TLC plates were stained as follows:

for amino acids- the paper was dipped in 0.5% (w/v) ninhydrin in acetone, dried and placed in an oven at 105°C for 5 min. Amino acids stain pink/purple whilst the imino acids, hydroxyproline and proline, stain yellow/brown.

for sugars- a solution of 16 g phthalic acid dissolved in a mixture of 490 ml acetone, 490 ml diethyl ether and 20 ml water was used. Aniline (0.5 ml) was added to 100 ml of solution prior to use; the paper was dipped, dried and placed in an oven at 105°C for 5 min. Pentose sugars stain red/brown and 6-deoxyhexose sugars stain chocolate brown.

for phenolics- the paper was sprayed with Folin and Ciocalteu's reagent and exposed to ammonia vapour in a closed glass tank, until the yellow background had decolorised. The paper was dried and examined; phenolic compounds stain blue.

Assays

for hexose sugars (Dische, 1962)- sucrose was used as a low M_r marker for column chromatography experiments and its presence was tested for with a solution of 0.2% (w/v) anthrone in concentrated sulphuric acid. Sample (0.5 ml) was added to 1.0 ml anthrone solution and vortexed. Hexose sugars turned the yellow anthrone solution blue/green.

for ascorbate (Dawson et al., 1986)- ascorbate in solution was assayed using 2,6-dichlorophenolindophenol (DCPIP). To 1 ml of sample one drop of 6 M- hydrochloric acid was added and this was titrated with a solution of 1 mM-DCPIP. The deep blue solution turned colourless in the presence of ascorbate and the titration end point was taken as that at which the colourless solution turned pink, and remained pink after standing for 0.5 min. A calibration curve was constructed for concentrations of 0-10 mM ascorbate (Fig. 3).

Hydrolysis

acid hydrolysis- peptide bonds were hydrolysed in 6 M-hydrochloric acid and 10 mM-phenol, with 1 ml acid added to approx. 4 mg dry sample. Hydrolysis was conducted in sealed tubes (either sealed glass tubes or Pyrex tubes with Teflon seal caps) at 110°C overnight. Tubes were cooled and acid was removed by drying the samples under vacuum on the Speed-Vac.

alkaline hydrolysis- this method was used to cleave peptide bonds and generate hydroxyproline-oligoarabinoside fragments. 0.18 M-barium hydroxide was added to the dry sample (approx. 4 mg/ml) and hydrolysis was conducted in a sealed tube at 105°C overnight. The

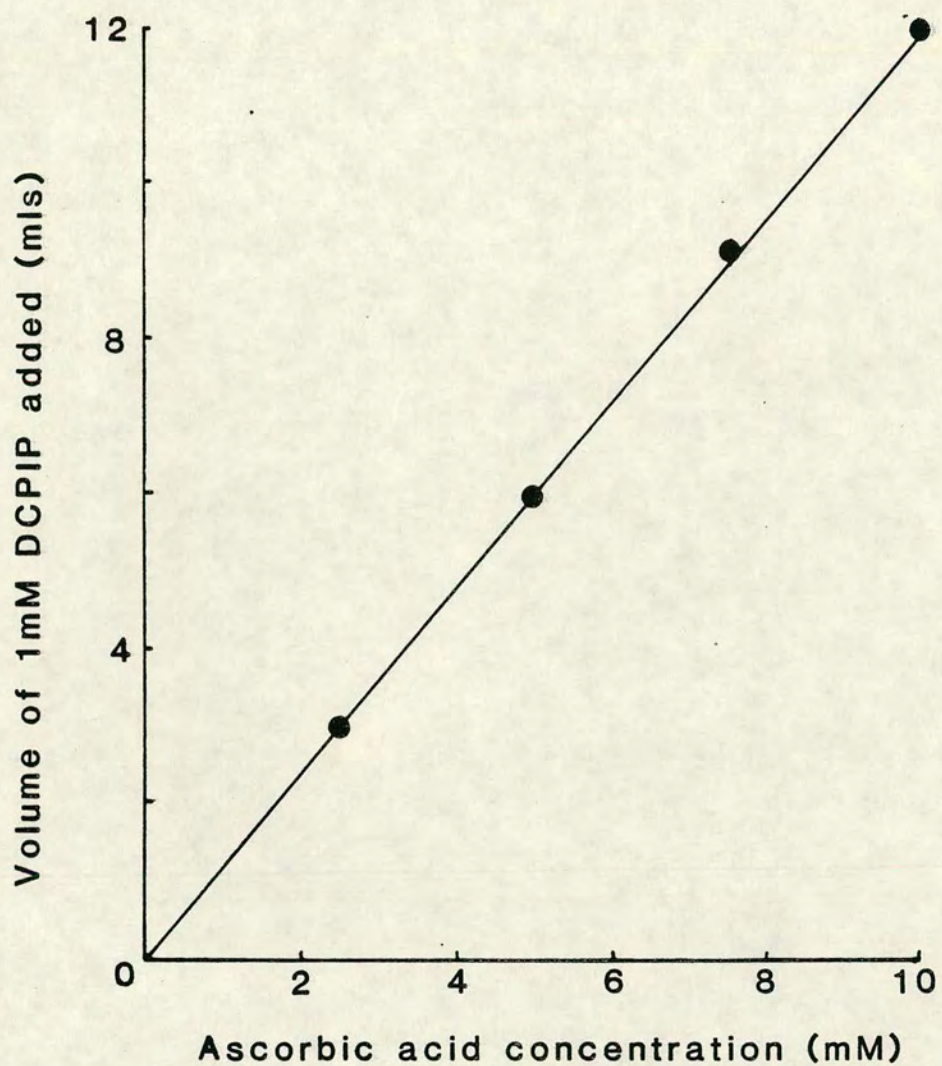


Fig. 3. Calibration graph to determine concentration of ascorbic acid. Known concentrations of ascorbic acid were titrated with 1 mM-DCPIP. End-point = colourless solution turned pale pink.

mixture was cooled and unreacted barium hydroxide precipitated with CO₂ produced from dry ice. A drop of bromothymol blue added to the sample acted as an indicator: colour change from blue to yellow indicated that all hydroxide had been neutralised. The sample was centrifuged at 2000 rpm for 5 min and the supernatant, containing hydrolysed fragments, collected.

Deglycosylation using TFMS acid

This method was used to deglycosylate extensin without causing peptidyl cleavage (Edge *et al.*, 1981; Sojar & Bahl, 1987). Samples were thoroughly dried under vacuum on the Speed-Vac. Exercising due care, 0.25-0.5 ml TFMS acid was added to each tube and nitrogen was layered over the surface. Samples were reacted in sealed tubes at 0°C for 2 h with occasional shaking. The reaction tubes were then cooled to about -20°C on dry ice/ethanol and unreacted TFMS acid was neutralised by one of the following procedures.

- A) 60% pyridine in water was gradually added dropwise to give a final pH of 7 (tested using indicator paper).
- B) Samples were diluted with twice the sample volume of diethyl ether; then an equal volume of 50% pyridine in water was carefully added and the tubes were vortexed to mix the layers. Once the layers had separated again the upper ether layer was removed. This extraction procedure was repeated and the lower aqueous layer retained.

High Voltage Paper Electrophoresis

Samples containing hydroxyproline oligoarabinosides that had been prepared by alkaline hydrolysis were separated by HVPE at pH 2.

Samples were loaded on to Whatman 3MM paper, with yellow ϵ -DNP lysine as marker, and electrophoresed from cathode to anode at 2 kV for 2-3 h over an electrical path length of 80 cm. The buffer used was formic acid/acetic acid/water (1:4:45 by vol).

Paper Chromatography

Paper chromatography was performed by the descending method on Whatman 3MM paper in the solvent system butan-1-ol/acetic acid/water (12:3:5 by vol).

Thin Layer Chromatography

TLC was performed by the ascending method on aluminium backed, silica-gel TLC plates cut to 10 cm height. Plates were developed in one of the following solvent systems:

- (a) 80% (w/v) phenol;
- (b) propan-1-ol: 25% (v/v) aqueous ammonia (7:3 v/v).

Spots on dried, stained TLC plates were quantified using the Quicksan R&D densitometer; this measured the intensity of colour of each spot and the area under the peaks produced was compared to a (previously constructed) calibration graph of peak area against amount of stained standard (e.g. isodityrosine), so that the amount of isodityrosine in each sample could be calculated.

two dimensional thin layer chromatography-the sample was loaded as a spot in the bottom left-hand corner of the silica gel TLC plate. The plate was developed by the ascending method in solvent (b) and allowed to dry before it was turned through 90° and then developed in solvent (a).

Scintillation Counting

Aqueous radioactive samples were assayed for radioactivity with a scintillation cocktail of 0.33% (w/v) PPO and 0.033% (w/v) POPOP in toluene/Triton X-100 (2:1 v/v) and a ratio 1:10 for sample : scintillant volume.

Non-aqueous samples (e.g. strips of chromatography paper) were assayed for radioactivity with a non-Triton scintillation cocktail containing 0.5% (w/v) PPO and 0.05% (w/v) POPOP in toluene. Sufficient cocktail was added to wet the sample (e.g. 1 ml for a 5 cm x 1 cm strip of Whatman 3MM).

Autoradiography

TLC plates bearing radioactive material were exposed to sheets (30 cm x 40 cm) of X-ray film and kept in the dark for 4 weeks at room temperature. The X-ray film was developed in an automatic developer, Gevomatic 60, to show the position of radioactive spots.

High Performance Liquid Chromatography

gel permeation HPLC- this was performed on a gel permeation column, Zorbax Bioseries Column-GF 250 (9.4 mm i.d. x 25 cm), with a mobile phase of 0.1 M-sodium phosphate (NaH_2PO_4), adjusted to pH 7, and pumped at a flow rate of 1.0 ml/min. 20 μl samples were injected from an injection loop and peaks monitored using a refractive index (R.I.) detector with mobile phase buffer in the reference cell. All buffers were made with HiPerSolv water for HPLC (BDH Chemicals Ltd.) and were filtered and degassed before use.

amino acid analysis- this was performed on a Waters Pico-Tag system

(White et al., 1986) which involved hydrolysis and pre-column derivatisation of the sample followed by reverse phase HPLC.

Samples were hydrolysed in 6 M-hydrochloric acid and 10-mM phenol and dried under vacuum on the Speed-Vac. Dried samples were dissolved in a known volume of water with a known amount of 20 mM taurine added as internal standard and then filtered through 0.45 μ m filters. Aliquots of samples (50-200 μ l) and amino acid standards (5 μ l) were placed in small glass tubes and dried under vacuum. Samples were redried under vacuum from 10-20 μ l of a solution containing ethanol/water/triethylamine (2:1:1 by vol). Derivatisation was with phenylisothiocyanate (PITC) and a fresh batch of derivatisation reagent was prepared for each analysis; the reagent consisted of ethanol/triethylamine/water/PITC (7:1:1:1 by vol). Reagent (20 μ l) was added to each sample, mixed thoroughly and allowed to stand for 20 min. Samples were then thoroughly dried under vacuum; at this point they may be stored in the freezer for up to several weeks if necessary.

When required for analysis samples were dissolved in 100-200 μ l diluent, which consisted of 5 mM-sodium phosphate (Na_2HPO_4), titrated to pH7.4 with 10% H_3PO_4 /acetonitrile (19:1 v/v). Prepared samples were inserted into the sample rack of the HPLC system and two automated injections of 10-25 μ l from each sample were separated by gradient elution using the following mobile phases:

A) 940 ml of 0.14 M-sodium acetate, titrated to pH 6.1 with acetic acid, and containing 0.05% (v/v) triethylamine, mixed with 60 ml acetonitrile.*

B) acetonitrile/water (3:2 by vol).

Details of the gradient profile are shown in Table 1.

Table 1. Gradient profile of eluents for amino acid analysis.

TIME (min)	FLOW (ml min ⁻¹)	% A	% B
0.0	1.0	100	0
10.0	1.0	54	46
10.5	1.0	0	100
11.5	1.0	0	100
12.0	1.5	0	100
12.5	1.5	100	0
20.0	1.5	100	0
20.5	1.0	100	0
25.0	1.0	100	0

Total run time = 25 min, of which sample run time = 13 min and equilibration time = 12 min.

Eluent A = 940 ml of 0.14 M-sodium acetate, titrated to pH 6.1 with acetic acid, and containing 0.05% (v/v) triethylamine, mixed with 60 ml acetonitrile.

Eluent B = acetonitrile/ water (3:2 by vol.)

Detection of the derivatised samples was by u.v. absorption at 254 nm. The integrator was calibrated to calculate the amount of each amino acid in the sample hydrolysate by comparison with the peaks in an amino acid standard mixture.

*An alternative mobile phase A [composed of 940 ml of 0.03 M-sodium acetate titrated to pH 6.4 with acetic acid, and containing 0.05% (v/v) triethylamine, mixed with 60 ml acetonitrile] was used to separate amino acids that could not be resolved using the above system.

Ion Chromatography

The purity of sodium chlorite, as supplied by BDH, was monitored on a Dionex Series 2000i ion chromatography system. Anions were separated on an ion-exchange column HPIC-AS3 and 2 mM-sodium carbonate was used as eluent at a flow rate of 15 ml/min. Ions were subsequently detected by a conductivity detector.

Samples and standards were made up in 2 mM-sodium carbonate and de-ionised, distilled water used throughout the process. The system (Fig. 4) also included a suppressor column which used 0.012 M-sulphuric acid as eluent.

Recrystallisation of Sodium Chlorite

Technical grade sodium chlorite (40 g) was dissolved in 50 ml water at 80°C to give a saturated solution. This was filtered through a warm funnel into a warm beaker to remove undissolved sodium chlorite. The filtrate was cooled on ice with stirring; if recrystallisation did not occur at this point, the solution was

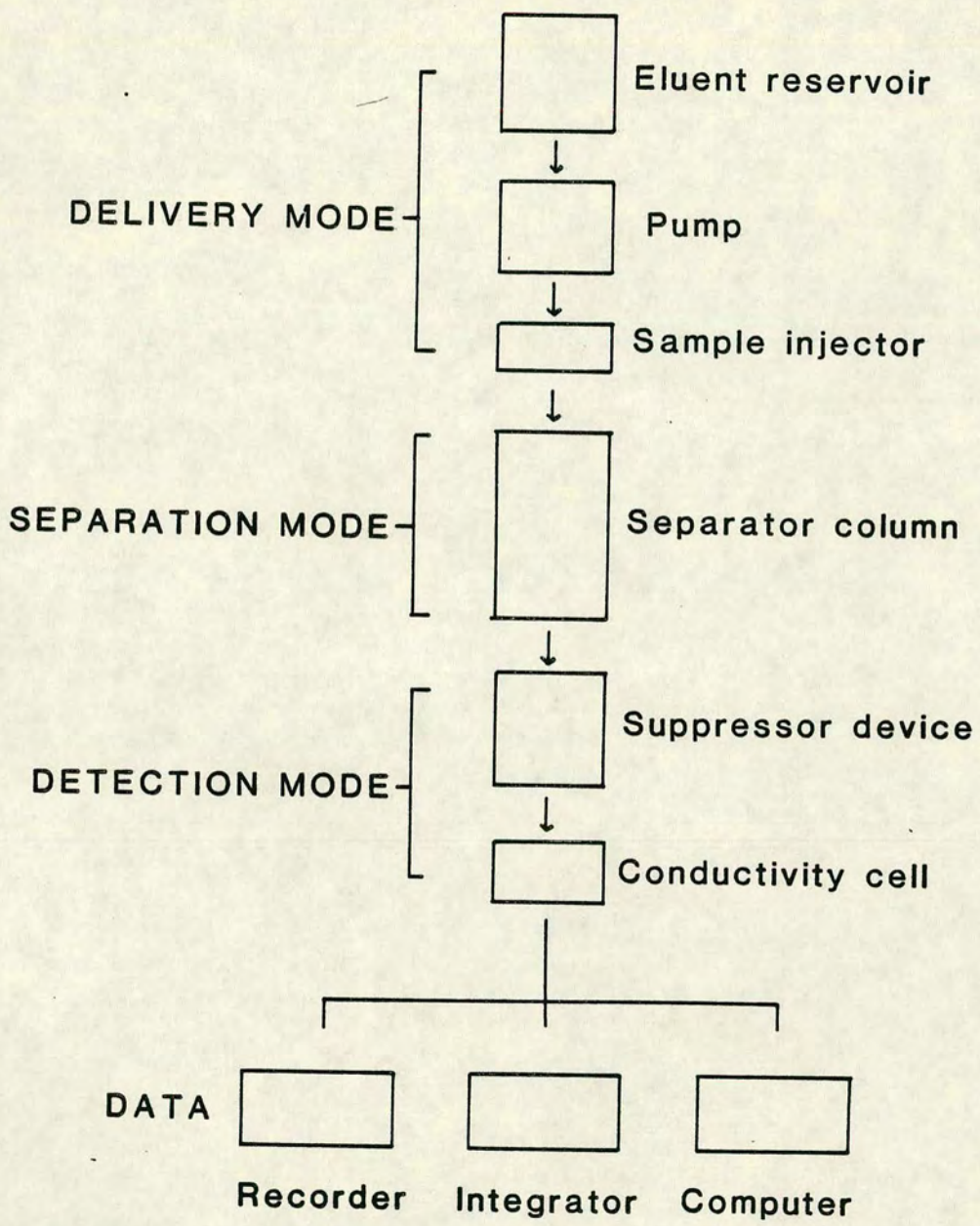


Fig. 4. Components of Dionex ion chromatograph.

'seeded' by addition of a crystal of sodium chlorite. The recrystallised sodium chlorite was filtered on a Büchner funnel and the crystals were left to dry completely at room temperature in the dark.

Column Chromatography

gel permeation chromatography- samples of radioactive, salt-soluble glycoprotein were analysed on a Sephacryl S-400 column (1.5 cm x 158 cm) in 1 M-sodium chloride/20 mM-Mes, adjusted to pH 6.1 with sodium hydroxide, at a flow rate of 20 ml/hr. For radioactive samples a portion was taken for scintillation counting, whilst the remainder was applied to the top of the column; this allowed the % recovery of radioactivity to be calculated for each experiment. Blue dextran and sucrose, each 1% (w/v), were added to the sample and used to determine the void volume (V_0) and included volume (V_t); sucrose was monitored in the fractions by the anthrone assay (see Assays). Fractions (1.5 ml) were collected and a portion was assayed for radioactivity on the scintillation counter.

ion-exchange chromatography- the following buffer solutions were made:

- (a) 0.1 M-sodium hydroxide titrated to pH 7.5 with solid Hepes
- (b) 0.1 M-sodium hydroxide titrated to pH 8.0 with solid Hepes
- (c) 0.1 M-sodium hydroxide titrated to pH 8.5 with solid Taps
- (d) 0.1 M-sodium hydroxide titrated to pH 9.0 with solid Taps
- (e) 0.1 M-sodium hydroxide titrated to pH 9.5 with sodium hydrogen carbonate
- (f) 0.1 M-sodium hydroxide titrated to pH 10.0 with sodium hydrogen carbonate

- (g) 0.1 M-sodium hydroxide titrated to pH 10.5 with sodium hydrogen carbonate
- (h) 0.1 M-sodium hydroxide titrated to pH 11.0 with sodium hydrogen carbonate
- (i) 0.1 M-sodium hydroxide titrated to pH 11.5 with phosphoric acid
- (j) 0.1 M-sodium hydroxide.

A 10 ml column of SP-50 Sephadex, cation exchanger, was packed into a 10 ml pipette and equilibrated with buffer (a). The radioactive glycoprotein sample to be applied to the column was dissolved in buffer (a) and a portion was taken for scintillation counting. The remainder of the sample was passed through the column followed by 10 mls of buffer (a) and a sample of the pooled eluate was taken for scintillation counting; from this it was possible to calculate how much of the sample had bound to the column. A stepwise pH gradient at constant ionic strength was then applied with the following volumes of buffers: 7.5 ml of (b) and (c); 15.0 ml of (d) and (e); and 7.5 ml of (f), (g), (h), (i) and (j). Fractions (2.5 ml) were collected throughout and afterwards monitored for (i) pH; (ii) radioactivity; and (iii) protein content by u.v. absorbance at $A_{280\text{nm}}$.

Preparation of [^{14}C]isodityrosine

L-[U- ^{14}C]tyrosine (1.85 MBq) and 20 mg non-radioactive tyrosine were dissolved in 0.5 ml 9 M-ammonia solution and 33 μl 0.75 M-potassium ferricyanide was added. The solution was incubated at approx. 20°C for 3 h in a capped tube. The whole sample was then loaded on to Whatman 3MM paper as a 40 cm streak, with external markers of tyrosine, isodityrosine and dityrosine, and analysed by descending paper chromatography in the solvent system butan-1-

ol/acetic acid/water (12:3:5 by vol). The paper was dried and then re-dried twice from methanol. The external markers, stained with ninhydrin, were used to locate the region of radioactive isodityrosine. This region was divided into eighteen 40 x 1 cm strips, each of which was eluted in 1 M-ammonia solution. A small portion of each eluate was scintillation counted and the remainder dried under vacuum.

Fractions containing the peak of radioactivity were dissolved in a total of 1 ml 1 M-ammonia solution, loaded as a 20 cm streak on Whatman 3MM, with external markers as above, and chromatographed in butan-1-ol/pyridine/water (4:3:4 by vol). The isodityrosine region was again located by staining the external markers and the region divided into twelve 20 x 1 cm strips. These were eluted in 1 M-ammonia solution; a small portion was scintillation counted and the remainder dried under vacuum.

Appropriate fractions were dissolved in a total of 1 ml 1 M-ammonia solution and chromatographed as a 20 cm streak on Whatman 3MM in 80% (w/v) phenol. The region co-chromatographing with the external marker isodityrosine was divided into twelve 20 x 1 cm strips which were eluted in 1 M-ammonia solution. A portion of each fraction was scintillation counted and the remainder dried under vacuum.

Fractions containing the peak of radioactivity were dissolved in a total of 1 ml 1 M-ammonia solution; this sample was considered to be pure isodityrosine. It was divided into 50 μ l portions, each of which was dried under vacuum and stored frozen.

Difference in counting efficiency between solubilised glycoprotein and cell wall pellets

It was necessary to make an estimation of the difference in counting efficiency between solubilised glycoprotein and cell wall pellets that were scintillation counted in Triton scintillant, so that the levels of radioactivity could be directly compared. Duplicate samples of radioactive cell walls, isolated from a Capsicum frutescens suspension culture that had been fed with [³H]arabinose, were suspended in 0.25 ml water and scintillation counted in Triton scintillant. The walls were washed with toluene to remove the Triton scintillant, rinsed with methylcyclohexane and dried. Then they were hydrolysed in 0.5 ml trifluoroacetic acid (TFA) in sealed tubes at 120°C for 1 h, in order to solubilise the arabinose residues. The hydrolysates were collected, the residual wall pellets were washed several times to remove all of the soluble radioactive material and the washings were pooled with the hydrolysates. These samples were dried under vacuum to remove the TFA which would interfere with the counting efficiency, and then they were counted as aqueous samples in Triton scintillant. The residual wall pellets were also counted to show that radioactivity had been solubilised.

From the cpm values obtained it was calculated that scintillation counting of solubilised radioactive material was 2.3 x more efficient than scintillation counting of the same radioactivity in cell wall pellets. This factor was used in later experiments to obtain a value of the radioactivity in cell wall pellets.

RESULTS

Analysis of suspension culture cell walls

Introduction

A survey of a range of dicotyledonous plant cell suspension cultures was conducted in order to see which species would take up [³H]proline most efficiently, and then incorporate the precursor into hydroxyproline units of cell wall glycoprotein. Investigations were carried out on plant cell suspension cultures because these have been shown to have high levels of HRGPs (Lamport, 1965) and also because radioactive labelling experiments are easily performed upon suspension culture cells where the radioactive precursor can be dissolved in the culture medium. The advantages of radioisotope labelling are (i) it is a sensitive method allowing small amounts of material to be detected and (ii) radioactive molecules are easy to assay by scintillation counting. Suspension cultures of dicotyledonous species were used because they typically contain 2-10% extensin whilst monocotyledonous species have lower amounts, typically 0.2-1%.

The suspension cultures that appeared most efficient at incorporating the label were then further investigated to see at which stages during the growth cycle maximum uptake and incorporation of [³H]proline occurred.

Uptake and incorporation of [³H]proline by cell suspension cultures

Duplicate cell suspension cultures (5 ml) of Rosa sp. (Paul's Scarlet), Spinacia oleracea, Atropa belladonna, Hyoscyamus niger, Catharanthus roseus and Capsicum frutescens m, all in the late

log phase of growth, were each inoculated with 0.074 MBq [³H]proline and incubated for 6 h. Uptake of [³H]proline was monitored during the incubation period by scintillation counting a portion of the culture medium at hourly intervals. Then cell walls were isolated as outlined in the Methods and the amount of radioactivity incorporated was taken as that remaining in the walls after PAW extraction (Table 2).

Partitioning of the incorporated [³H]proline into hydroxyproline and proline in PAW-insoluble wall material was investigated by acid hydrolysis of samples of the isolated walls in 6 M-hydrochloric acid and separation of the hydrolysis products by paper chromatography. This analysis showed that virtually all of the [³H]proline had been incorporated into proline or hydroxyproline (Fig. 5), and the % incorporation as hydroxyproline for each of the species is shown in Table 2.

As can be seen from Table 2 there was variation between species as to the extent of incorporation of [³H]proline into insoluble glycoprotein, despite high radioactivity uptake levels of at least 50% for all cultures. Capsicum frutescens m appeared most efficient at incorporating [³H]proline into hydroxyproline units of insoluble wall glycoprotein, suggesting that these cells have a high extensin content in their walls.

The ratios in Table 2 are comparative rather than absolute values because there will be differences in counting efficiency between soluble and extracted protein samples in Triton scintillant, and insoluble wall glycoprotein samples in non-Triton scintillant. However, the values remain valid because counting efficiency will not vary between the species tested. The values of hydroxyproline content in insoluble glycoprotein are based upon the assumption that all the

Table 2. Incorporation of radioactivity from [³H]proline into cell wall glycoprotein.

Suspension culture	$\frac{[{}^3\text{H}]\text{glycoprotein}}{[{}^3\text{H}]\text{proline supplied}} \times 100\%$	$\frac{[{}^3\text{H}]\text{hydroxyproline}}{[{}^3\text{H}]\text{glycoprotein}} \times 100\%$
<u>C. roseus</u>	0.4	22
<u>S. oleracea</u>	2.5	45
<u>Rosa sp.</u>	3.4	47
<u>A. belladonna</u>	3.5	42
<u>H. niger</u>	6.5	50
<u>C. frutescens m</u>	18.2	60

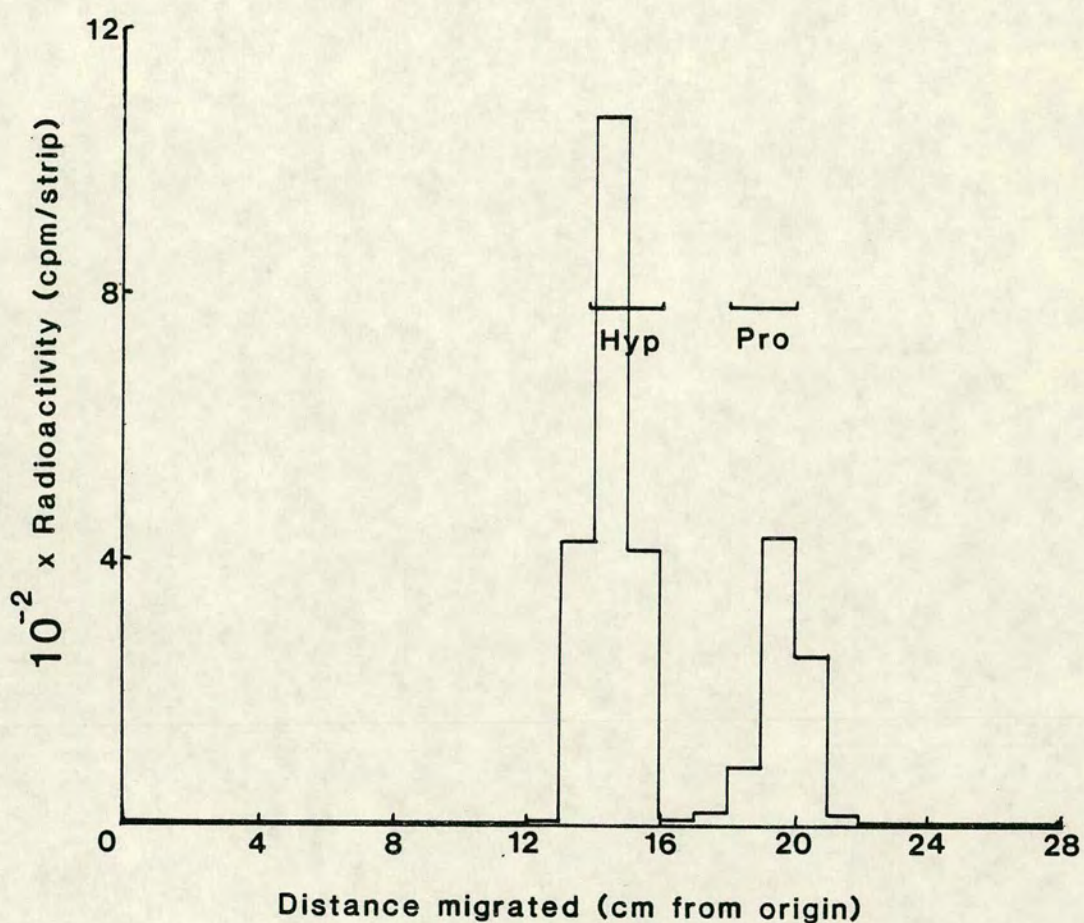


Fig. 5. Partitioning of [³H]proline between proline and hydroxyproline residues in cell wall glycoprotein of Capsicum frutescens m. Cell wall samples were hydrolysed in 6 M-hydrochloric acid and the hydrolysis products were separated by paper chromatography in butan-1-ol/acetic acid/water (12:3:5 by vol.).

radioactivity incorporated was present as hydroxyproline or proline.

Variation in [³H]proline incorporation during the growth cycle of Capsicum cultures

In addition to the Capsicum frutescens m suspension culture used in the previous experiment a more friable Capsicum frutescens f₁ culture (kindly donated by Robert Hall) was also used. Duplicate 50 ml flasks of each culture were maintained under the conditions outlined in the Methods and at days 0, 1, 2, 3, 5, 7, 9 and 11 after subculture, 5 ml samples of suspension were removed aseptically from each flask and incubated with 0.074 MBq [³H]proline for 6 h. At the end of this period the packed cell volume was measured as a record of cell growth and the % uptake of radioactivity by the cells was calculated from the amount remaining in the culture medium. These values are shown in Fig. 6a & b.

The increase in packed cell volume over the 11 days can be clearly seen, but growth may have been better expressed as an increase in tissue fresh weight with time because it was difficult to get an accurate packed cell volume for the 'lumpy' cultures. Uptake of radioactivity was at least 90% throughout the experiment and little difference in uptake between the two cultures was observed.

Cell walls were isolated from each of the samples and a portion was acid hydrolysed and used for paper chromatography to partition the radioactivity into proline and hydroxyproline; from this the incorporation into hydroxyproline in insoluble cell wall glycoprotein was calculated (Table 3). The proportion of [³H]proline incorporated into Capsicum frutescens f₁ wall glycoprotein as hydroxyproline was slightly higher than that incorporated into the

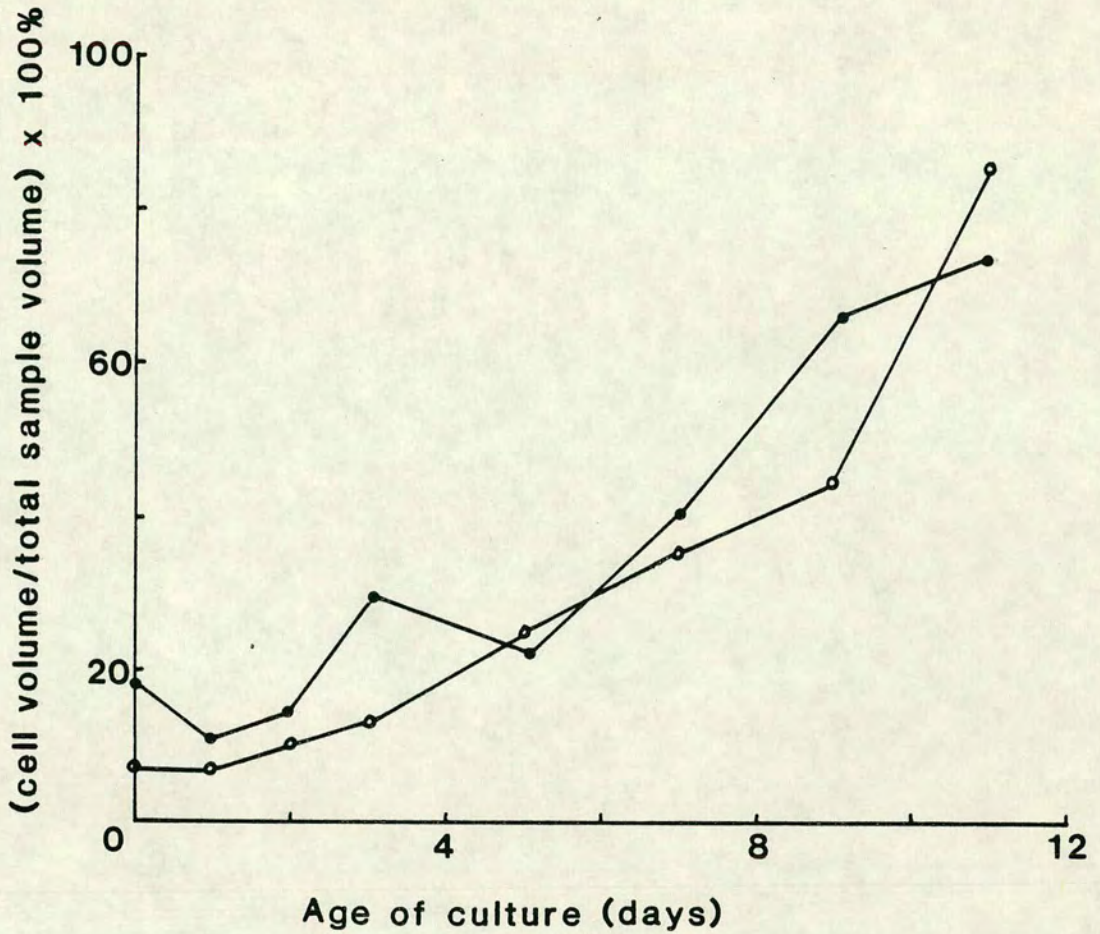


Fig. 6a. Growth of Capsicum cell suspension cultures. Growth was measured as the packed cell volume of each 5 ml sample of suspension culture. ○, Capsicum frutescens m. ●, Capsicum frutescens f₁.

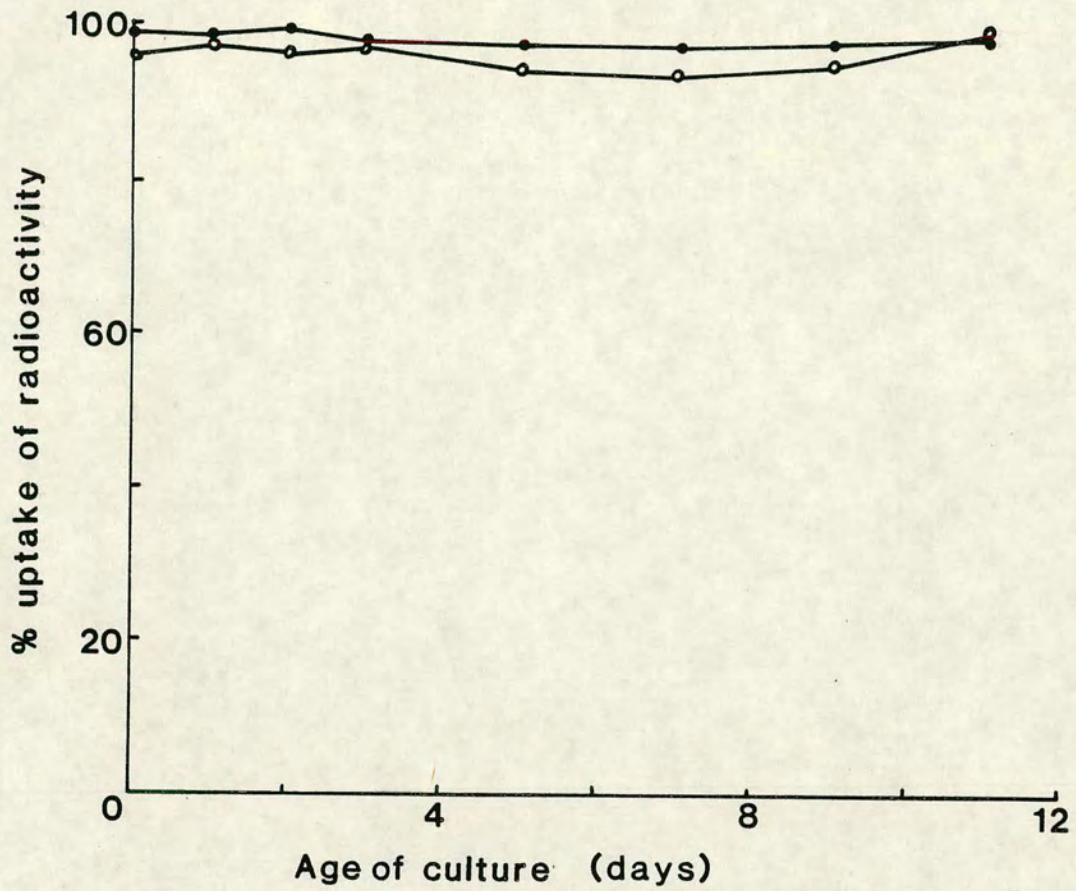


Fig. 6b. Uptake of [^3H]proline by Capsicum frutescens cell suspension cultures. Uptake of radioactivity by the suspension cultures after a 6 h incubation period was calculated from the amount of [^3H]proline that remained in the culture medium. o, Capsicum frutescens m. ●, Capsicum frutescens f₁.

Table 3. Incorporation of radioactivity from [³H]proline into [³H]hydroxyproline residues of cell wall glycoprotein of Capsicum frutescens.

$$\frac{[{}^3\text{H}]\text{hydroxyproline}}{[{}^3\text{H}]\text{glycoprotein}} \times 100\%$$

Day	<u>Capsicum frutescens</u> m	<u>Capsicum frutescens</u> f ₁
0	53	52
1	56	69
2	51	70
3	40	73
5	35	69
7	42	73
9	45	-
11	44	66

Capsicum frutescens f₁ was a more friable suspension culture than Capsicum frutescens m.

less friable culture and suggests that these cells are rich in extensin. The proportion of radioactivity incorporated as hydroxyproline into wall glycoprotein varied little over the 11 day growth period suggesting that the cells' ability to use [³H]proline as a precursor for wall hydroxyproline is consistent. Variation in amount of hydroxyproline becoming wall-bound over the time course was calculated from the total radioactivity in each PAW extracted cell wall pellet x mean % incorporated as [³H]hydroxyproline; the values (Table 3) suggested that maximum incorporation was 2-3 days after subculture. It would appear that extensin is being covalently bound to the cell wall most rapidly at this time.

Conclusion

It was concluded from these investigations that Capsicum frutescens was most efficient at incorporating radioactivity into hydroxyproline rich wall glycoprotein and that Capsicum f₁ cultures, 2-3 days after subculture, were potentially the most suitable system for maximising incorporation of [³H]proline into cell wall glycoprotein.

Optimisation of gel permeation chromatography system

Introduction

Radioactive, salt-soluble extensin precursors and extensin fragments solubilised from cell wall glycoprotein were separated into molecules of different sizes (e.g. dimers and monomers) by gel permeation chromatography. In this process molecules are graded by the sieving properties of the gel matrix; large molecules that are excluded from the gel elute first in the void volume (V_0), whilst smaller molecules that are retained within the gel elute in the included volume (V_t).

Various workers (Stuart & Varner, 1980; Smith et al., 1984) have shown that certain extensins cannot be resolved upon SDS-polyacrylamide gels but success in separating extensin on a cationic-urea PAGE system, devised by Stuart & Varner (1980), has been achieved by Stafstrom & Staehelin (1986a). The reasons for choosing gel permeation chromatography, rather than cationic-urea PAGE, as the separation method to be used in this project were: (a) that peaks of radioactive molecules could be easily detected by scintillation counting of a portion of each fraction collected, (b) lower amounts of radioactive material could be detected by scintillation counting than by autoradiography and (c) separated molecules are in solution.

This chapter outlines the gel permeation media and buffer solutions tested in order to find a suitable system for separating extensin and also mentions some of the problems encountered in interpreting data obtained from gel permeation experiments.

Selection of gel permeation media and eluents

A column of Sepharose CL-6B (1.5 cm x 49 cm) with 10 mM-sodium hydroxide (measured pH approx. 11.6) as eluent was the first system tested but this gave variable results for the elution of extensin molecules and monomer extensin eluted near the void volume (Fig. 7a) with an apparently high M_r . It was decided that sodium hydroxide was unsuitable; the high pH of the eluent is near to the isoelectric point (pI) of extensin (pI greater than 10) and because proteins are least soluble at their pI values it is possible that extensin may be aggregating and/or partially precipitating.

The Sepharose CL-6B column was re-equilibrated with 0.1 M-sodium phosphate (NaH_2PO_4), adjusted to pH 7. A sample of monomer extensin analysed on the column with this eluent eluted in the included volume (Fig. 7b) and there was a low % recovery of radioactivity (13%), so this eluent was also considered unsuitable.

The next buffer used was 1 M-sodium chloride, as used by Smith et al. (1986) to separate tomato extensin precursors on Sepharose CL-6B. With this buffer, monomer extensin eluted as a single, partially included peak and there was a good % recovery of radioactivity (greater than 90%). Columns packed with Sepharose CL-2B and CL-4B, each 1.5 cm x 49 cm, were also equilibrated with 1 M-sodium chloride and used to analyse monomer extensin. Whilst a high % recovery was achieved with all three columns, it was decided that the Sepharose CL-6B column had the most suitable fractionation range; for the fractionation range of all gel media used see Table 4. In subsequent work, the eluent was buffered at a fairly neutral pH, so the final eluent composition was 1 M-sodium chloride/20 mM-Mes, adjusted to pH 6.1 with sodium hydroxide; this did not affect the

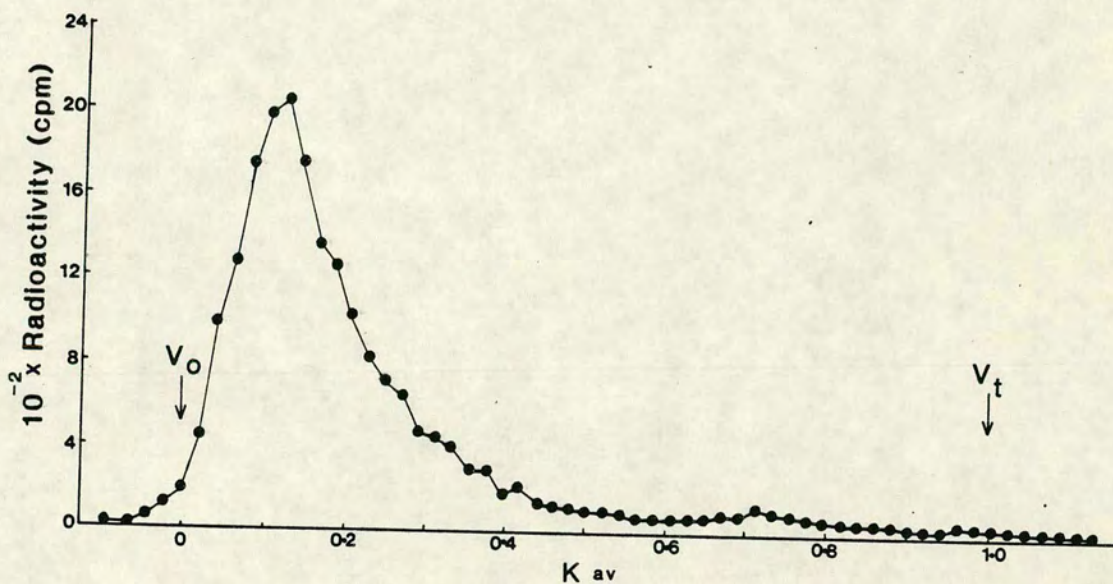


Fig. 7a. Gel permeation chromatography (on Sepharose CL-6B) of monomer extensin. The column of Sepharose CL-6B (1.5 cm x 49 cm) was eluted with 10 mM-sodium hydroxide (pH approx. 11.6). •, [U- ^{14}C]extensin. Radioactivity = cpm/0.5 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.



Fig. 7b. Gel permeation chromatography (on Sepharose CL-6B) of monomer extensin. The column of Sepharose CL-6B (1.5 cm x 49 cm) was eluted with 0.1 M-sodium phosphate (pH 7.0). ●, [U-¹⁴C]extensin. Radioactivity = cpm/0.25 ml. Recovery of radioactivity = 13%. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

Fig. 7c. Gel permeation chromatography (on Sephacryl S-400) of monomer extensin. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). ●, [arabinosyl-³H]extensin. Radioactivity = cpm/0.25 ml. Recovery of radioactivity = 95%. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

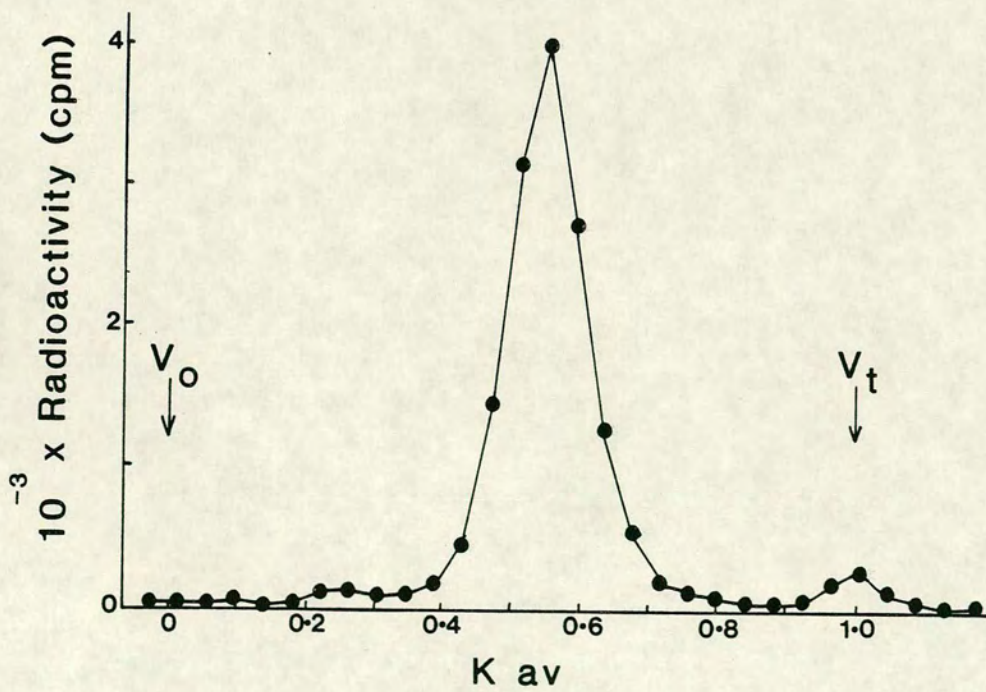
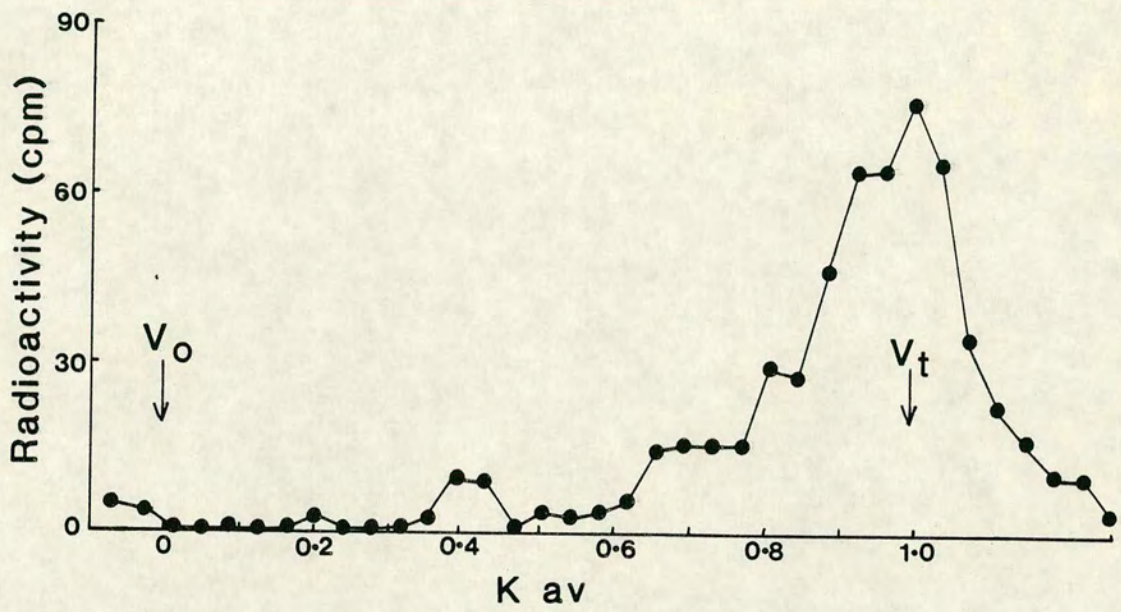


Table 4. Fractionation ranges of gel permeation chromatography media. Molecular weight fractionation ranges for proteins and polysaccharides, reproduced from Pharmacia Handbooks: Gel filtration theory and practice.

	Proteins	Polysaccharides
Sepharose CL-2B	$7 \times 10^4 - 4 \times 10^7$	$1 \times 10^5 - 2 \times 10^7$
Sepharose CL-4B	$6 \times 10^4 - 2 \times 10^7$	$3 \times 10^4 - 5 \times 10^6$
Sepharose CL-6B	$1 \times 10^4 - 4 \times 10^6$	$1 \times 10^4 - 2 \times 10^6$
Sephacryl S-400	$2 \times 10^4 - 8 \times 10^6$	$1 \times 10^4 - 2 \times 10^6$

elution profile of extensin monomer.

A final improvement to the system was tested by use of a column of Sephacryl S-400 (1.5 cm x 48 cm). This gel has a broader fractionation range than Sepharose CL-6B and so molecules should elute in a narrower peak. A sample of monomer extensin analysed on this column with 1 M-sodium chloride/20 mM-Mes (pH 6.1) as eluent did give a slightly narrower peak and again showed a high % recovery (greater than 90%) of radioactivity (Fig. 7c). This system gave reproducible results; monomer extensin always eluted with a k_{av} of 0.55-0.59 (see below, column calibration).

Stafstrom & Staehelin (1986a) used an eluent of 150 mM-Tris hydrochloride (pH 8) to separate salt-extractable extensin from carrot cell walls by gel permeation chromatography on a Sephacryl S-400 column. A sample of Capsicum extensin monomer was analysed on the Sephacryl S-400 column with this eluent; as can be seen from Fig. 7d this buffer caused extensin to elute as a broader peak and the % recovery of radioactivity was lower (60%). It was concluded that this eluent was not suitable for good resolution of extensin molecules.

Having selected a Sephacryl S-400 column and an eluent of 1 M-sodium chloride/20 mM-Mes (pH 6.1) as the most suitable gel permeation chromatography system, the process was routinely carried out as described in the Methods.

Column calibration

The column was calibrated with a series of dextran standards. A solution of 0.025% (w/v) each of blue dextran, dextran of known M_r and sucrose was made in 1 M-sodium chloride/20 mM-Mes (pH 6.1) and a 2 ml sample chromatographed on the Sephacryl S-400 column.

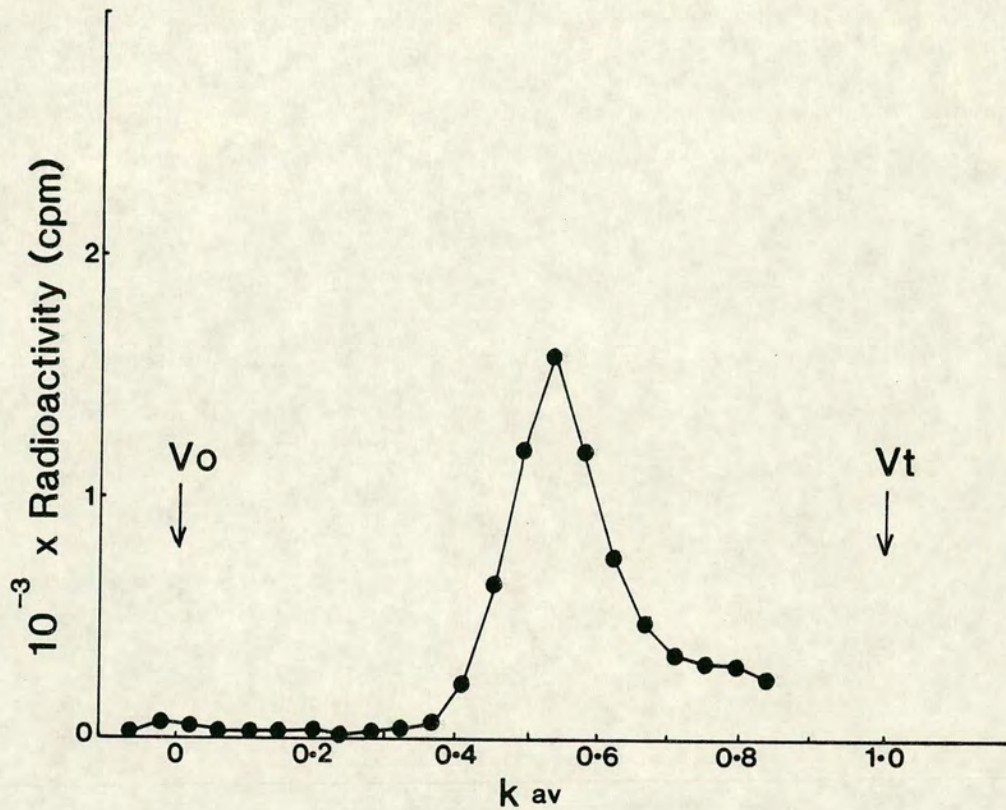


Fig. 7d. Gel permeation chromatography (on Sephacryl S-400) of monomer extensin. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 150 mM-Tris hydrochloride (pH 8.0). ●, [arabinosyl-³H]extensin. Radioactivity = cpm/0.75 ml. Recovery of radioactivity = 60%. V₀, elution volume of Blue Dextran; V_t, elution volume of sucrose.

Fractions (60 x 1 ml) were assayed by the anthrone assay and from the elution profile the values V_0 (void volume), V_e (sample elution volume) and V_t (included volume) were calculated and then used to obtain the k_{av} value from the equation:

$$k_{av} = \frac{V_e - V_0}{V_t - V_0}$$

A graph of k_{av} against M_r of dextran markers was plotted and the line of best fit was calculated by linear regression. The elution positions of blue dextran and sucrose were not used in this calculation because these molecules have M_r values outside the fractionation range of the gel. This calibration graph (Fig. 8) was used to predict the M_r and elution position of extensin monomers and dimers.

Non-globular dextrans (Burton & Brant, 1983) were used to calibrate the column rather than globular protein markers because extensin molecules are thought to adopt a rod shaped conformation; some evidence for this comes from recent transmission electron micrographs (Stafstrom & Staehelin, 1986a) of soluble extensin precursors which show the molecules to be kinked rods of approx. 80 nm length. The strong positive charge of extensin, provided by the lysine and histidine residues in the polypeptide backbone, is suggested to be partly responsible for holding the molecule in a rigid rod conformation. The true behaviour of extensin molecules upon a gel permeation column is difficult to determine with accuracy because, as a glycoprotein, it is likely that extensin molecules may behave intermediately between proteins and polysaccharides. In a recent paper, Heckman et al. (1986) discuss the anomalous behaviour of extensin precursors upon gel filtration columns and they suggest that

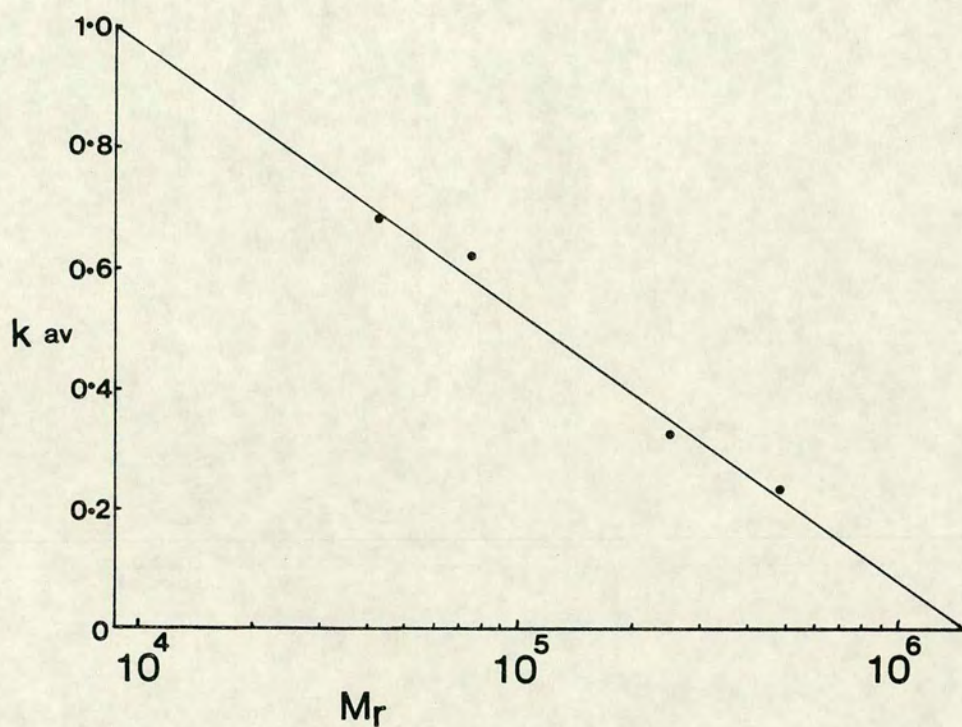


Fig. 8. Calibration graph of k_{av} against M_r for Sephacryl S-400. Dextran standards were eluted from the column of Sephacryl S-400 (1.5 cm x 48 cm) with 1 M-sodium chloride/20 mM-Mes (pH 6.1). The anthrone assay was used to show the elution volume of each dextran and k_{av} values were calculated.

extensin monomers, as rigid rodlike molecules, are likely to reptate (end on migration of a linear polymer) through the column matrix. Such movement leads to inaccurate estimations of M_r compared with protein standards; their extensin monomers co-eluted with thyroglobulin ($M_r = 669$ kD). The dextran calibration used in this project appears to give fairly accurate estimations of M_r ; Capsicum extensin monomers eluted with apparent $M_r = 80-100$ kD which compares favourably with an estimated $M_r =$ approx. 90 kD, based on measurements using pore gradient gel electrophoresis or linear sucrose gradients (Stuart & Varner, 1980).

Role of molecular conformation in data interpretation

Another problem arising from the use of gel permeation chromatography to separate extensin molecules is knowing whether a shift in peak position is due to a change in M_r or a change in conformation; for example, a molecule could be cleaved to yield a peak of smaller M_r , or a rod shaped molecule could become more globular and this would also give an apparently smaller M_r . This issue will be further discussed in a later chapter.

Also on the issue of molecular conformation is the question of how extensin dimers would elute from the column. The simplest answer is to assume that a dimer, having twice the M_r of an extensin monomer, would behave as a rod of twice the size, but dimer elution may depend upon where the two monomers are cross-linked and consequently, upon the shape of the resulting dimer. In this project it has been assumed that a dimer would appear to the gel matrix as twice the size of a monomer and so the peak elution position of dimers can be predicted from the column calibration graph. To improve the

resolution of potential dimers from monomers a longer Sephacryl S-400 column was used (1.5 cm x 158 cm), but with the same eluent and operating at the same flow rate as the short column.

Conclusion

The most satisfactory gel permeation chromatography system for separating extensin molecules was a column of Sephacryl S-400 and an eluent of 1 M-sodium chloride/20 mM-Mes (pH 6.1); this gave reproducible results and a high % recovery of radioactivity. Monomer extensin molecules eluted as a narrow peak of partially included material, and after the column had been calibrated with dextrans of known M_r , the M_r of monomer extensin was estimated as approx. 90 kD. From this value it was possible to predict that a peak of extensin dimer could be resolved from a peak containing extensin monomer.

Cleavage reagents for isodityrosine

Introduction

As discussed earlier in the main introduction the approach being taken to demonstrate the existence of intermolecular isodityrosine cross links in extensin is to cleave network or dimer molecules to monomers by specifically breaking isodityrosine. This involves identification of a suitable cleavage reagent and conditions under which it will specifically cleave isodityrosine. In this chapter the results obtained from using two reagents, sodium chlorite and N-bromosuccinimide, are discussed. The ability of each chemical to solubilise glycoprotein from cell walls, to cleave isodityrosine and its effect upon monomer extensin was investigated. The effects of sodium chlorite upon isodityrosine and cell wall glycoprotein have been reported; Fry (1982) demonstrated that a 2-5 min treatment with a solution of warm, acidified sodium chlorite cleaved isodityrosine to tyrosine, and O'Neill & Selvendran (1980) used a similar treatment to solubilise glycoprotein from bean cell walls. Sodium chlorite would seem to be a suitably specific cleavage reagent but the possibility of using N-bromosuccinimide, which may cleave dityrosine cross links in resilin (C. Neville, [Zoology Dept., University of Bristol] personal communication), was also considered.

Solubilisation of glycoprotein from cell walls

(1) Acidified sodium chlorite

Cell walls, isolated from Capsicum frutescens f₁ cell suspension culture that had been fed with [³H]proline, were treated with a solution of 0.3% (w/v) sodium chlorite, adjusted to pH 3.4 with

acetic acid, at 70°C for varying times. The effects of giving repeated 15 min treatments with acidified chlorite solution and having 50 mM-lanthanum chloride present in the solution were also investigated; the treatments given are summarised in Table 5. All reactions were halted by the addition of 1.8 M-ammonia solution (aq.) to make the solution alkaline (pH approx. 9) and so prevent the chlorite from reacting further. Samples were centrifuged to pellet insoluble wall material; equal portions of each supernatant, and the cell wall pellets, were scintillation counted.

The results (Table 5) show that glycoprotein was solubilised from the cell walls by acidified chlorite because the controls (samples 13-15) had much lower levels of radioactivity in the supernatant. Acidified chlorite had solubilised glycoprotein within 15 min treatment; the variability in cpm values between 15-60 min treatment is probably due to error in measuring out cell wall material. Comparison of the cpm values of the wall pellets for the controls with the treated samples showed that 85-90% glycoprotein was solubilised. No further glycoprotein was solubilised by subsequent 15 min treatments with acidified chlorite. Lanthanum chloride was added to samples 9-12 in an attempt to release any glycoprotein solubilised by acidified chlorite but remaining ionically bound to polyanionic components of the cell wall; however, formation of a white precipitate, alongwith the results obtained, showed that the salt interfered with solubilisation, suggesting that a reaction was occurring between lanthanum chloride and sodium chlorite.

This experiment confirmed the finding by O'Neill & Selvendran (1980) that acidified chlorite solutions can solubilise glycoprotein from cell walls.

Table 5. Acidified chlorite treatments used to solubilise glycoprotein from cell walls of Capsicum frutescens f1 suspension culture

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Number of treatments	1	1	1	1	1	2	3	4	1	2	3	4	-	-	-
Treatment time (min)	15	30	45	60	15	30	45	60	15	30	45	60	0	0	0
50 mM-lanthanum chloride present	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-
Results															
10-3 x Radioactivity (cpm in 2.5 ml)	47.4	47.7	40.3	33.9	39.7	37.6	38.5	39.6	14.9	4.7	6.7	2.5	1.4	0.7	0.5

Cell walls were treated with 0.3% (w/v) sodium chlorite, adjusted to pH 3.4 with acetic acid, at 70°C. Reactions were halted by the addition of 1.8 M-ammonia solution, to give a final pH of 9.0. Samples 13-15 acted as controls: 13 was incubated at pH 3.4 at 70°C for 15 min before adjustment to pH 9; 14 was incubated as an aqueous suspension at 70°C for 15 min before adjustment to pH 9 and 15 was incubated as an aqueous suspension at 70°C for 15 min.

(2) N-bromosuccinimide

Another sample of isolated, radioactive cell walls was treated with N-bromosuccinimide in solutions of 0.2 M-acetic acid, adjusted to pH 4, 5, 6 or 7 with sodium hydroxide. Concentrations of N-bromosuccinimide used were 0, 1, 5, 10 and 25 mM and treatment times were 5, 15, 30, 60 and 120 min. All reactions were carried out at about 20°C. At the end of each treatment a portion of the supernatant was scintillation counted in order to see whether glycoprotein had been solubilised. The wall pellets were also assayed for radioactivity. Samples where only acetate buffer had been added to the cell walls acted as controls.

The values (Fig. 9) showed that some glycoprotein was solubilised by the action of N-bromosuccinimide, with solubilisation occurring most effectively at pH 4 but with very little solubilisation occurring in the neutral buffer. At pH 4 glycoprotein solubilisation increased with the length of treatment and with the concentration of N-bromosuccinimide up to 10 mM; no further increase was noted with 25 mM-N-bromosuccinimide. Comparison of the cpm for the total glycoprotein solubilised by treatment with 10 mM-N-bromosuccinimide at pH 4 and the cpm in the wall pellet showed that 5-6% glycoprotein had been solubilised. This experiment showed that N-bromosuccinimide could be a potential cleavage reagent under acidic conditions, although it appeared far less effective at solubilising glycoprotein from cell walls (5-6%) than acidified chlorite (85-90%).

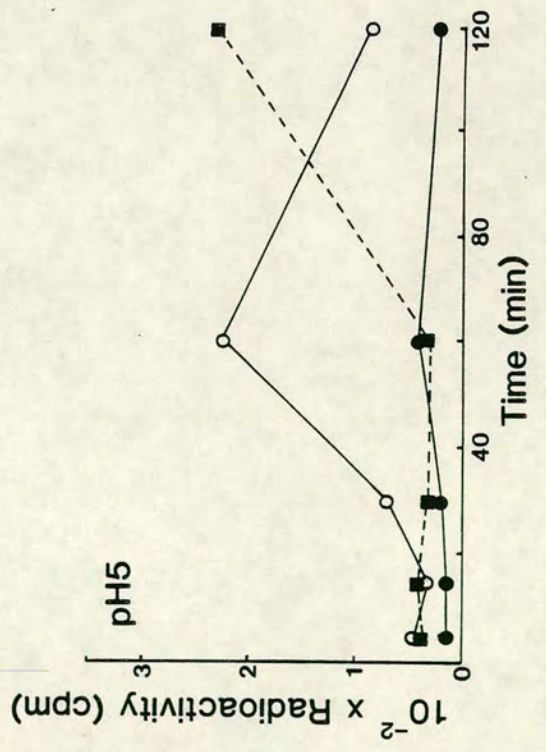
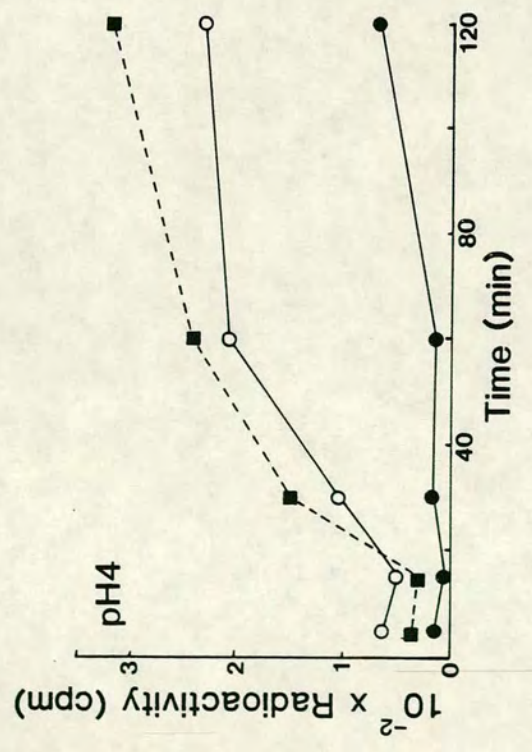
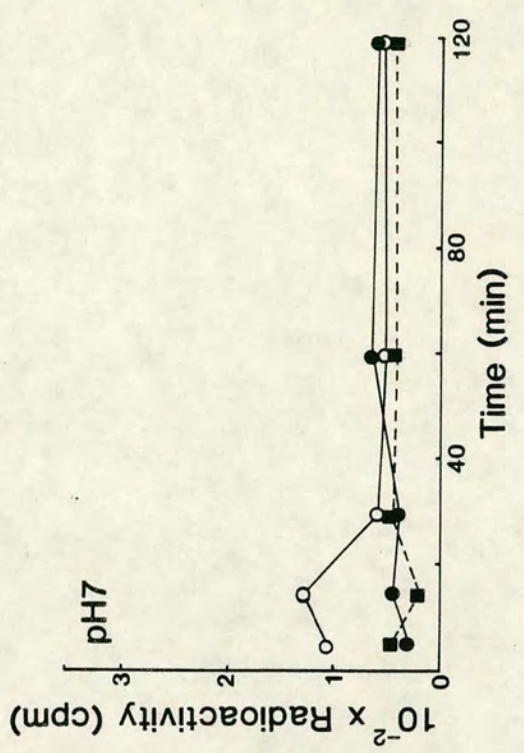
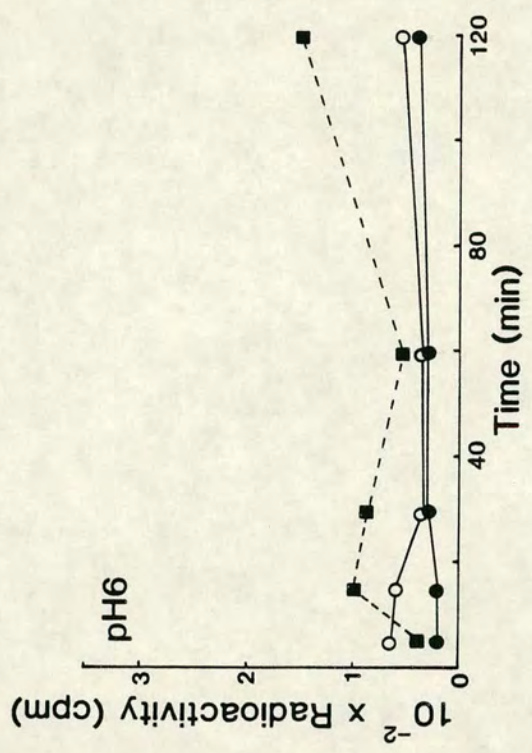
Effects upon monomer extensin

(1) Acidified sodium chlorite

The chemical used to break isodityrosine cross links must

Fig. 9. Solubilisation of radioactive glycoprotein from cell walls by treatment with N-bromosuccinimide. Cell walls were treated with varying concentrations of N-bromosuccinimide (0-25 mM) in solutions of 0.2 M-acetic acid, adjusted to pH 4, 5, 6 or 7 with sodium hydroxide. The graphs show the amount of radioactive material (cpm/0.15 ml) solubilised after each treatment time.

●—● 0 mM, ○—○ 5 mM, ■--■ 25 mM N-bromosuccinimide.



not affect the backbone of the extensin molecules if it is to be a specific reagent. One way of checking this is to treat monomer extensin with the reagent and look for any change in elution profile following gel permeation chromatography. O'Neill & Selvendran (1980) reported that acidified sodium chlorite had no apparent effects upon lysozyme, other than to convert lysine residues to α -aminoadipic acid residues, and so it appeared that acidified chlorite would not cleave peptide bonds.

[U- 14 C]extensin (salt-soluble) prepared from spinach cell suspension cultures was analysed by gel permeation chromatography on a Sepharose CL-6B column with an eluent of 1 M-sodium chloride. Another sample of the monomer extensin was treated with chlorite (final concentration of sodium chlorite = 0.4% (w/v)) acidified to pH 3.4, at 60°C for 5 min. The reaction was halted with 1.8 M-ammonia solution and the neutralised sample analysed by the above gel permeation chromatography system.

The elution profiles (Fig. 10) showed that untreated monomer extensin eluted as a narrow, partially included peak whilst the acidified chlorite treated monomer eluted as a broader peak of lower apparent M_r . It was concluded that acidified chlorite did affect monomer extensin molecules and two interpretations of the result seemed possible: (a) ionically bound spinach extensin is not monomeric and has already been cross linked so that the acidified chlorite did cleave these links or (b) acidified sodium chlorite is able to cleave the peptide backbone of extensin, either at a particularly labile bond or by random cleavage. The broad peak obtained from acidified chlorite treatment suggests that the latter explanation is more probable and further evidence to show that this interpretation is

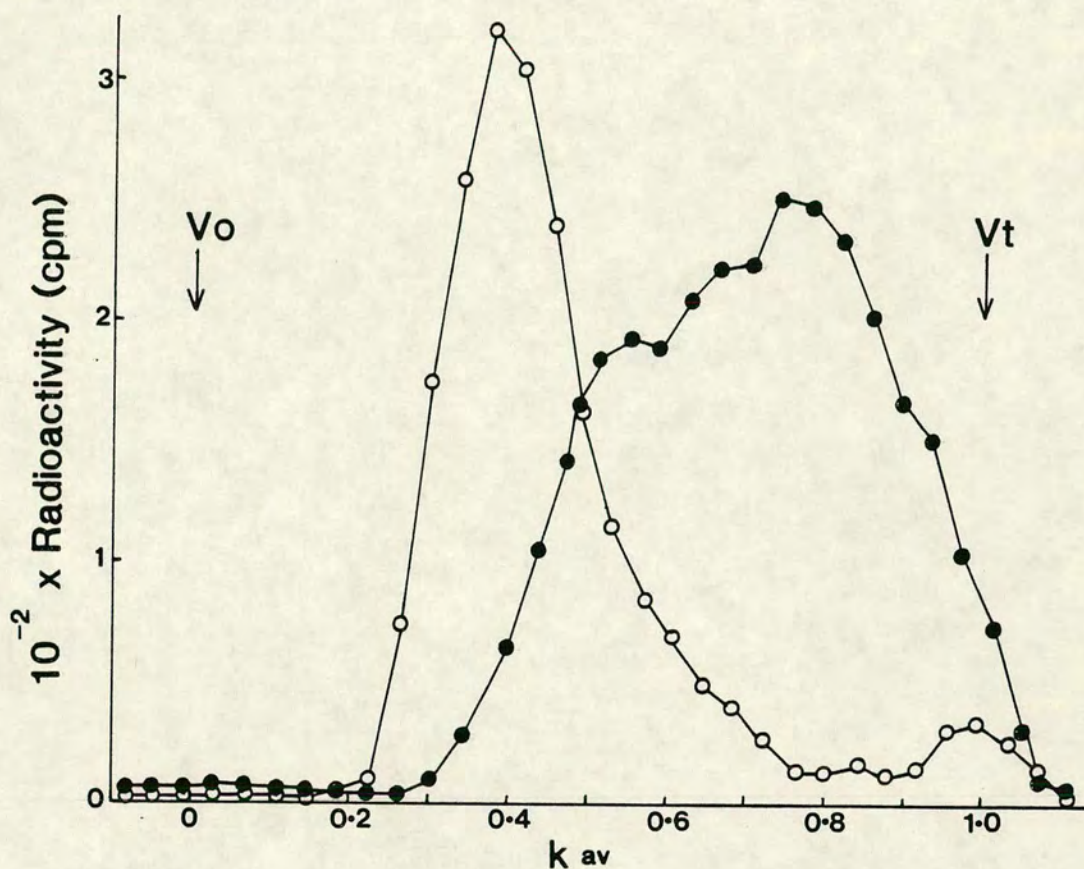


Fig. 10. Gel permeation chromatography (on Sepharose CL-6B) of monomer extensin, before and after acidified chlorite treatment. The column of Sepharose CL-6B (1.5 cm x 49 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). \circ , $[U-^{14}C]$ monomer extensin (untreated); \bullet , $[U-^{14}C]$ monomer extensin (treated with 0.4% (w/v) sodium chlorite, acidified to pH 3.4, at 60°C for 5 min.) Radioactivity = cpm/0.1 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

correct will be presented in later chapters.

(2) N-bromosuccinimide

Extensin monomer from Capsicum frutescens f₁ cell suspension cultures, fed with [³H]proline, was analysed by gel permeation chromatography on a Sephacryl S-400 column with an eluent of 1 M-sodium chloride/20 mM-Mes (pH 6.1). A further sample was treated with 5 mM-N-bromosuccinimide in pH 4 acetate buffer for 2 h at about 20°C and the reaction was halted by adjusting the solution to pH 7 with 1 M-sodium hydroxide. This sample was analysed on the same column and the elution profiles (Fig. 11) compared. Treatment with N-bromosuccinimide caused the extensin to elute near the included volume, suggesting that peptide bonds had been cleaved.

There are various reports in the literature that N-bromosuccinimide is a powerful oxidising agent used to cleave peptide bonds, especially tryptophanyl, tyrosyl and histidyl bonds (Ramachandran & Witkop, 1967). Consequently it appeared that N-bromosuccinimide was unlikely to be a suitably specific cleavage reagent for isodityrosine without affecting the peptide backbone of extensin molecules and so it was decided not to experiment further with this chemical.

Cleavage of isodityrosine

The experiment performed by Fry (1982) was repeated to investigate the effects of acidified chlorite upon isodityrosine. Aliquots (10 µl) of 1.7 mM-isodityrosine were treated with 10 µl of 0.3% (w/v) sodium chlorite (pH 3.4) and incubated at 60°C for 0, 0.5, 1, 2, 5 and 15 min; the reaction was halted by addition of 10 µl of

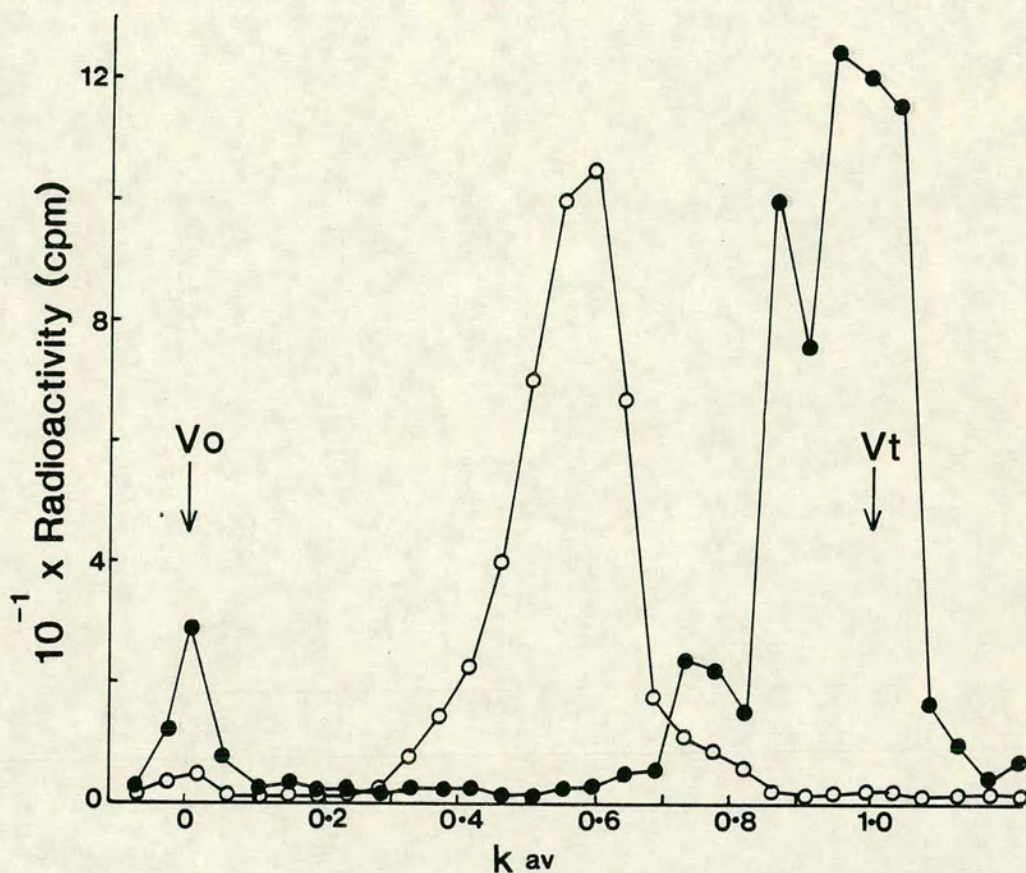
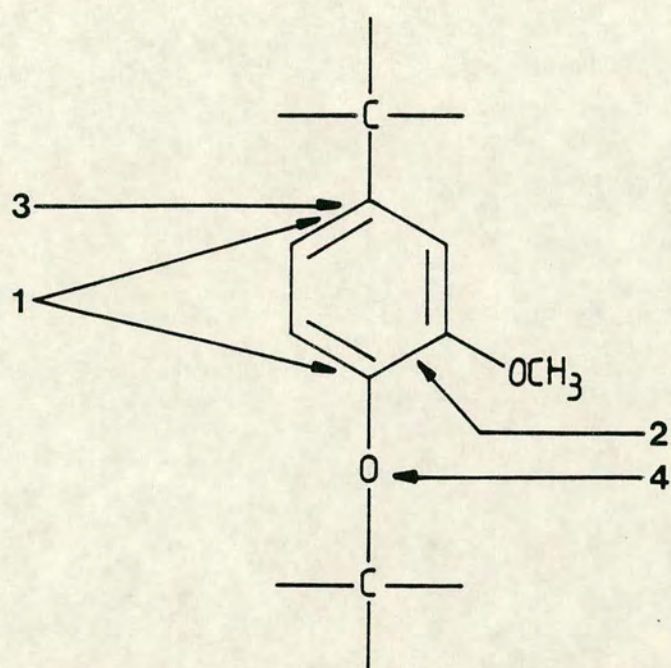


Fig. 11. Gel permeation chromatography (on Sephacryl S-400) of monomer extensin, before and after treatment with N-bromosuccinimide. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). \circ , [hydroxypropyl-³H]extensin monomer (untreated); \bullet , [hydroxypropyl-³H]extensin monomer (treated with 5 mM-N-bromosuccinimide in pH 4 acetate buffer, at 20°C for 2 h.) Radioactivity = cpm/0.3 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

1.8 M-ammonia solution. The samples were dried under vacuum, re-dissolved in 10 μ l of ammonia solution and the products separated by thin layer chromatography as described in the Methods. The plates were stained with ninhydrin. The control lane (0 min treatment) showed a spot of isodityrosine but in all lanes where acidified chlorite treatment had been given, amino acid breakdown products were visible. One product co-chromatographed with tyrosine suggesting that isodityrosine could be cleaved to tyrosine by acidified chlorite. The amount of isodityrosine breakdown increased from 0.5 to 5 min treatment and by 15 min no isodityrosine remained. The results of such an experiment can be seen in Fig. 19, where the % isodityrosine remaining in each sample has been quantified by the Quickscan densitometer.

Acidified chlorite is commonly used in the paper industry as a bleaching agent to solubilise lignin phenolic components in wood and various mechanisms have been postulated for the action of sodium chlorite upon lignin (Wise et al., 1946; Sarkanen et al., 1962; Yang & Goring, 1978). The active components of an acidified chlorite solution are chlorine dioxide and chlorine, which are able to oxidise both phenolics with free hydroxyl groups and phenolic esters and ethers. Dence et al. (1962) proposed a general mechanism for the possible sites of degradative attack by chlorine dioxide and chlorine on lignin; this is reproduced in Fig. 12a. From this model it is possible to propose reaction mechanisms for the action of acidified chlorite upon isodityrosine (Fig. 12b), either by quinone formation leading to production of serine and a quinone, or by ring opening with cleavage to form tyrosine and a muconic acid derivative. Detection of tyrosine as a breakdown product suggests that the ring opening

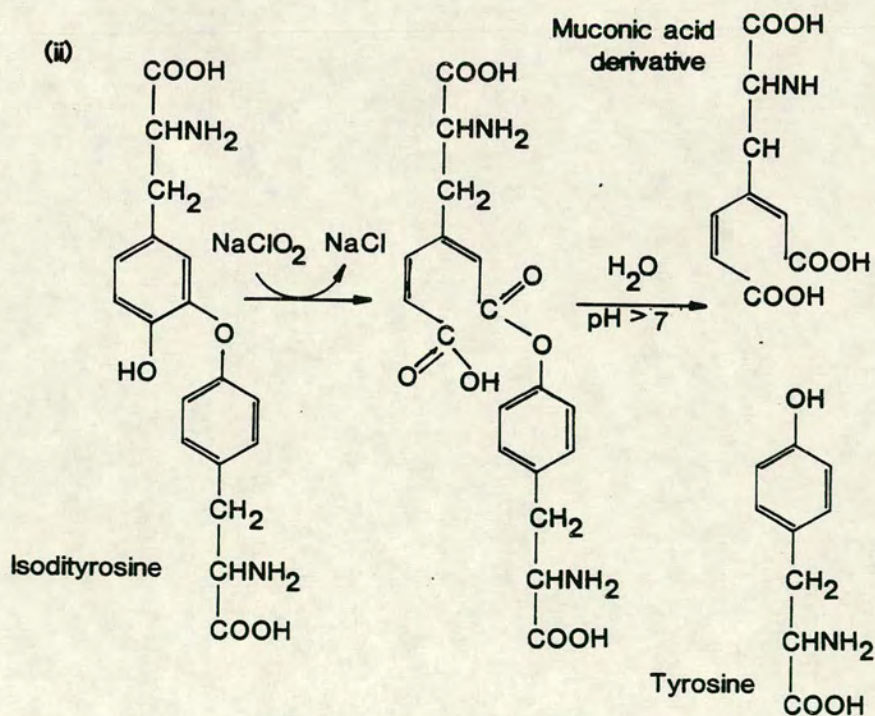
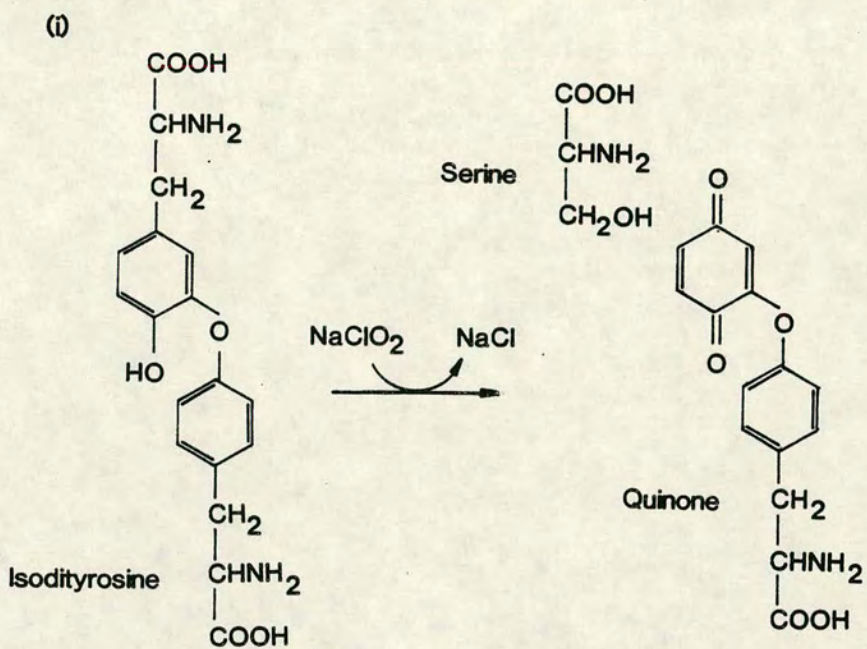


1 = Quinone formation 2 = Ring opening

3 = Electrophilic displacement 4 = Dealkylation

Fig. 12a. Possible sites of degradative attack by chlorine dioxide and chlorine on lignin; mechanisms proposed by Dence, Gupta & Sarkanen (1962).

Fig. 12b. Proposed reaction mechanisms for the action of acidified chlorite upon isodityrosine. Mechanism (i) is via quinone formation leading to the production of serine and a quinone; (ii) is via ring opening leading to the generation of tyrosine and a muconic acid derivative.



reaction does occur but this does not preclude the quinone formation reaction from also occurring.

In another experiment investigating the action of acidified chlorite upon isodityrosine, serine was used as an external marker but ninhydrin staining of the TLC plate did not reveal any spots of serine in the sample lanes. The action of acidified chlorite upon both serine and tyrosine was investigated by substituting each amino acid in the place of isodityrosine in the above experiment. The developed TLC plates showed that acidified chlorite had no apparent effects upon serine, even after 15 min treatment, whilst the tyrosine had been degraded after 15 min treatment. These results support the suggestion that isodityrosine breakdown occurs by the ring opening mechanism.

To investigate more comprehensively the breakdown products resulting from acidified chlorite treatment of isodityrosine, [U-¹⁴C] isodityrosine was treated with 0.3% (w/v) sodium chlorite (pH 3.4) at 60°C for 0, 0.5, 1, 2, 5, 7.5, 10 and 15 min. Reactions were halted with 1.8 M-ammonia solution and each sample was developed by two dimensional TLC as outlined in the Methods. The plates were autoradiographed for 4 weeks. Development of the autoradiographs showed, in the control sample (0 min treatment with sodium chlorite), that the radioactive isodityrosine was impure. From the position of non-radioactive, marker isodityrosine on the TLC plate it was estimated that a barely detectable amount of [U-¹⁴C]isodityrosine was present and consequently, no breakdown products were detected. Further purification and concentration of the [U-¹⁴C]isodityrosine preparation would be necessary before the experiment could be repeated.

Purity of sodium chlorite

The sodium chlorite supplied by BDH Chemicals Ltd. is of technical grade and is claimed to be 80% pure. BDH Chemicals Ltd. list the impurities as sodium chloride (12%), sodium carbonate (2-3%) and sodium chlorate but it is possible that other trace impurities may also be present which could be responsible for cleaving the peptide bonds of extensin monomer. Sodium chlorite was analysed by ion chromatography as described in the Methods to see what impurities were present. Sodium chlorite purified by recrystallisation was also analysed and compared to the crude chemical; the results are shown in Fig. 13. The number of peaks detected in the crude chlorite varied between 3 and 5; a small peak of sodium carbonate, eluting at 2.00 min, was sometimes present as was a small peak of an unidentified component eluting at 11.00 min. Sodium chloride, eluting at 4.30 min, and a peak eluting at 14.60 min, tentatively identified as sodium chlorate, were always present as contaminants. Analysis of the recrystallised sodium chlorite gave a single peak eluting at 5.00 min; in the crude reagent the major peak (assumed to be sodium chlorite) eluted at 3.50 min suggesting that recrystallisation did not yield sodium chlorite. However, as shown below, the recrystallised chemical reacted in the same way as technical grade sodium chlorite.

Recrystallised sodium chlorite was used to cleave isodityrosine in a repeat of the experiment described earlier. Comparison of the results with those obtained from crude sodium chlorite showed no difference in the ability to cleave isodityrosine to tyrosine and to degrade further the tyrosine.

The purified chemical was used to treat monomer extensin and the results of this treatment also showed no difference from treatment

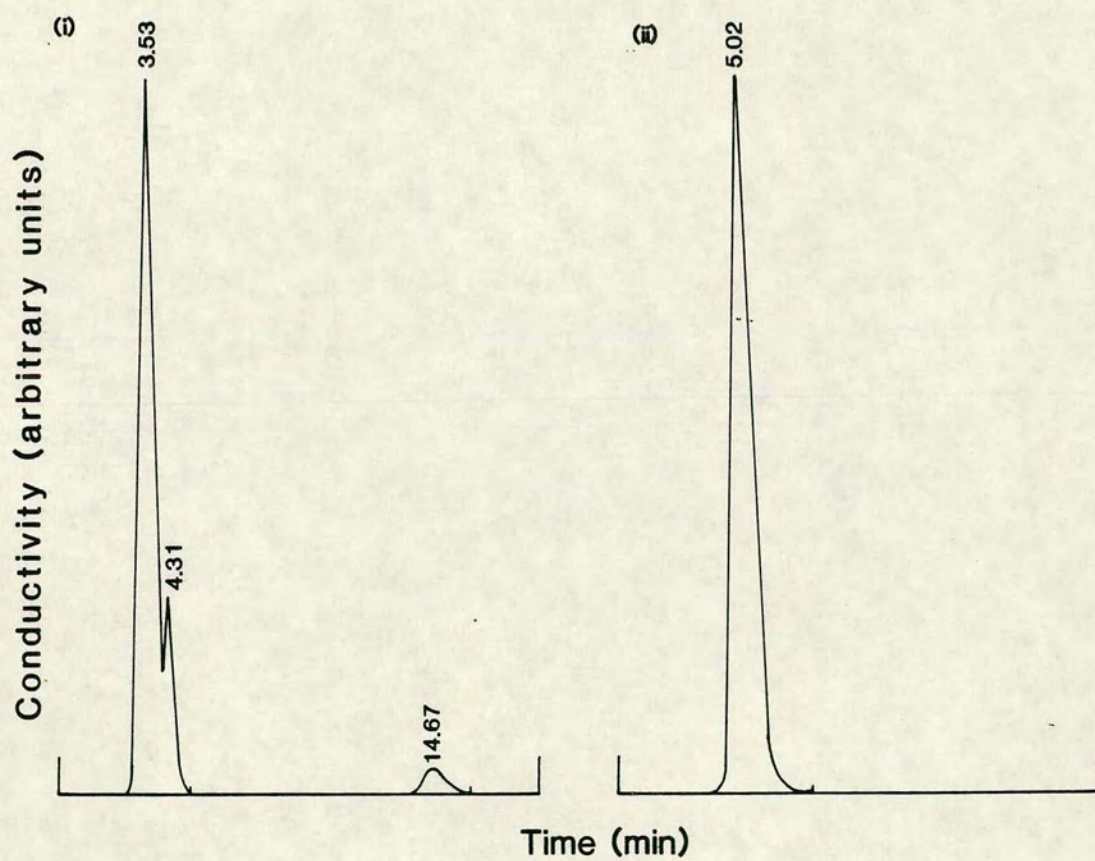


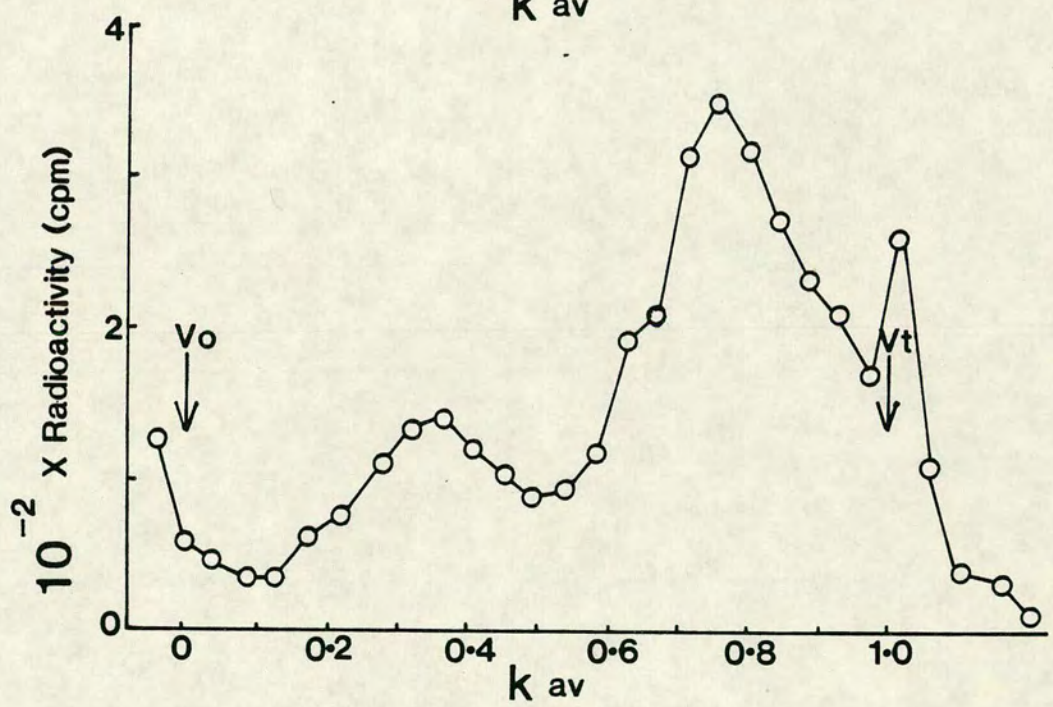
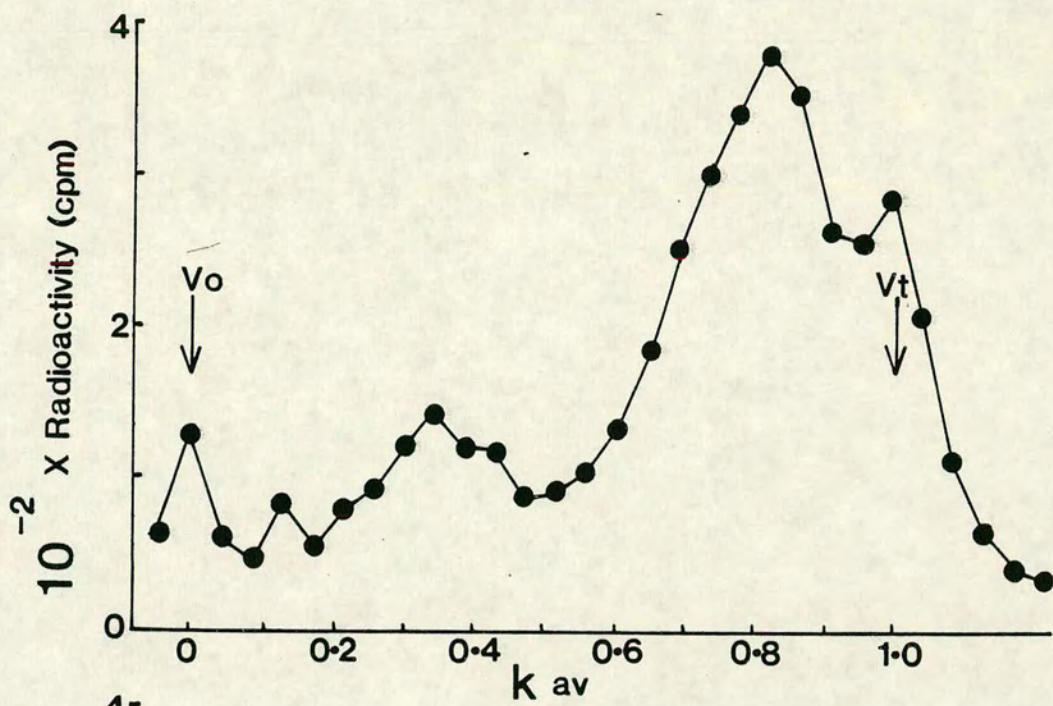
Fig. 13. Ion chromatography of sodium chlorite, (i) unpurified and (ii) purified by recrystallisation. Retention times: sodium chlorite = 3.53 min, sodium chloride = 4.31 min, sodium chlorate (?) = 14.67 min, purified sodium chlorite = 5.02 min.

with crude sodium chlorite (Fig. 14). Because both crude and purified sodium chlorite had the same effects upon isodityrosine and monomer extensin, it was concluded that the effects were probably due to sodium chlorite and not to any impurities present. In future experiments technical grade sodium chlorite was used.

Conclusion

Of the two reagents investigated, it was decided that a solution of acidified sodium chlorite has the greater potential to be a suitably specific cleavage reagent for isodityrosine cross links. It has been shown to cleave free isodityrosine to tyrosine, possibly by the reaction mechanism postulated in Fig. 11b. Its effects upon peptide bonds are relatively unknown, although treatment of monomer extensin showed that some bonds are cleaved and this is considered further in the next chapter. Acidified sodium chlorite can solubilise glycoprotein from cell walls and although it has been suggested that this occurs by cleavage of phenolic cross links (O'Neill & Selvendran, 1980) this remains to be demonstrated.

Fig. 14. Gel permeation chromatography (on Sephacryl S-400) of monomer extensin, treated with purified and unpurified sodium chlorite. The column of Sephacryl S-400 (1.5 cm x 49 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). ●—● [hydroxyprolyl]-³H]extensin monomer treated with 0.15% (w/v) unpurified sodium chlorite, acidified to pH 3.4, at 60°C for 5 min. ○—○, [hydroxyprolyl]-³H]extensin monomer treated as above with purified sodium chlorite. Radioactivity = cpm/0.3 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.



Investigation into conditions for specific cleavage of isodityrosine

Introduction

In the last chapter it was concluded that acidified sodium chlorite is able to cleave peptide bonds; this is contrary to the conclusion reached by O'Neill & Selvendran (1980). They investigated the effects of acidified chlorite upon lysozyme by treating a 1% (w/v) lysozyme solution with 0.3% (w/v) sodium chlorite and 0.12% (v/v) acetic acid at 70°C for 30 min. Recovery of 95% of the material after 24 h dialysis suggested that it had not been cleaved into small fragments. Gel permeation chromatography of untreated and treated samples on Sephadex G-100 showed a slight decrease in M_r of the treated lysozyme, and an increase in peak width which they attributed to a possible change in molecular conformation by modification of functional groups, such as conversion of lysine residues to α -aminoadipic acid residues. Although they did not fully exclude the possibility of peptidyl cleavage O'Neill & Selvendran concluded that peptide bond breakage probably did not occur. However, closer examination of their gel permeation chromatography data showed that 2.5-3.0 x the column bed volume of eluent was required to elute lysozyme, whilst tyrosine, used as a low M_r marker, eluted at 4.5 x the bed volume (see Fig. 1 of O'Neill & Selvendran [1980]). This suggests that both molecules were interacting with the gel matrix, and thus makes any interpretation about changes in molecular size invalid.

In view of this doubt, it was decided to investigate the effects of acidified chlorite treatment upon another protein, bovine serum albumin (B.S.A.), in order to assess the extent of peptide bond cleavage. An attempt was also made to identify conditions under

which peptidyl cleavage could be avoided without inhibition of isodityrosine cross link cleavage.

Acidified chlorite treatment of B.S.A.

A sample of 1% (w/v) B.S.A. dissolved in 0.1 M-sodium phosphate, adjusted to pH 7, was analysed by the gel permeation HPLC system, as described in the Methods. Peaks were monitored with the refractive index (R.I.) detector which measures the bulk property of a solute, rather than with the uv detector which measures the light absorption at 280 nm by aromatic amino acid residues including tyrosine. The R.I. detector was used because protein treated with acidified chlorite may not always be detected by the uv monitor, as a result of degradation of tyrosine residues by acidified chlorite action. The elution profile (Fig. 15a) showed that untreated B.S.A. eluted as a sharp peak at 16.3 min, with some minor contaminants present at 15.3 and 23.8 min.

A solution of blue dextran and sucrose, each at 1% (w/v), was used to determine the void and included volumes of the column; blue dextran eluted at 14.2 min (V_0) and sucrose eluted at 23-24 min (V_t).

A sample of 1% (w/v) B.S.A. was treated with 0.1% (w/v) sodium chlorite (pH 3.4) at 70°C for 15 min and the reaction halted by addition of 1.8 M-ammonia solution to give pH 7-8. The products were then analysed by the HPLC system and the elution profile is shown in Fig. 15b. Comparing this trace with that of the untreated B.S.A. (Fig. 15a) there were no detectable differences in the B.S.A. peak at 16.3 min. However, it was not possible to quantify a limit of detection for peptidyl cleavage because the area of the B.S.A. peak in

Fig. 15. Gel permeation HPLC chromatograms of B.S.A. treated with acidified chlorite. The Zorbax Bioseries Column-GF 250 (9.4 mm i.d. x 25 cm) was eluted with 0.1 M-sodium phosphate [NaH_2PO_4] (pH 7). Sensitivity of the R.I. detector was the same for all traces.

(a) 1% (w/v) B.S.A.

(b) 1% (w/v) B.S.A. treated with 0.1% (w/v) sodium chlorite (pH 3.4) at 70°C for 15 min.

(c) 1% (w/v) B.S.A. treated with 1.0% (w/v) sodium chlorite (pH 3.4) at 70°C for 15 min.

V_0 , elution volume of Blue Dextran = 14.2 min; V_t , elution volume of sucrose = 23-24 min; B.S.A. = 16.3 min.

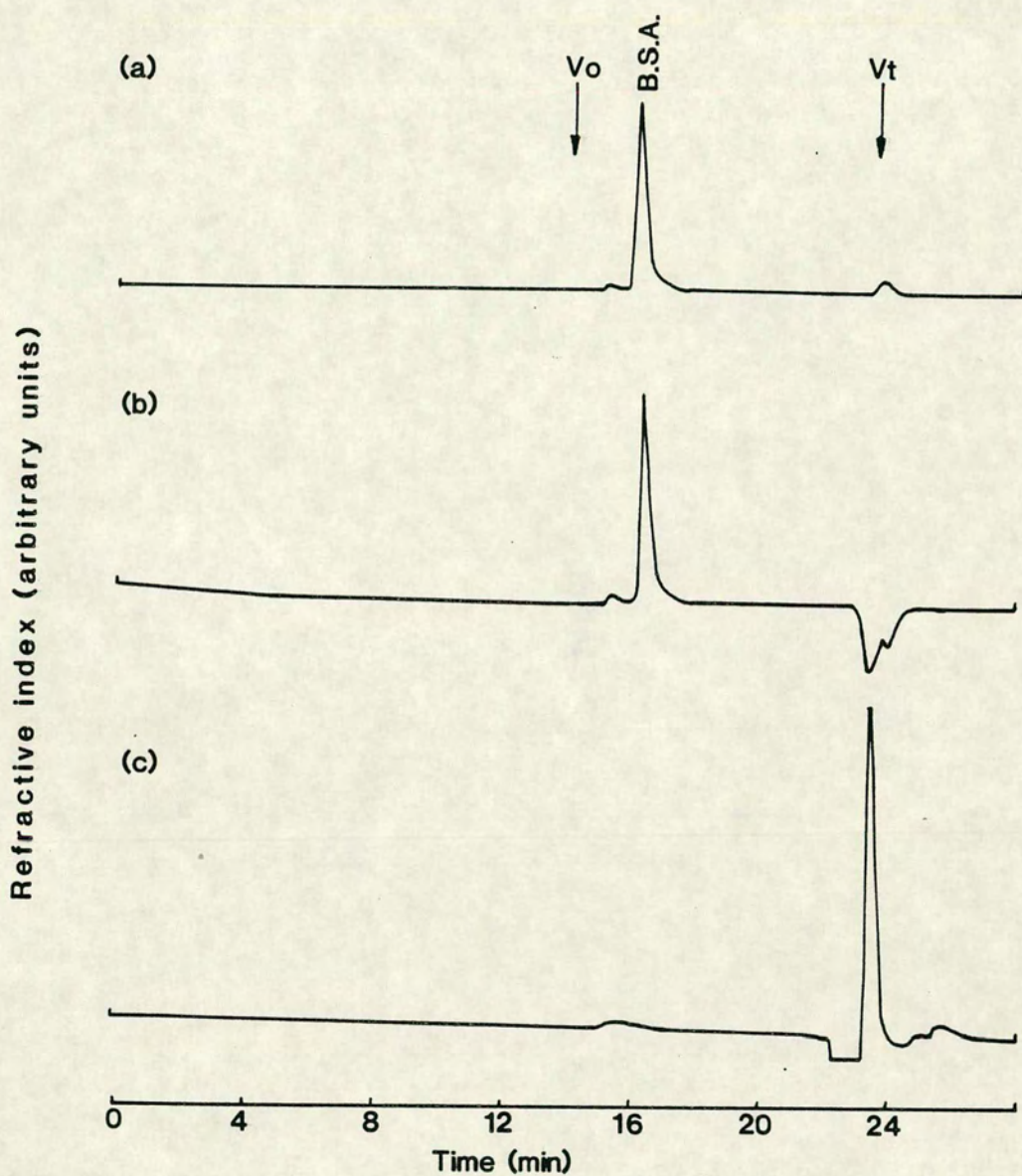


Fig. 15b was slightly greater than that in Fig. 15a; this discrepancy is probably due to variation produced by the HPLC system. The sodium chlorite, acetic acid and ammonia eluted as a trough at 23-25 min (V_t). These profiles suggested that acidified chlorite treatment did not cleave peptide bonds in B.S.A. under these conditions of a high protein:sodium chlorite ratio. In the earlier experiments radioactive monomer extensin had been treated with an excess of sodium chlorite; therefore an investigation was conducted to see whether the ratio of B.S.A. to acidified chlorite was important in predicting peptidyl cleavage.

A sample of 0.1% (w/v) B.S.A. was treated with 1% (w/v) sodium chlorite (pH 3.4) at 70°C for 15 min and the reaction halted with 1.8 M-ammonia solution to give a neutral solution. Analysis by HPLC gel permeation chromatography (Fig. 15c) showed that the peak at 16.3 min had disappeared and several peaks of smaller M_r had been produced; this suggested that the excess of acidified chlorite had caused peptidyl cleavage. It was therefore concluded that the ratio of B.S.A.:acidified chlorite was important and so a range of experiments was carried out in order to identify suitable conditions to avoid peptidyl cleavage. The ratio of B.S.A.:sodium chlorite, the reaction temperature and reaction time were all varied; a summary of treatments is given in Table 6. Solutions of B.S.A. and acidified sodium chlorite were made as % (w/v) solutions for experimental purposes but in Table 6 they have been converted to molarity values; for B.S.A. this was calculated in terms of amino acyl residues of approx. $M_r = 100$. The molar ratio of B.S.A.:sodium chlorite could then be estimated and this value allows a better comparison of the number of amino acyl residues available to the number of sodium

Table 6. Investigation into peptidyl cleavage of B.S.A. during acidified chlorite treatment.

B.S.A. (M)	NaClO ₂ (M)	Molar ratio B.S.A.:NaClO ₂	Temperature °C	Treatment time (min)	Cleavage
0.010	0.11	0.09	60	5	+
0.025	0.11	0.23	60	5	+
0.050	0.11	0.45	60	15	+
0.075	0.11	0.68	60	15	-
0.100	0.11	0.91	60	15	-
0.100	0.011	9.09	60	15	-
0.010	0.11	0.09	70	5	+
0.025	0.11	0.23	70	5	+
0.050	0.11	0.45	70	5	+
0.075	0.11	0.68	70	15	+
0.100	0.11	0.91	70	15	-
0.100	0.016	6.09	70	15	-

Molarity of B.S.A. was calculated in terms of amino acyl residues (approx. M_r of an amino acyl residue = 100).

+ = cleavage detectable by gel permeation HPLC.

- = no cleavage detectable.

chlorite molecules present.

From the results summarised in Table 6 it can be seen that variation in ratio of B.S.A.:sodium chlorite does affect the cleavage of peptide bonds; an excess of acidified chlorite over protein can cause peptidyl cleavage after just 5 min treatment whereas, with an excess of protein over acidified chlorite there did not appear to be any peptidyl cleavage, even after 15 min treatment. It was concluded from this series of experiments that where protein (amino acid residues) and sodium chlorite concentrations were equimolar, or where an excess of protein was present, there was no detectable cleavage of peptide bonds but with ratios below 0.75:1 for B.S.A.:sodium chlorite some cleavage was detectable.

Reactions conducted at 60°C generally resulted in less peptidyl cleavage of B.S.A. than those carried out at 70°C. Variation in length of treatment time with acidified chlorite suggested that 5 min treatments were optimal in ensuring that peptide bonds were not cleaved.

Representative elution profiles for samples that were (a) cleaved (b) partially cleaved and (c) not cleaved by acidified chlorite treatment are shown in Fig. 16a, b & c. The breakdown of B.S.A. is apparent from the decrease in height of the peak at 16.2 min in (a) and (b); some lower M_r fragments have been produced but an increase in the V_0 peak (14.3 min) suggests that some protein molecules may have aggregated or undergone a change in conformation. The elution profile shown in Fig. 16c shows additional peaks of low M_r material which is not present in the other two profiles. This is because the higher B.S.A.:sodium chlorite ratio used in this experiment caused the B.S.A. to precipitate during acidified chlorite

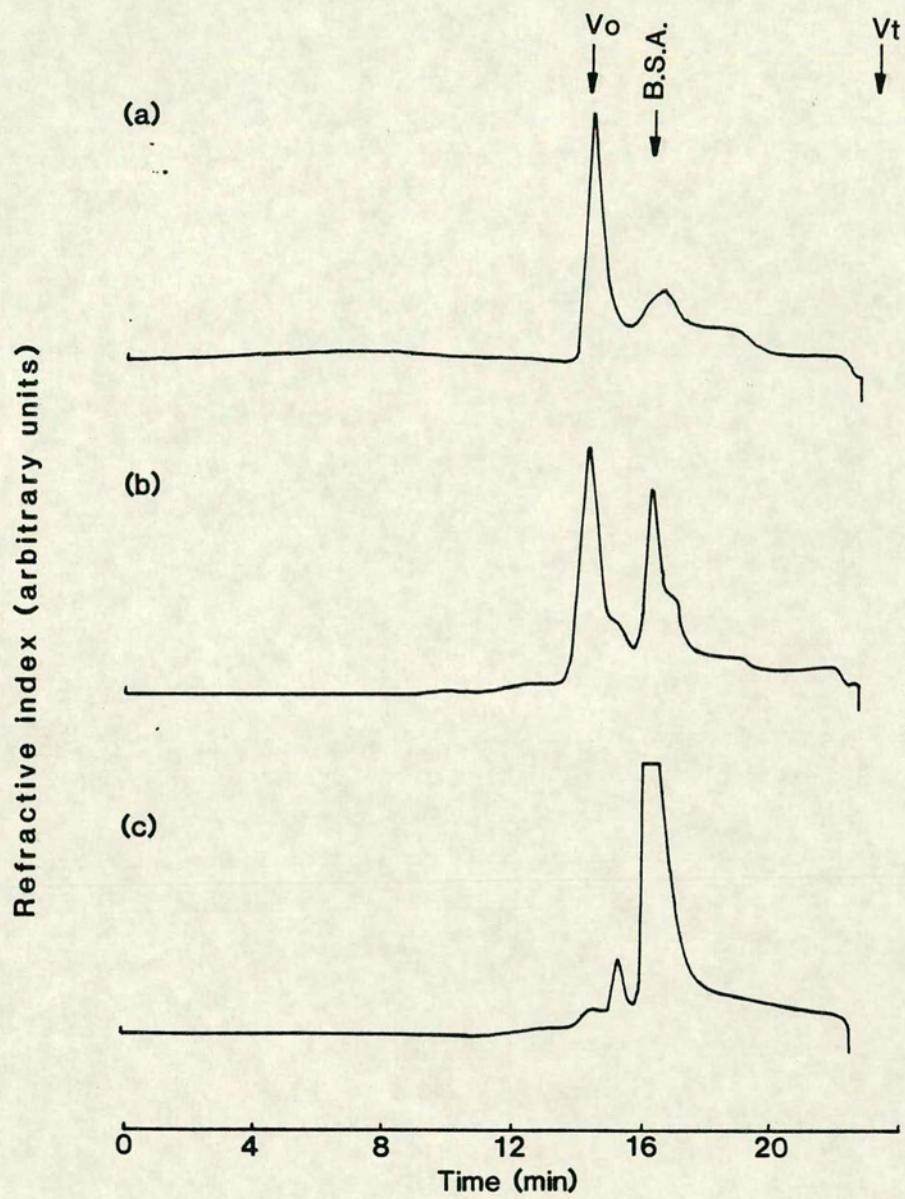
Fig. 16. Gel permeation HPLC chromatograms of B.S.A. treated with acidified chlorite. The Zorbax Bioseries Column-GF 250 (9.4 mm i.d. x 25 cm) was eluted with 0.1 M-sodium phosphate [NaH_2PO_4] (pH 7). Sensitivity of the R.I. detector was the same for all traces.

(a) B.S.A. cleaved: 0.25% (w/v) B.S.A. treated with 1% (w/v) sodium chlorite (pH 3.4) at 60°C for 15 min.

(b) B.S.A. partially cleaved: 0.5% (w/v) B.S.A. treated with 1% (w/v) sodium chlorite (pH 3.4) at 60°C for 15 min.

(c) B.S.A. not cleaved: 0.75% (w/v) B.S.A. treated with 1% (w/v) sodium chlorite (pH 3.4) at 60°C for 15 min.

V_0 , elution volume of Blue Dextran = 14.3 min; V_t , elution volume of sucrose = 23-24 min; B.S.A. = 16.2 min.



treatment and it was found that the protein could be re-dissolved by addition of a drop of 18 M-ammonia solution; thus, a large peak of ammonia was also detected in this sample. The addition of excess ammonia means that it is not possible to directly compare Fig. 16c with Fig. 16a & b, but the elution profile (Fig. 16c) suggests that no breakdown of B.S.A. had occurred.

Acidified chlorite treatment of lysozyme

An attempt was made to extend the investigation of acidified chlorite treatment upon proteins by re-considering the effects upon lysozyme. Lysozyme is a basic protein and, in terms of charge, is therefore more comparable to basic extensin than B.S.A., which is a neutral protein. A sample of 0.25% (w/v) lysozyme was analysed by HPLC gel permeation chromatography with the same buffer as used above. The elution profile (Fig. 17a) showed that lysozyme eluted as a sharp peak at 17.0 min with minor contaminants eluting at 15.8 and 24.0 min.

Lysozyme solutions of varying concentration (Table 7) were treated with acidified chlorite at 60°C for 5 min and reactions halted with 1.8 M-ammonia solution. The products were separated by the HPLC system and, as shown in Fig. 17b, there was no detectable cleavage of peptide bonds, even when an excess of acidified chlorite over lysozyme was present. The experiment was repeated at the higher temperature of 70°C with treatment for 15 min, to see whether peptide bonds were still stable under harsher conditions; however, lysozyme precipitated during treatment at 70°C, presumably because the protein had become denatured. Addition of urea, to give a final concentration of approx. 3 M-urea, re-dissolved the precipitates and the samples were analysed by the HPLC system. A representative trace (Fig. 17c) showed that no

Fig. 17. Gel permeation HPLC chromatograms of lysozyme treated with acidified chlorite. The Zorbax Bioseries Column-GF 250 (9.4 mm i.d. x 25 cm) was eluted with 0.1 M-sodium phosphate [NaH_2PO_4] (pH 7). Sensitivity of the R.I. detector was the same for all traces.

(a) 0.25% (w/v) lysozyme.

(b) 0.5% (w/v) lysozyme treated with an excess of acidified chlorite at 60°C for 5 min.

(c) 1.0% (w/v) lysozyme treated with an excess of acidified chlorite at 70°C for 15 min.

V_0 , elution volume of Blue Dextran = 14.3 min; V_t , elution volume of sucrose = 23-24 min; lysozyme = 17 min.

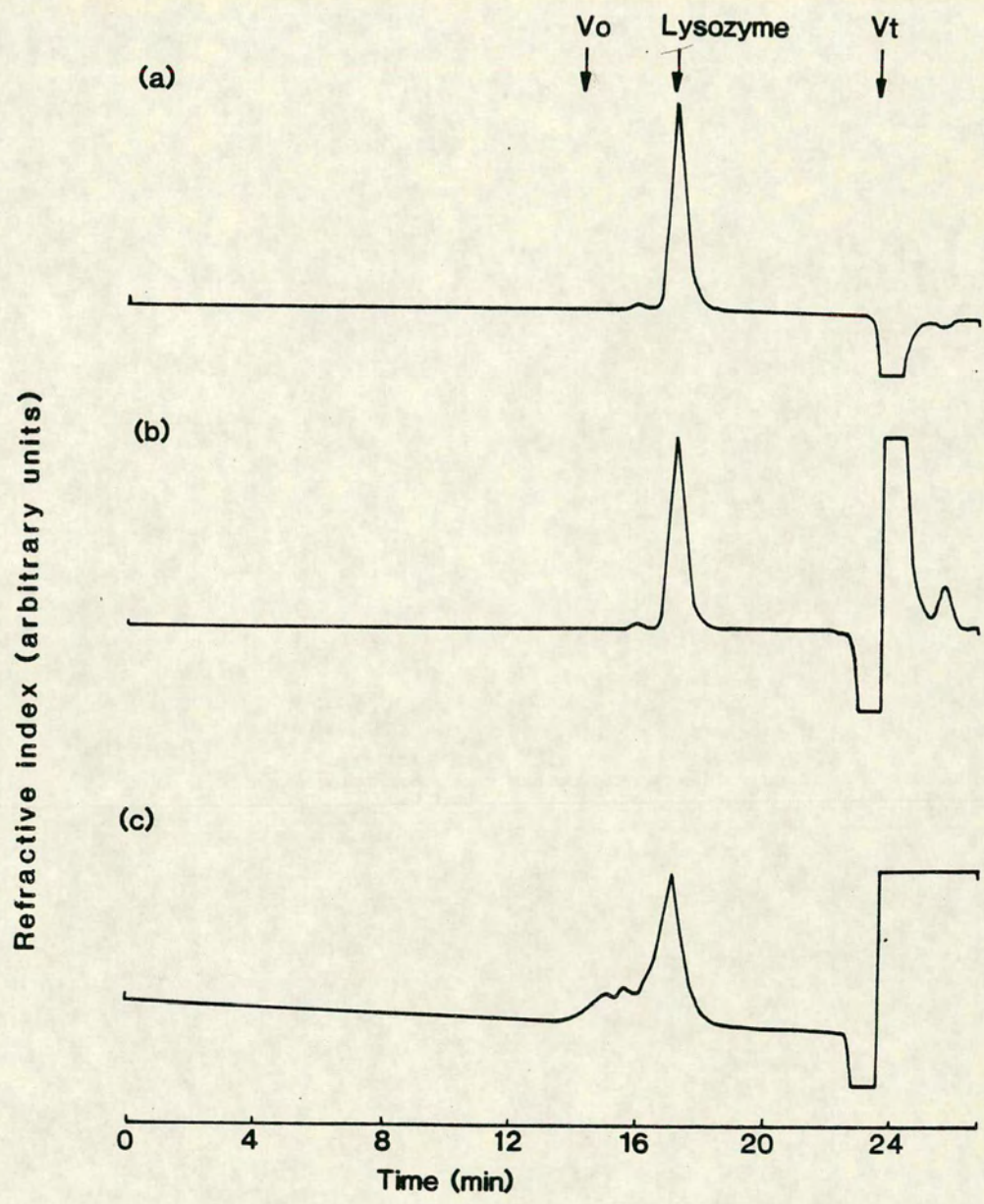


Table 7. Investigation into peptidyl cleavage of lysozyme during acidified chlorite treatment.

Lysozyme (M)	NaClO ₂ (M)	Molar ratio Lysozyme:NaClO ₂	Temperature °C	Treatment time (min)	Cleavage
0.01	0.11	0.09	60	5	-
0.05	0.11	0.45	60	5	-
0.10	0.11	0.91	60	5	-
0.01	0.11	0.09	70	15	ppt.
0.05	0.11	0.45	70	15	ppt.
0.10	0.11	0.91	70	15	ppt.

Molarity of lysozyme was calculated in terms of amino acyl residues (approx. M_r of an amino acyl residue = 100).

+ = cleavage detectable by gel permeation HPLC.

ppt. = lysozyme precipitated in these samples but was re-dissolved by addition of urea (final concentration = 3 M), prior to gel permeation HPLC. No cleavage was detectable but high M_r peaks were detected.

low M_r products were detectable but detection of peaks of higher M_r (15.1 and 15.6 min) suggested that the protein molecules had aggregated. As a consequence of these problems, no further attempts were made to investigate the effects of acidified chlorite upon lysozyme. However, the results were compatible with the conclusion that at a protein:sodium chlorite ratio of 1:1 peptide bond cleavage is not detectable.

Acidified chlorite treatment of monomer extensin in the presence of B.S.A.

As suggested above, monomer extensin may be cleaved by acidified chlorite treatment because of the excess of sodium chlorite molecules over that of amino acid residues. It may be possible to add a non-radioactive protein, such as B.S.A., to samples of extensin monomer to protect extensin backbones against peptidyl cleavage during acidified chlorite treatment. A sample of [$U-^{14}C$]salt-soluble extensin, prepared from spinach cell suspension culture, acted as an untreated control and was analysed on a Sepharose CL-6B column with 1 M-sodium chloride, 20 mM-Mes (pH 6.1) as eluent (further details in Methods); the elution profile is shown in Fig. 18a.

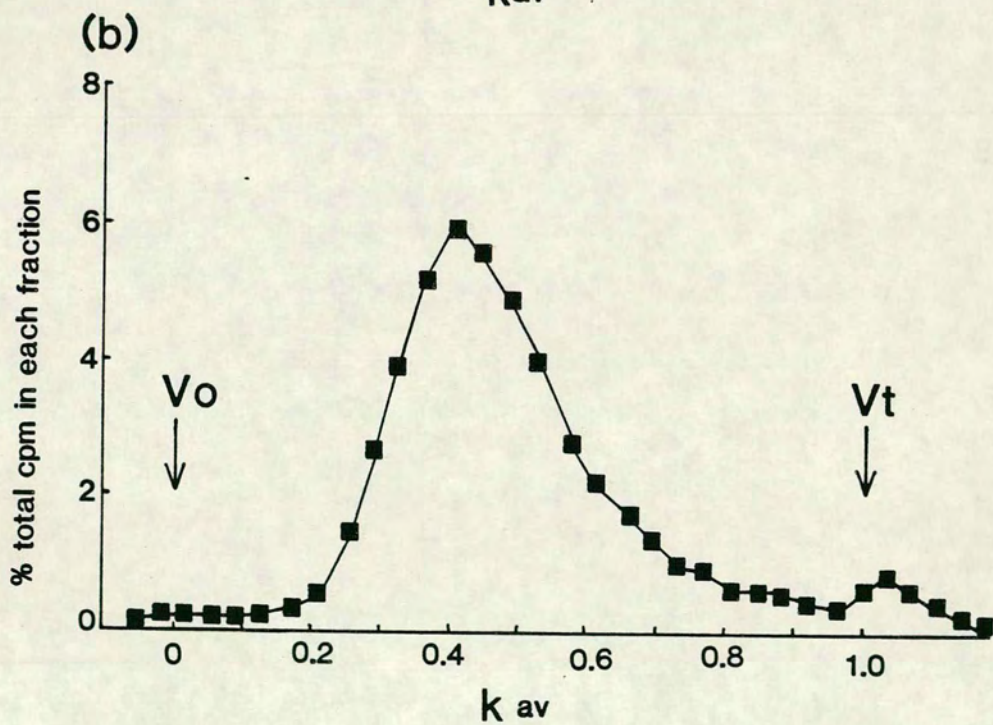
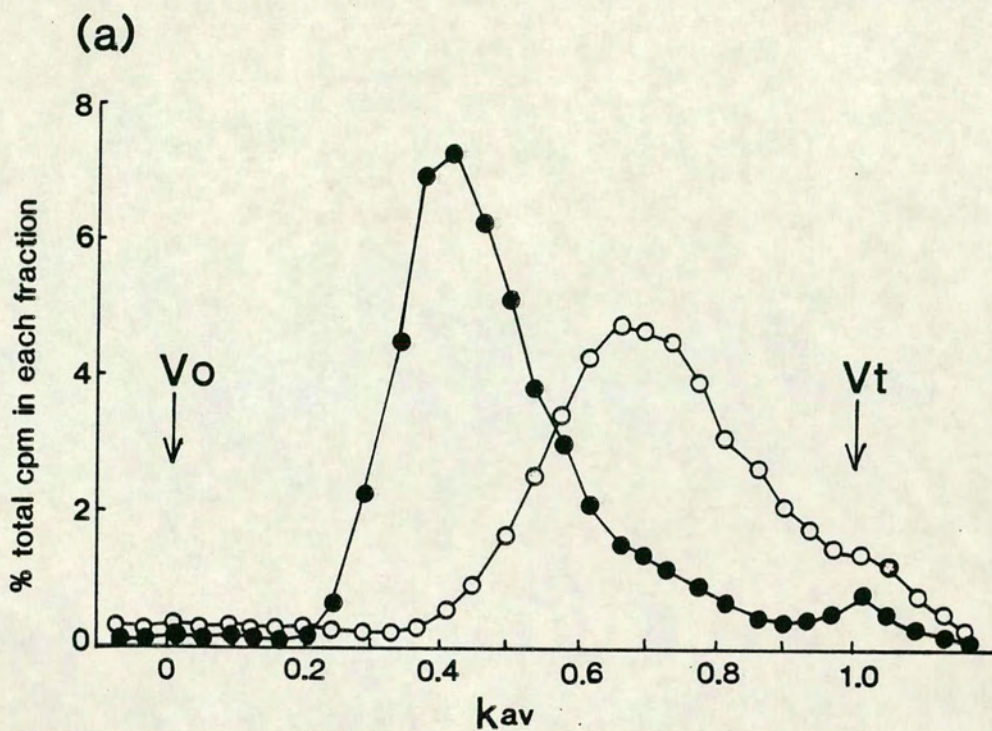
A second sample was treated with sodium chlorite (final concentration = 0.4% (w/v)), adjusted to pH 3.4 with acetic acid, at 60°C for 5 min and the reaction was halted with 1.8 M-ammonia solution. The reaction products were analysed under the same conditions as above and the elution profile (Fig. 18b) shows that acidified chlorite treatment had cleaved the peptide backbone of extensin to give a broad peak of lower apparent M_r products. It should be noted that when extensin is treated with acidified chlorite

Fig. 18. Gel permeation chromatography (on Sepharose CL-6B) of monomer extensin treated with acidified chlorite in the absence and presence of B.S.A.

(a) acidified chlorite treatment of monomer extensin in the absence of B.S.A. ●, [U-¹⁴C]extensin monomer (untreated); ○, [U-¹⁴C]extensin monomer treated with 0.4% (w/v) sodium chlorite, acidified to pH 3.4, at 60°C for 5 min.

(b) acidified chlorite treatment of monomer extensin in the presence of B.S.A. ■, [U-¹⁴C]monomer extensin treated with 0.4% (w/v) sodium chlorite, acidified to pH 3.4, at 60°C for 5 min, in the presence of 0.4% (w/v) B.S.A.

The column of Sepharose CL-6B (1.5 cm x 49 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.



there is no generation of higher M_r products as was found for B.S.A. treatment.

The third sample was given an identical acidified chlorite treatment but in the presence of 0.4% (w/v) B.S.A. Analysis by gel permeation chromatography (Fig. 18c) showed that in this experiment the treated extensin monomer eluted with a profile identical to that of the untreated control. It is concluded that B.S.A. can protect extensin from peptidyl cleavage during acidified chlorite treatment; the simplest explanation for this effect is that the large excess of peptide bonds in the non-radioactive protein, compared to the much lower number of peptide bonds in the [^{14}C]monomer extensin, are more likely to be cleaved by the acidified chlorite. This does not entirely preclude cleavage of any extensin backbone peptide bonds but it makes the probability of such cleavage extremely unlikely.

The fact that B.S.A. protects against extensin peptidyl cleavage means that conditions have been identified whereby acidified sodium chlorite can act as a specific reagent to cleave isodityrosine cross links, provided that the presence of B.S.A. does not inhibit breakdown of isodityrosine by acidified chlorite.

Acidified chlorite treatment of isodityrosine in the presence of B.S.A.

To investigate the effects of the presence of B.S.A. upon breakdown of isodityrosine by acidified chlorite treatment, experiments were performed in which a solution of 1.7 mM-isodityrosine was treated with acidified chlorite solutions of varying concentration in the absence and presence of varying concentrations of B.S.A. The reactions were conducted at 60°C and halted after 0, 0.5, 1, 2, 5 and

15 min by addition of 1.8 M-ammonia solution to give a final pH of approx. 9. The samples were analysed by thin layer chromatography as described in the Methods, the plates stained with ninhydrin and the spots monitored with the Quicksan R&D densitometer. The final molarities and molar ratios of the components in each experiment are summarised in Table 8 and an indication as to whether isodityrosine cleavage had occurred is also given. From this table it can be seen that in the absence of B.S.A. complete cleavage of isodityrosine took place within 5 min and that with a higher sodium chlorite concentration present breakdown was complete within 1 min. Complete cleavage was taken as disappearance of the isodityrosine spot; degradation of tyrosine took longer.

In the presence of B.S.A., isodityrosine was still cleaved to tyrosine by acidified chlorite treatment. With a low concentration of B.S.A. present (i.e. at B.S.A.:sodium chlorite ratios of 0.15 where it had previously been shown that cleavage of peptide bonds does occur) there was little effect upon the rate of isodityrosine breakdown; with a higher sodium chlorite concentration, complete cleavage of isodityrosine had still occurred after 1 min treatment but with a lower sodium chlorite concentration complete cleavage took 15 min. In the presence of equimolar B.S.A.:sodium chlorite (ratio of 0.91), where it had previously been shown that peptidyl cleavage did not occur, there was only partial cleavage of isodityrosine with some remaining after 15 min treatment. This can be seen from Fig. 19, where the % isodityrosine remaining after the milder (0.1%) acidified chlorite treatment at the various time points was calculated from measurements made on the Quicksan densitometer. With equimolar B.S.A.:sodium chlorite about 60% isodityrosine remained after 15 min

Table 8. Acidified chlorite treatment of isodityrosine in the presence of B.S.A.

Idt (mM)	NaClO ₂ (mM)	B.S.A. (mM)	Molar ratio Idt:NaClO ₂	Molar ratio B.S.A.:NaClO ₂	Temp. °C	Cleavage*
1.7	33	-	0.052	-	60	5 min.
1.7	33	5	0.052	0.15	60	15 min.
1.7	33	30	0.052	0.91	60	partial
1.7	110	-	0.016	-	60	1 min.
1.7	110	5	0.016	0.045	60	1 min.
1.7	110	100	0.016	0.91	60	partial

Molarity of B.S.A. was calculated in terms of amino acyl residues (approx. M_r for amino acyl residue = 100).

* For samples where a time is given, this indicates that Idt cleavage was complete by this time. The entry 'partial' indicates that some Idt remained after 15 min treatment.

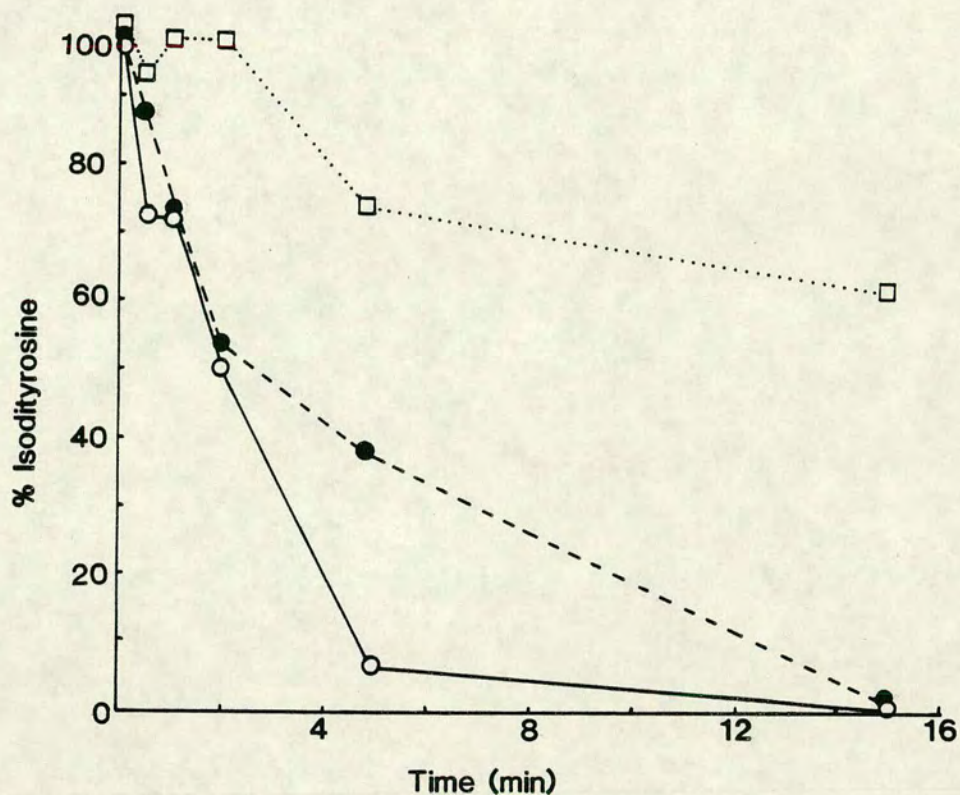


Fig. 19. Acidified chlorite treatment of isodityrosine in the presence of B.S.A. The % of isodityrosine remaining was calculated from measurements made on the Quicksan densitometer. ○, isodityrosine treated with acidified chlorite in the absence of B.S.A.; ●, isodityrosine treated with acidified chlorite at a B.S.A.:sodium chlorite ratio = 0.15:1.00 (mol/mol); □, isodityrosine treated with acidified chlorite at a B.S.A.:sodium chlorite ratio = 0.91:1.00 (mol/mol).

treatment.

Conclusion

It was shown that acidified chlorite treatment can cleave peptide bonds in B.S.A. and that the molar ratio of amino acid residues: sodium chlorite is important in determining whether peptidyl cleavage will occur; at equimolar concentrations of B.S.A.:sodium chlorite peptidyl cleavage was not detected. In this project the mechanism of acidified chlorite action upon peptide bonds was not investigated but it would be interesting to know whether the reagent acts upon certain labile peptide bonds and, if so, which bonds these are.

A non-radioactive protein, such as B.S.A., could be used to prevent the peptide backbone of tracer levels of radioactive extensin molecules from being cleaved during acidified chlorite treatment, whilst allowing cleavage of isodityrosine to occur. It was therefore concluded that conditions do exist whereby the ether bond of isodityrosine can be specifically cleaved by acidified chlorite treatments. A suitable treatment would appear to be in the presence of equimolar B.S.A.:sodium chlorite at a temperature of 60°C for about 5 min, although it should be remembered that these conditions are based upon reactions with free isodityrosine and soluble extensin monomers. Cleavage of isodityrosine cross links in insoluble network extensin may require different cleavage conditions.

Amino acid analysis of extensin

Introduction

Amino acid analysis was carried out on material obtained from spinach suspension culture cell walls by elution with salt in order to confirm that the protein present was a HRGP and had an amino acid profile characteristic of extensin. Salt-soluble extensin precursors have been isolated from various other plants, e.g. tomato cell suspension cultures (Smith et al., 1984; Smith et al., 1986) and carrot root discs (Stuart & Varner, 1980), and so a comparison of the data available from these analyses with data obtained from this investigation could be made. The absence or presence of isodityrosine in monomer extensin was investigated and the results were used to reconsider the interpretations drawn from acidified chlorite treatment of monomer extensin, where it was suggested that ionically bound extensin may already have been cross linked or that the peptide backbone may have been cleaved.

A survey was also made of the amino acid profiles of cell wall hydrolysates from a range of whole plant tissues and from Capsicum frutescens f₁ cell suspension culture in order to monitor the levels of HRGP and to demonstrate the presence of isodityrosine in whole plant tissues.

Purification and amino acid analysis of monomer extensin

Material leached from the cell walls of 4-d old spinach cell suspension cultures with 25 mM-lanthanum chloride and 10 mM-ascorbic acid was used; spinach was chosen because the initial experiments investigating the effects of acidified chlorite treatment upon monomer

extensin used salt-soluble extensin from spinach cultures and so to draw any valid conclusions it was necessary to use the same material. The leached material was purified by cation-exchange chromatography on SP-50 Sephadex with a stepwise pH gradient at constant ionic strength, as outlined in the Methods. A small sample of [arabinosyl-³H]extensin from spinach was added to the non-radioactive preparation in order to determine binding to, and recovery from, the ion-exchange column.

It was calculated that 98% of the material had bound to the SP-50 Sephadex column and 59% of the material was recovered as a single, sharp peak eluting at pH 9.0-9.5 (Fig. 20). Fractions containing the peak of material were pooled and analysed by gel permeation chromatography on Sepharose CL-6B with 1 M-sodium chloride as eluent. Fig. 21 shows that 1-2 peaks were present and appropriate fractions were pooled; these were dialysed, dried under vacuum and taken for amino acid analysis.

Amino acid profiles are shown in Fig. 22; the elution profile for an amino acid standard mixture (Fig. 22a), containing known amounts of each amino acid, allowed a response factor to be calculated by the integrator for each amino acid and these values were then used to estimate the amount of each amino acid in the sample by reference to taurine added as an internal standard. Taurine was added to the samples after acid hydrolysis and so it was not possible to make corrections for any losses during hydrolysis. Figs. 22b & 22c show the profiles for the spinach monomer extensin peaks 1 and 2, and Table 9 shows the amino acid composition of each peak as the mol % of each amino acid. It can be seen that both samples are rich in hydroxyproline, although peak 2 contained less hydroxyproline than peak 1. It is debatable as to whether peak 2 is a separate peak or

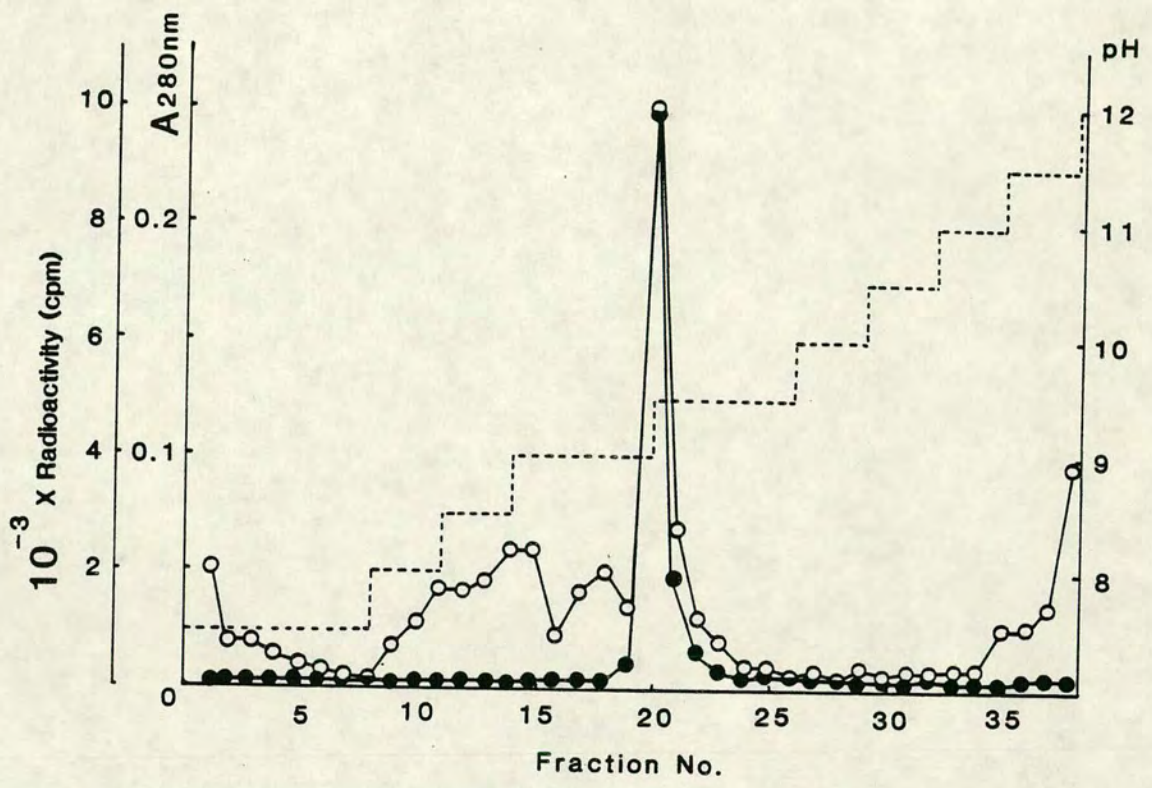


Fig. 20. Purification of monomer extensin by cation-exchange chromatography on SP-50 Sephadex with a stepwise pH gradient at constant ionic strength. For experimental details see Methods (ion-exchange chromatography). A small sample of [arabinosyl]-³H]extensin monomer was added to the preparation to determine binding to, and recovery from, the column. ----, pH gradient; ●—●, radioactivity (cpm/0.25 ml) ○—○, A₂₈₀ nm. Fraction volume = 2.5 ml. pH values represent those applied to the top of the column while the fraction in question was being loaded.

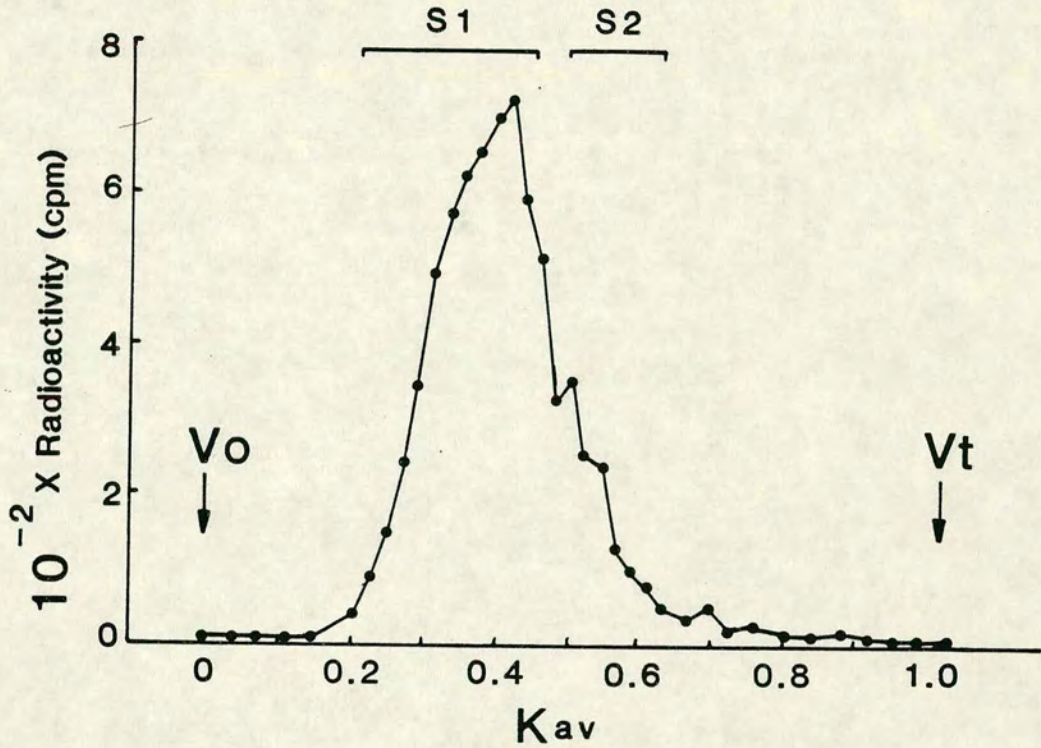
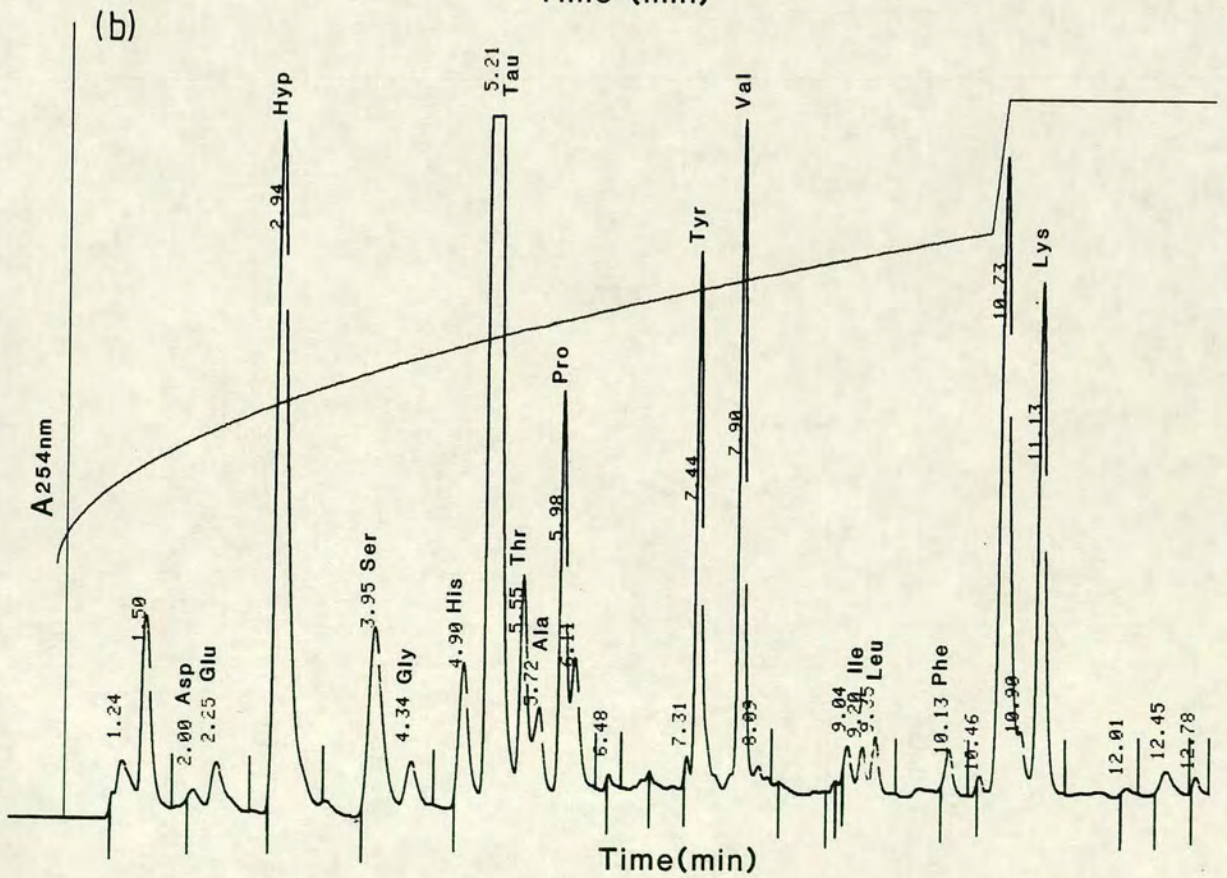
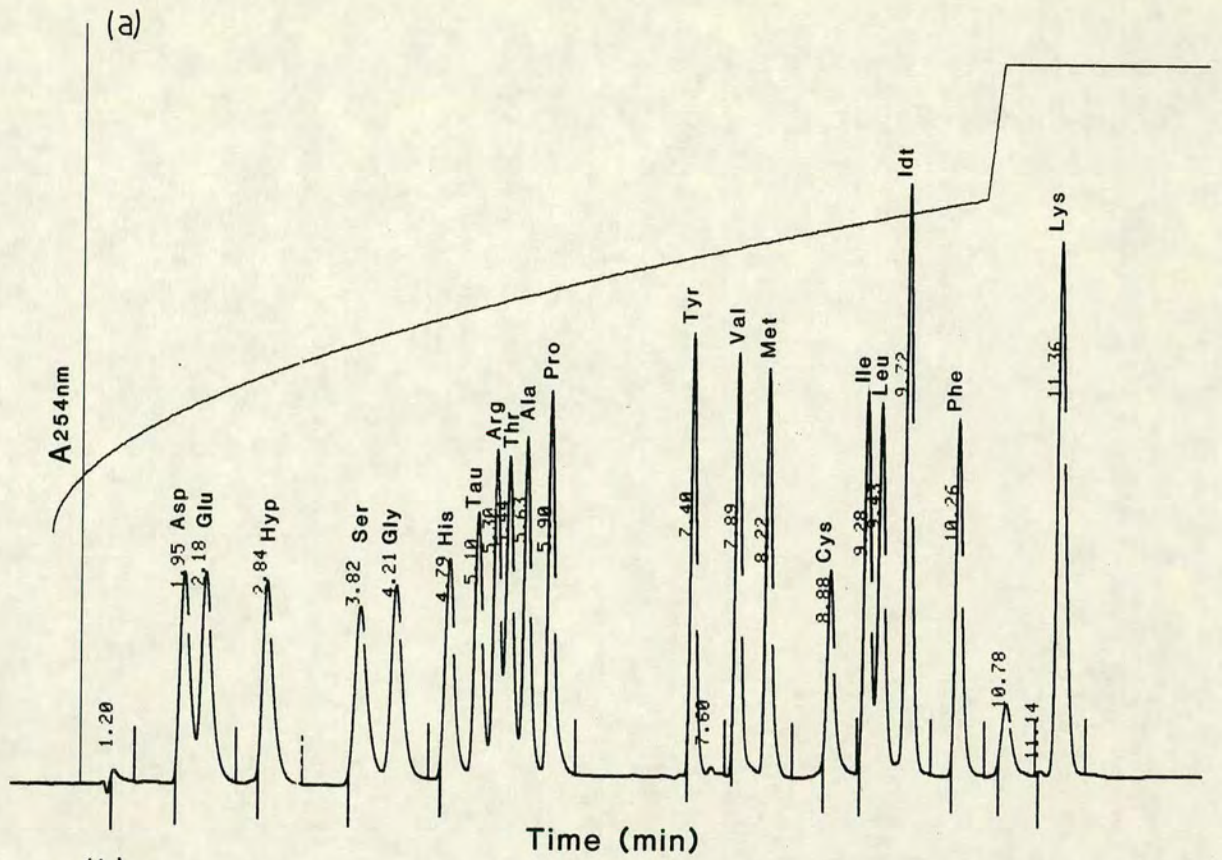


Fig. 21. Gel permeation chromatography of purified monomer extensin on Sepharose CL-6B. The column of Sepharose CL-6B (1.5 cm x 49 cm) was eluted with 1 M -sodium chloride.

•, [arabinosyl-³H]extensin. Radioactivity = cpm/0.1 ml.

(S1), (S2) = fractions pooled for further analysis.

V₀, elution volume of Blue Dextran; V_t, elution volume of sucrose.



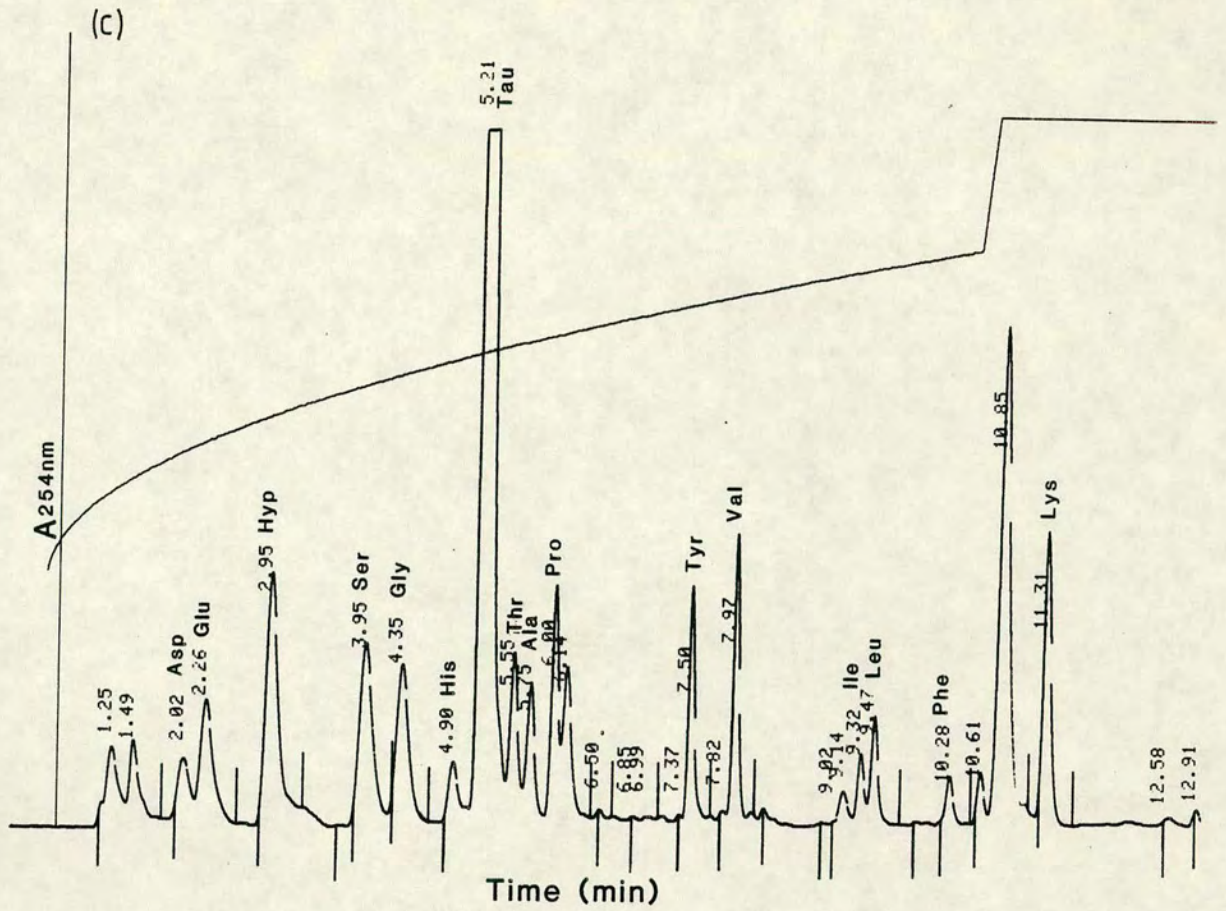


Fig. 22. Amino acid profiles of (a) an amino acid standard mixture, (b) hydrolysed spinach monomer extensin peak S1 and (c) hydrolysed spinach monomer extensin peak S2.

Tau = internal standard.

merely the tail of peak 1, although the amino acid compositions would suggest that it is a separate peak. Comparing peak 1 to the amino acid composition data from other sources (Table 9) it can be seen that the profile is close to that of P1 precursor from tomato suspension culture (Smith et al., 1986). As well as being rich in hydroxyproline the material contained significant amounts of serine, tyrosine, valine, histidine and lysine, all of which are characteristic of extensin. It was therefore concluded that the material leached from the cell walls of spinach suspension culture was soluble, monomer extensin. Recently gene sequencing techniques have allowed the amino acid sequence of various extensins to be predicted (Chen & Varner, 1985 a,b; Tierney et al., 1988) and this work suggests that extensins are a family of molecules, with fundamental similarities, but some differences, in the abundance of various amino acids; this has been more fully discussed in the main introduction.

No isodityrosine was detected in spinach monomer extensin (Fig. 22b) and it was calculated that, if present, there were less than 0.06 mol isodityrosine/ 100 mol amino residues. Assuming $M_r = 36000$ for the polypeptide backbone of extensin (Stuart & Varner, 1980) it was estimated that there would be less than 0.4 half-isodityrosine residues per extensin molecule which would clearly be insufficient to cross link soluble spinach extensin. Consequently, it was concluded that acidified chlorite treatment of monomer extensin resulted in peptide bond cleavage and not cleavage of cross links, as previously suggested. The only report of isodityrosine presence in salt extracted extensin to-date is made by Stafstrom & Staehelin (1986a) who show by amino acid analysis that isodityrosine is present in extensin-1 from carrot root discs. They show that the isodityrosine

Table 9. Amino acid composition of salt-soluble extensin precursors.

Amino acids are expressed as mol %.

Amino acid	Spinach culture		Tomato culture*		Carrot discs**
	S1	S2	P1	P2	
Asp	0.73	4.44	1.80	0.70	0.35
Glu	2.40	10.07	1.90	0.30	0.42
Hyp	31.80	17.27	33.50	41.80	50.78
Ser	7.22	9.11	9.50	12.10	12.76
Gly	1.09	5.64	1.60	0.30	0.69
His	6.27	4.44	7.10	1.00	11.39
Arg	-	-	0.70	0.10	0.16
Thr	5.18	6.59	7.20	1.00	1.09
Ala	1.97	3.84	2.00	0.50	0.58
Pro	6.78	6.35	8.30	0.80	1.13
Tyr	14.51	9.47	8.90	14.90	10.08
Val	11.16	7.43	5.00	5.10	2.96
Met	-	-	-	-	-
Cys	-	-	-	-	-
Ile	0.80	2.40	0.90	0.90	0.26
Leu	0.95	3.72	0.80	0.20	0.34
Idt	-	-	-	-	-
Phe	1.31	2.40	0.60	0.20	-
Lys	7.80	6.83	10.10	20.10	6.88

* From Smith et al. (1984). ** From Stuart & Varner (1980).

disappeared in samples treated with acidified sodium chlorite.

Amino acid analysis of cell wall extensin

Cell walls were isolated from a variety of whole plant organs (onion bulb, lettuce leaves, celery stalks and Capsicum fruit) and from the cells of Capsicum frutescens f₁ suspension culture as outlined in the Methods. Hydrolysates of the isolated walls were taken for amino acid analysis in order to look for the presence of isodityrosine and to compare it with the hydroxyproline content. The amino acid compositions (Table 10) show that the cell walls contained low levels of hydroxyproline; the greatest amount was present in the cell suspension culture and this result is in agreement with the general finding that plant cell suspension cultures contain elevated levels of HRGP. All of the values for mol % hydroxyproline in whole plant walls were lower than that of 12 mol % hydroxyproline found by O'Neill & Selvendran (1980) for depectinated bean parenchyma cell walls; this difference may be due to variations in hydroxyproline content between plant and tissue types.

Table 10 also shows that isodityrosine appeared to be present in all the cell walls although it was only quantifiable for lettuce, Capsicum fruit and Capsicum frutescens f₁ suspension culture; the amino acid profile for the suspension culture is shown in Fig. 23. The ratios of hydroxyproline:isodityrosine were calculated for the samples where isodityrosine was quantified; for Capsicum fruit (ratio 15:1) and Capsicum frutescens f₁ suspension culture (ratio 19:1) the ratios were consistent with the value of 15:1 obtained by Fry (1982) for a variety of suspension culture cell wall hydrolysates. This further supports the suggestion that isodityrosine is an important

Table 10. Amino acid composition of cell wall hydrolysates.

Amino acids are expressed as mol %.

Amino acid	Onion	Lettuce	Celery	<u>Capsicum</u> <u>fruit</u>	<u>C. frutescens</u> <u>F₁ culture</u>
Asp	13.74	11.64	14.22	10.38	8.52
Glu	11.95	12.82	12.82	9.44	8.73
Hyp	1.99	0.97	1.56	4.67	13.15
Ser	4.45	3.75	4.27	4.61	6.17
Gly	4.25	5.51	5.31	12.06	4.20
His	2.19	1.87	2.12	2.57	2.70
Arg	8.10	5.92	5.95	6.35	4.40
Thr	5.05	4.83	4.59	4.30	4.35
Ala	7.17	6.77	6.79	6.03	4.90
Pro	5.05	6.10	4.87	4.51	5.20
Tyr	4.58	4.14	5.11	4.20	4.40
Val	5.44	6.99	6.55	5.45	6.67
Met	0.66	1.82	1.24	0.16	1.33
Cys	0.86	0.99	1.48	1.26	1.00
Ile	4.52	5.62	5.43	4.88	3.87
Leu	7.50	9.91	8.27	8.02	6.90
Idt	trace	0.20	trace	0.31	0.70
Phe	3.25	4.40	4.07	4.35	3.74
Lys	8.76	5.74	5.35	6.45	9.00
Hyp/Idt	-	5:1	-	15:1	19:1

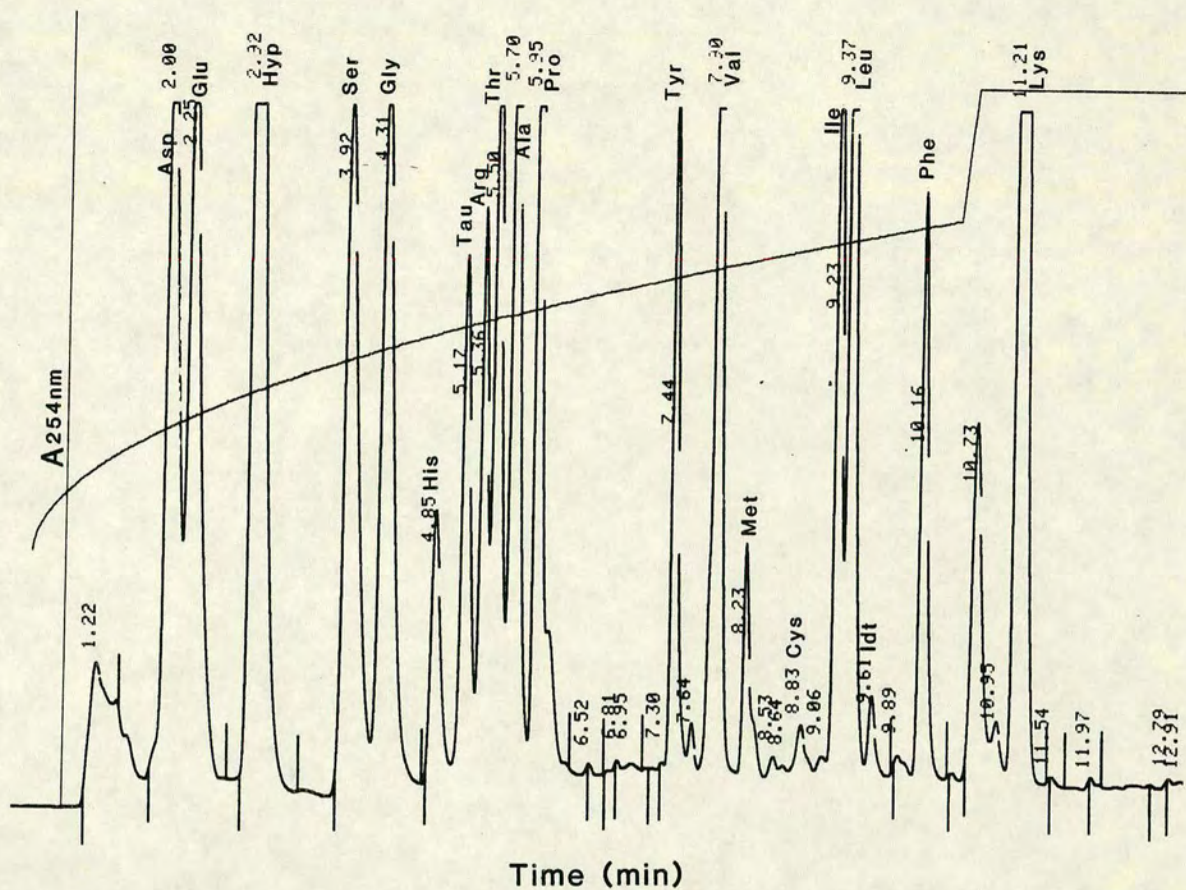


Fig. 23. Amino acid profile of cell wall hydrolysate from Capsicum frutescens f₁ suspension culture.

Tau = internal standard.

component of insoluble wall glycoprotein.

For the Capsicum frutescens f₁ suspension culture it was estimated that there were 5 half-isodityrosine residues per extensin molecule, based upon 0.7 mol isodityrosine/ 100 mol amino residues, and M_r values of 36000 for the polypeptide backbone of extensin (Stuart & Varner, 1980) and 100 for the average M_r of an amino acid residue. Stafstrom & Staehelin (1986a) suggest that for carrot root disc extensin there would be 7.75 half isodityrosine residues per monomer extensin, most of which would be involved in intramolecular links. It was not possible, however, to determine the relative quantities of intramolecular and intermolecular isodityrosine molecules present in the Capsicum frutescens f₁ extensin.

Conclusion

It was shown that material leached from spinach suspension culture cell walls was rich in hydroxyproline and had an amino acid profile characteristic of extensin; the amino acid composition was similar to that of extensin precursor isolated from tomato suspension culture walls (Smith et al., 1986). Isodityrosine was not detected in salt-soluble spinach extensin.

Amino acid analysis of cell walls from a variety of plant species showed that hydroxyproline and isodityrosine could both be detected, although hydroxyproline levels were lower than expected. Isodityrosine content was quantifiable for some samples and, in these cases, correlation was found with the hydroxyproline: isodityrosine ratio of 15:1 quoted by Fry for other cell wall hydrolysates.

Isolation of extensin dimers

Introduction

In order to test the hypothesis that extensin is cross linked in the cell wall by intermolecular isodityrosine residues it is necessary to isolate soluble, cross linked fragments of extensin, such as dimers. Two possible ways of producing extensin dimers were investigated. One was to leach soluble extensin precursors from the walls of living suspension culture cells because newly secreted precursor molecules are soluble in salt solutions such as lanthanum chloride and a proportion of dimer extensin molecules might be present in such leachates. The alternative way of attempting to obtain extensin dimers was to solubilise glycoprotein fragments from cell walls by the use of brief acidified chlorite treatments that could only cleave some cross links. As has been shown in previous chapters the material solubilised by acidified chlorite may suffer peptidyl cleavage and so B.S.A. was added to protect against peptide bond cleavage and thus facilitate the isolation of intact extensin dimers. Soluble extensin could then be separated into varying sized fragments by gel permeation chromatography and any potential dimers identified. Oligomers of extensin have been identified in salt leachates from carrot root disc cell walls (Stafstrom & Staehelin, 1986a, 1988) and from various suspension culture cell walls, including tomato (Heckman et al., 1988).

Salt elution of Capsicum frutescens f₁ suspension culture cell walls

In this initial experiment the aim was to build up a pool of radioactive extensin precursors in the culture and then identify the

optimum time at which cross linked dimers might be accumulated as intermediate products. A suspension culture of 3-d old Capsicum frutescens f₁ cells was incubated with [³H]arabinose in the presence of 10 mM-ascorbic acid, adjusted to pH 5.7 with sodium hydroxide, for 6 h; ascorbic acid has been shown to inhibit incorporation of soluble extensin into the wall network (Cooper & Varner, 1984) and so it will prevent any cross linking of radioactive extensin precursors. Ascorbic acid levels were monitored by the colorimetric assay described in the Methods, and the concentration was maintained at 10 mM by addition from a concentrated stock solution. At the end of the 6 h incubation the cells were filtered on muslin and washed extensively with culture medium to remove excess radioactivity and ascorbic acid. The cells were transferred to fresh medium and a time course of leaching in lanthanum chloride was carried out; approx. equal portions of cells were removed after 0, 1, 5, 10, 20, 40, 60, 120, 180 and 240 min and swirled in a small volume of 25 mM-lanthanum chloride and 10 mM-ascorbic acid. In the fresh medium, where no ascorbic acid was present, the radioactive extensin molecules had the opportunity to cross link to form salt-soluble oligomers, as well as becoming incorporated into the insoluble wall network. After being leached from the cell walls in the lanthanum chloride solution any salt-soluble extensin molecules will be prevented from cross linking further by the presence of the ascorbic acid.

A small portion of each leachate was taken for scintillation counting and the amount of radioactivity present at each point of the time course is shown in Fig. 24. The graph shows that, initially, a large amount of radioactive material is leached from the cell walls but this has dropped after 60 min incubation in culture medium; this

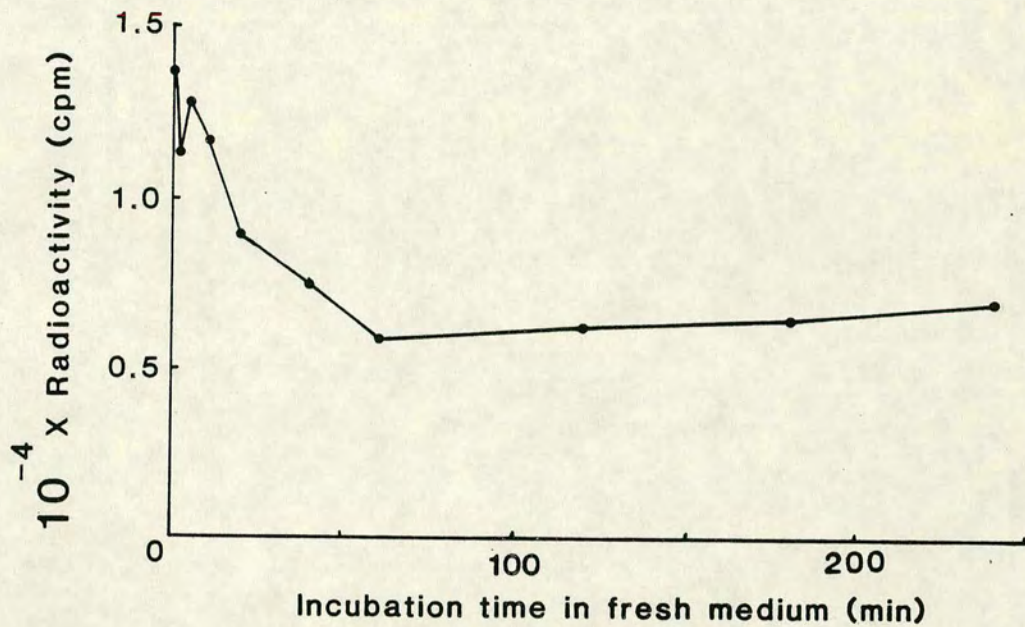


Fig. 24. Time course of leaching of radioactive material from cell walls of Capsicum frutescens f₁ suspension culture. Cells which had been fed with [³H]arabinose were incubated in fresh culture medium and portions of cells were removed at intervals and leached in 25 mM-lanthanum chloride and 10 mM-ascorbic acid. Radioactivity = cpm/0.1 ml.

suggests that a % of the salt-soluble extensin molecules are gradually cross linked into the wall network and can no longer be leached.

To separate the varying sized extensin molecules samples of the leachates were analysed by gel permeation chromatography on Sephacryl S-400 with 1 M-sodium chloride/20 mM-Mes (pH 6.1) as eluent. The results for samples incubated for 1 min and 60 min in culture medium (Fig. 25) show that most of the radioactivity eluted at V_i , probably as [^3H]arabinose, and that only a small peak of salt-soluble extensin ($k_{av} = 0.55$) was produced. This means that Fig. 24 really showed the elution of free arabinose, with soluble extensin forming only about 4% of the radioactivity detected. No peaks or shoulders of extensin oligomers were detected and there was little difference between the two profiles; an optimum time for dimer production could not be determined.

Effect of ascorbic acid pre-treatment

In this next experiment the suspension culture cells were given a pre-treatment with ascorbic acid to see whether this would lead to the accumulation of a large pool of unlabelled monomer extensin which would, in turn, increase the chance of formation of dimers when the ascorbic acid was removed.

Three flasks of 4-d old Capsicum frutescens f_1 suspension culture were set up as follows: (a) the cells were re-suspended in fresh medium and left for 3 d; (b) the cells were re-suspended in fresh medium at 24 h intervals; and (c) the cells were re-suspended in fresh medium containing 10 mM-ascorbic acid at 24 h intervals. All of these procedures were carried out aseptically. Flask (a) acted as an untreated control and flask (b) acted as a control to investigate the

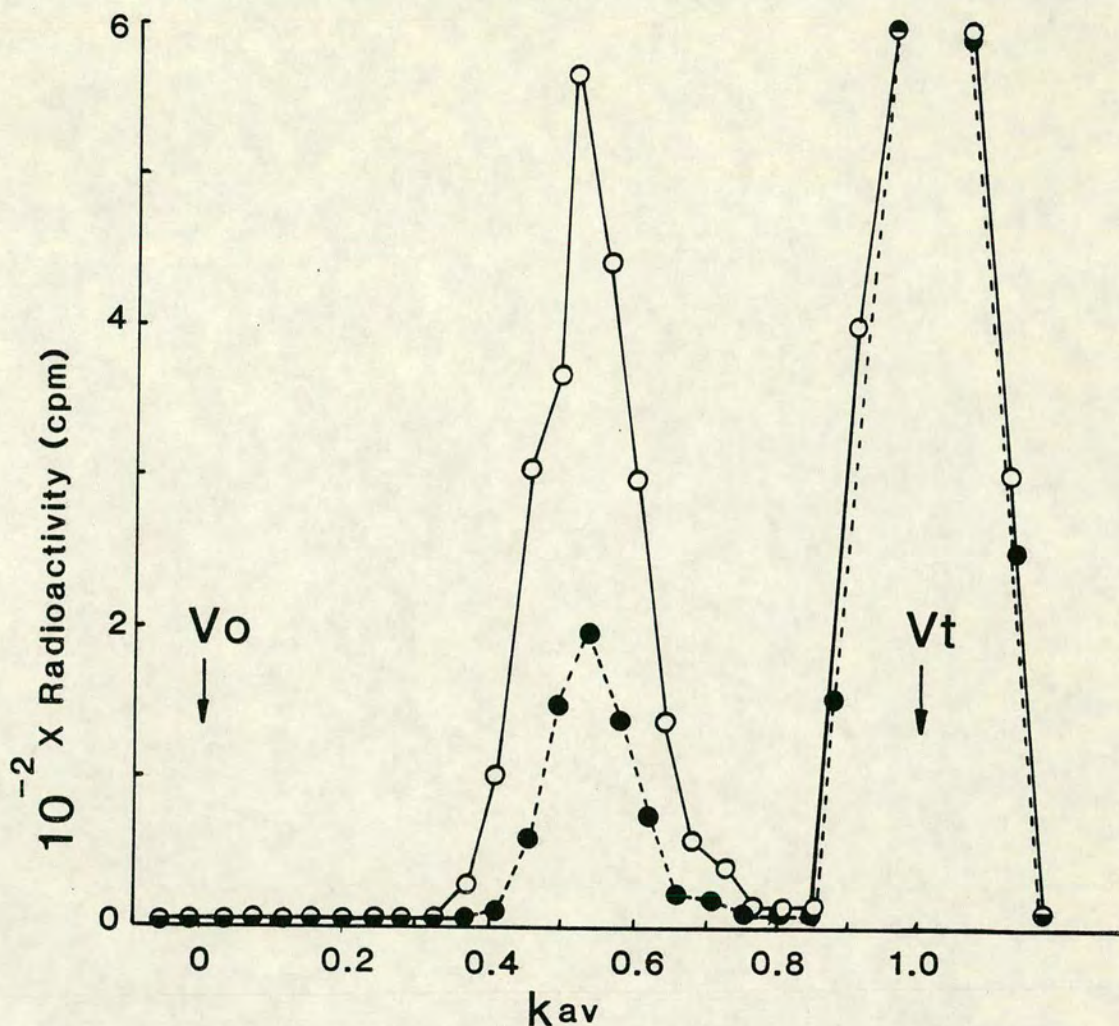


Fig. 25. Gel permeation chromatography (on Sephacryl S-400) of radioactive material leached from cell walls of Capsicum frutescens f₁. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactive material was from cells incubated in fresh culture medium for \circ - \circ , 1 min and \bullet - \bullet , 60 min prior to leaching. Radioactivity = cpm/0.7 ml. Extensin monomer = k_{av} 0.55. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

effect of daily re-suspension; this was necessary because flask (c) was maintained in an environment of 10 mM-ascorbic acid and in order to prevent a toxic level of ascorbic acid from accumulating the cells were transferred into fresh medium daily. Ascorbic acid levels were monitored and maintained at 10 mM as described in the previous experiment.

At the end of the 3-d pre-treatment the suspension cultures were incubated with [³H]arabinose for 10 h; flask (c) was still in the presence of 10 mM-ascorbic acid. Uptake of radioactivity was monitored by scintillation counting of small portions of culture medium at 0 h and 10 h; this showed that more than 98% of the radioactivity was removed from the culture medium in each flask. After incubation with [³H]arabinose the cells were washed extensively to remove excess radioactivity and ascorbic acid. Each culture was transferred into fresh medium and a time course was carried out where portions of cells were leached in 25 mM-lanthanum chloride and 10 mM-ascorbic acid at each time point. A portion of each leachate was analysed by paper chromatography, as described in the Methods, in order to assess partitioning of the radioactivity between polymer material and free arabinose (Fig. 26). Results of the paper chromatography showed that for cultures (a) and (b) most of the radioactivity had been incorporated into polymer material (radioactivity remaining at the origin), whilst for (c) the radioactivity was present as free [³H]arabinose and none had been incorporated into polymer. This suggested that the ascorbic acid pre-treatment had inhibited synthesis of extensin, although it did not appear to affect the uptake of arabinose by the cells. In contrast to the expected result, ascorbic acid pre-treatment did not lead to

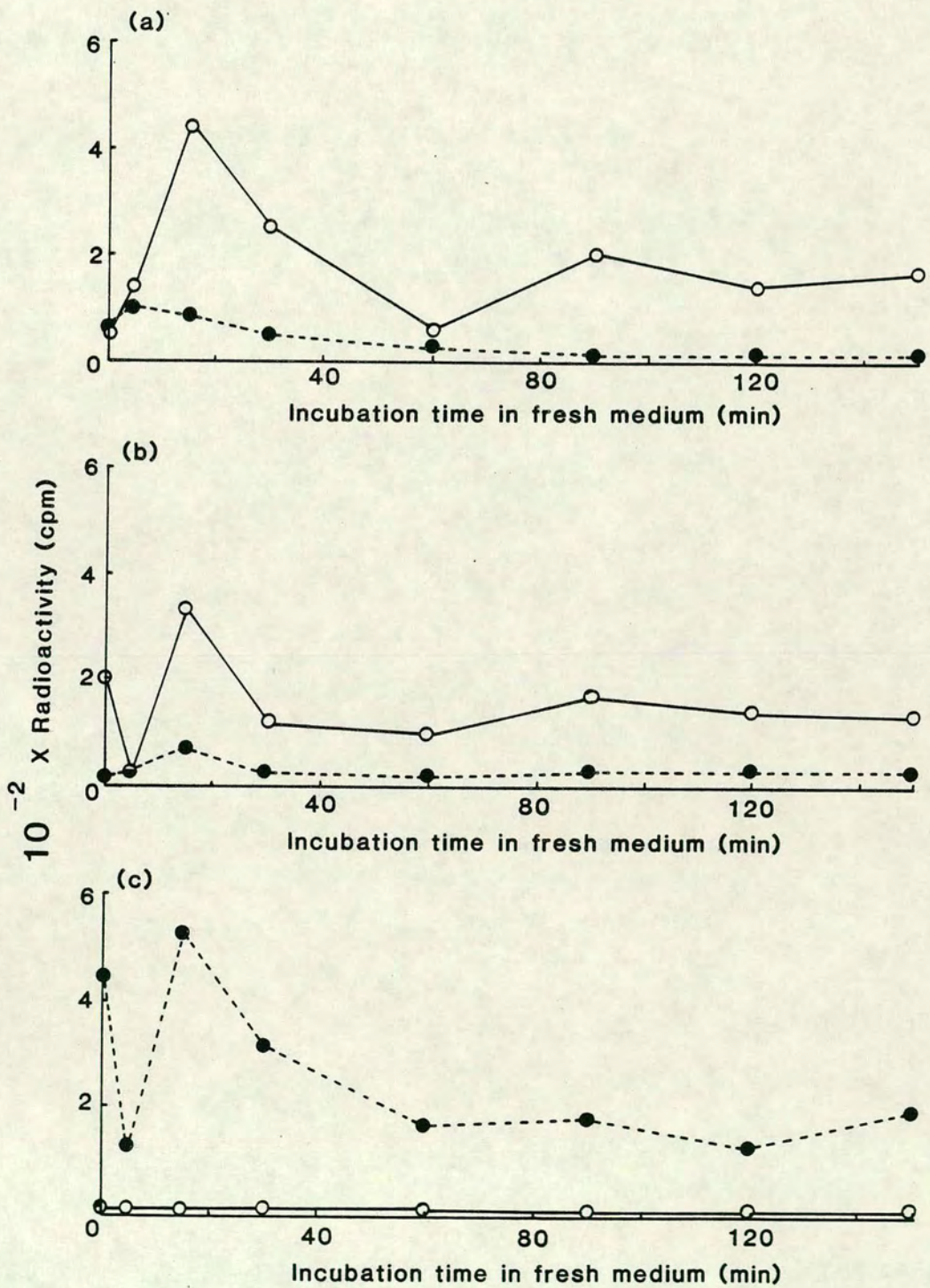
Fig. 26. Partitioning of radioactivity between polymer and free arabinose in lanthanum chloride leachates from cell walls of Capsicum frutescens f₁. An ascorbic acid pre-treatment was given to the cultures:

(a) cells were suspended in fresh culture medium and left for 3d.

(b) cells were re-suspended in fresh culture medium at 24 h intervals.

(c) cells were re-suspended in fresh culture medium containing 10 mM-ascorbic acid at 24 h intervals.

After incubation with [³H]arabinose the cells were incubated in fresh culture medium and portions of cells were removed at intervals and leached in 25 mM-lanthanum chloride and 10 mM-ascorbic acid. For further experimental details see text. Paper chromatography of leachate samples was performed in butan-1-ol/acetic acid/water (12:3:5 by vol). ○—○, radioactivity in polymer material; ●--●, radioactivity in free arabinose. Radioactivity = cpm/0.05 ml.



enhanced extensin oligomer production.

In the next investigation the cells were given a shorter 12 h pre-treatment with ascorbic acid to see whether this would lead to the accumulation of a pool of extensin monomers without causing inhibition of extensin synthesis. A suspension culture without ascorbic acid present acted as a control. Both cultures were incubated with [^3H]arabinose for 6 h and, at the end of this time, the cells were washed, transferred to fresh medium and a time course of incubation in the fresh medium prior to leaching in lanthanum chloride and ascorbic acid was carried out as for previous experiments. After leaching each cell sample in 25 mM-lanthanum chloride and 10 mM-ascorbic acid, an additional leaching of each cell sample in 100 mM-lanthanum chloride and 10 mM-ascorbic acid was performed to see whether a higher salt concentration was necessary to leach any soluble extensin oligomers from the cell walls. Oligomers will have a stronger positive charge and may, therefore, bind more strongly to the wall. Equal portions of each leachate were analysed by paper chromatography and the results are shown in Fig. 27.

The data in Fig. 27, where partitioning of radioactivity between polymer and free arabinose is shown, indicated that about 99% of the radioactivity had been incorporated into polymer. The cpm values for ascorbic acid pre-treated cells were about half those for the control which would imply that ascorbic acid had either inhibited the incorporation of [^3H]arabinose into extensin molecules or had inhibited the secretion of extensin molecules into the cell wall; however the effect was not as drastic as in the previous experiment.

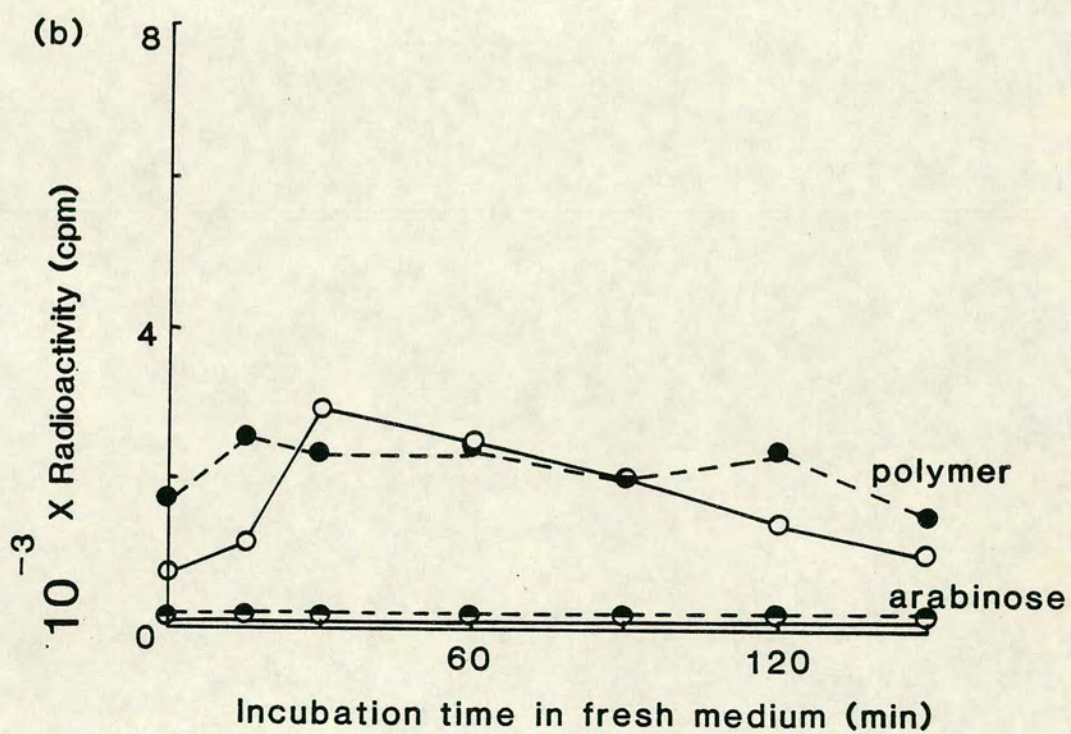
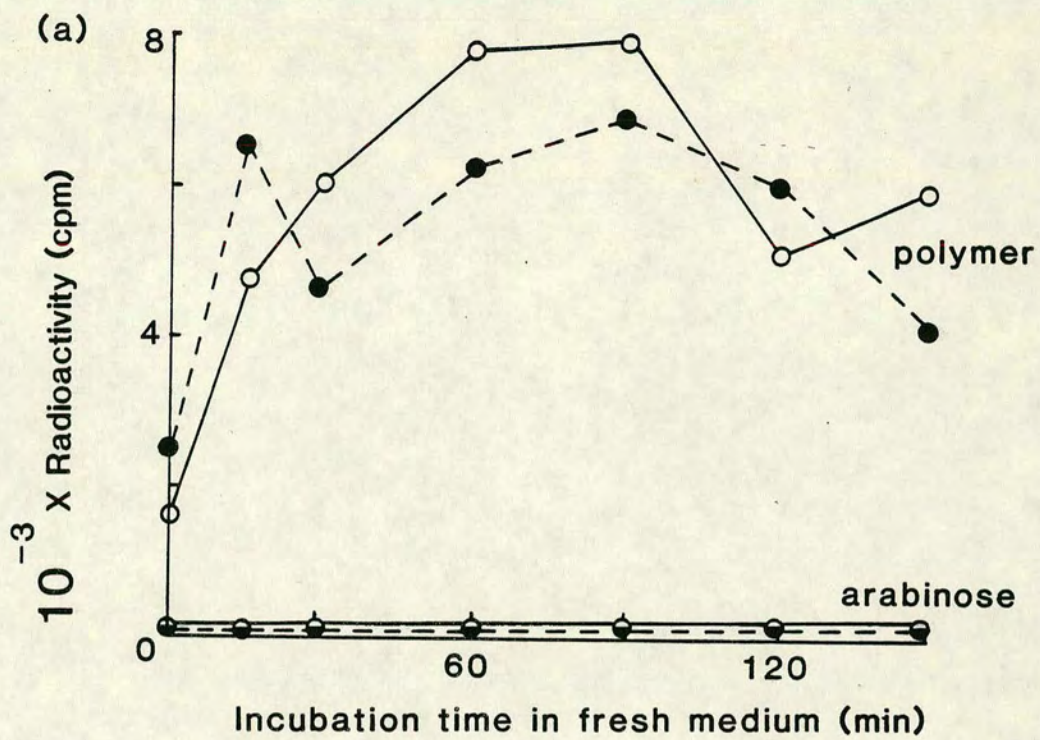
A sample of the 100 mM-lanthanum chloride leachate from the control culture, incubated in fresh medium without ascorbate for 15

Fig. 27. Partitioning of radioactivity between polymer material and free arabinose in lanthanum chloride leachates from cell walls of Capsicum frutescens f₁ that were given a short ascorbic acid pre-treatment.

(a) control culture.

(b) culture given a 12 h pre-treatment with ascorbic acid.

After incubation with [³H]arabinose the cells were incubated in fresh culture medium and portions of cells were removed at intervals and leached in 25 mM-lanthanum chloride and 10 mM-ascorbic acid and then in 100 mM-lanthanum chloride and 10 mM-ascorbic acid. Paper chromatography of leachate samples was performed in butan-1-ol/acetic acid/water (12:3:5 by vol). ○—○, radioactivity in 25 mM-lanthanum chloride leachate; ●—●, radioactivity in 100 mM-lanthanum chloride leachate. Radioactivity = cpm/0.05 ml.



min, was analysed by gel permeation chromatography to see whether any extensin oligomers could be detected. The results (Fig. 28) showed a large peak of [³H]polymer with the k_{av} (0.55) of monomer extensin and a small peak of higher M_r material which eluted with $k_{av} = 0.26$. It was possible that this small peak could have been oligomer extensin; one way of testing this was to analyse the material by ion-exchange chromatography on an SP-50 Sephadex column. If the radioactive material bound to the gel and could be eluted at pH 9-9.5 this would be an indication that it was extensin. The pool of putative oligomer extensin was dialysed, dried under vacuum and analysed by ion-exchange chromatography as described in the Methods. Scintillation counting of portions of the sample, before and after application to the column, showed that no binding had occurred, whilst a control experiment with the peak of monomer extensin, prepared in the same way, showed that 80% of the monomer had bound to the gel. The material in the high M_r peak lacked a strong positive charge and it was therefore concluded that it did not contain extensin oligomers.

Salt elution of tomato and Capsicum frutescens f₁ cultures

In this attempt to isolate extensin dimers ascorbic acid pre-treatments were omitted because they appeared to inhibit extensin synthesis. Incubation with [³H]arabinose for 6 h, with and without 10 mM-ascorbic acid present, was carried out with 2-d old Capsicum frutescens f₁ suspension cultures and tomato suspension cultures. Tomato cultures were used because Heckman et al. (1988) had isolated soluble extensin oligomers from such suspension cultures; it was considered possible that Capsicum frutescens f₁ suspension cultures may not produce oligomers as intermediate products of cross linking

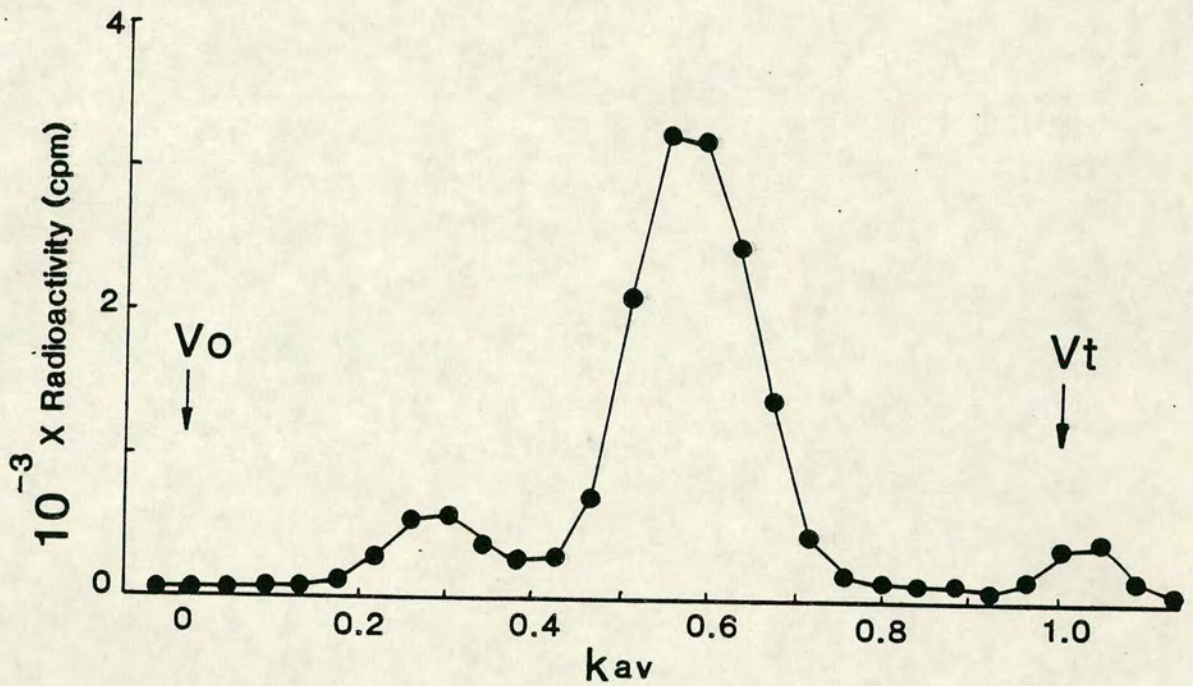


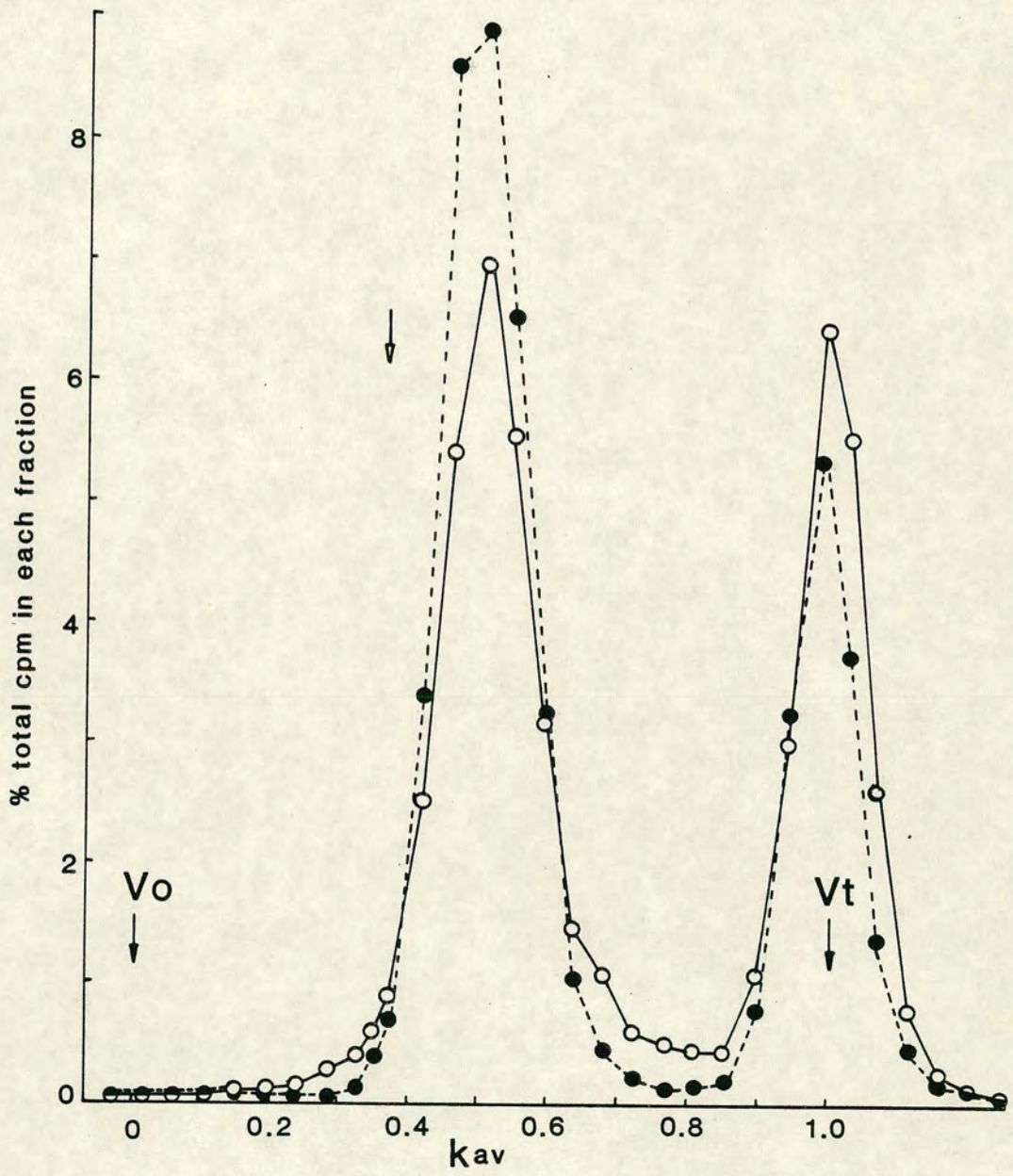
Fig. 28. Gel permeation chromatography (on Sephacryl S-400) of radioactive material leached from cell walls of Capsicum frutescens f₁. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactive material was from cells incubated in fresh culture medium for 15 min prior to leaching in 100mM-lanthanum chloride and 10 mM-ascorbic acid. Radioactivity = cpm/0.25 ml. Extensin monomer = k_{av} 0.55. Putative oligomer extensin = k_{av} 0.26. V₀, elution volume of Blue Dextran; V_t, elution volume of sucrose.

whilst tomato suspension cultures do. Preparation of the 25 mM-lanthanum chloride leachates was as for the above experiments and paper chromatography of leachate samples showed that more than 95% of the radioactivity in the leachate appeared to be incorporated into salt-soluble polymer for both cultures. Analysis of leachate samples from tomato cells, incubated with and without 10 mM-ascorbic acid for 60 min prior to leaching, by gel permeation chromatography on Sephacryl S-400 (Fig. 29) revealed peaks of monomer extensin ($k_{av} = 0.50$) but no dimer peaks were detected. It was estimated that dimers would elute with a $k_{av} = 0.37$ and in order to be resolved from the peak of monomer extensin, dimers would have to be present as 20-30% of the monomer peak. Comparison of the two monomer extensin peaks showed that in the presence of ascorbic acid slightly more salt-soluble extensin was present; this was in agreement with the observation that ascorbic acid inhibited cross linking into the cell wall (Cooper & Varner, 1984). Analysis of the leachate from Capsicum frutescens f_1 cells, incubated in fresh medium without ascorbic acid for 60 min prior to leaching, (Fig. 30), showed that only extensin monomer ($k_{av} = 0.52$) was detectable in this sample too. It was concluded from this experiment that extensin dimers were either not produced in the cell suspension cultures investigated, or that they were present but at levels too low to be detected (i.e. present as less than 20% of the monomer peak).

Effect of increasing radioactive precursor concentration

Several experiments were also carried out where [^3H]proline (0.334 μM) was fed to Capsicum frutescens f_1 suspension cultures to be incorporated into hydroxyproline residues in the peptide backbone of

Fig. 29. Gel permeation chromatography (on Sephacryl S-400) of radioactive material leached from cell walls of tomato suspension culture. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactive material was from cells incubated in fresh culture medium $\circ-\circ$, in the absence and $\bullet-\bullet$, presence of 10 mM-ascorbic acid for 60 min prior to leaching in 25 mM-lanthanum chloride and 10 mM-ascorbic acid. Extensin monomer = k_{av} 0.50. \downarrow , estimated k_{av} of extensin dimer. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.



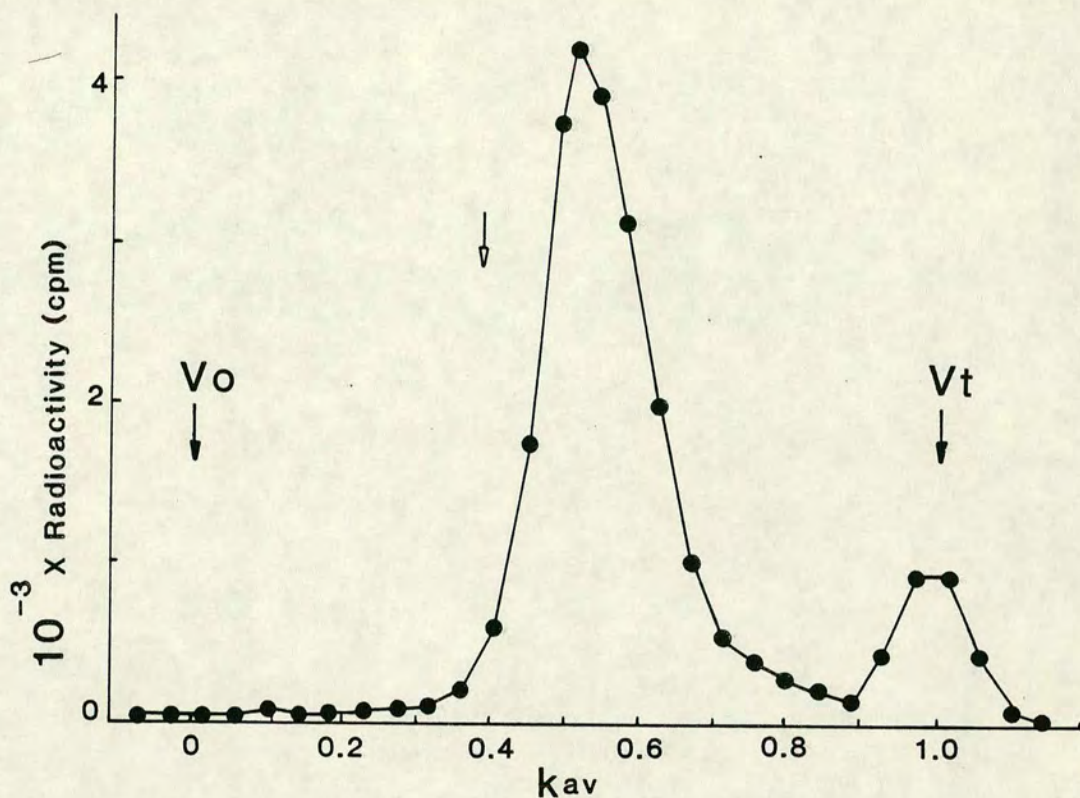


Fig. 30. Gel permeation chromatography (on Sephacryl S-400) of radioactive material leached from cell walls of Capsicum frutescens f₁. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactive material was from cells incubated in fresh culture medium in the absence of 10 mM-ascorbic acid for 60 min prior to leaching in 25 mM-lanthanum chloride and 10 mM-ascorbic acid. Radioactivity = cpm/0.45 ml. Extensin monomer = k_{av} 0.52. \downarrow , estimated k_{av} of extensin dimer. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

extensin; the cells were leached in lanthanum chloride and the leachates analysed by gel permeation chromatography in further attempts to detect peaks of dimer extensin. No oligomers were detected, however, and so, in the experiment discussed here, a higher concentration of radioactive precursor (3.34 μ M-proline) was used to see whether a detectable level of dimers could be produced. A small sample (2 g fresh weight) of 3-d old cells was used so that leaching could occur in a small volume (5 ml) of lanthanum chloride solution and the leachate could be applied directly on to the column. After 6 h incubation with the [3 H]proline, the cells were rinsed and then leached in 25 mM-lanthanum chloride and 10 mM-ascorbic acid. Uptake of radioactivity was calculated by scintillation counting samples of culture medium at 0 h and 6 h after addition of the radioactive precursor; it was found that 96% of the [3 H]proline had been taken up.

From $k_{av} = 0.55$ for monomer extensin it was estimated from the calibration graph of k_{av} against M_r (Fig. 8) that the k_{av} for dimers would be 0.42, and on the short (1.5 cm x 48 cm) Sephacryl S-400 column this difference is represented by only a few fractions, which means that it would be difficult to detect a small peak of dimer extensin. So, as mentioned in chapter 2, a long (1.5 cm x 158 cm) column of Sephacryl S-400 was used in an attempt to improve the resolution of dimers from monomers. Analysis of the leachate on the long Sephacryl S-400 column (Fig. 31) showed that the major peaks eluted with k_{av} values of 0.55 and 1.0, representing extensin monomer and free proline respectively. However, two minor peaks eluting with k_{av} values of 0.26 and 0.39 were also present and it was suggested that these could represent extensin oligomers. To see whether these peaks of radioactivity were extensin molecules two tests were carried

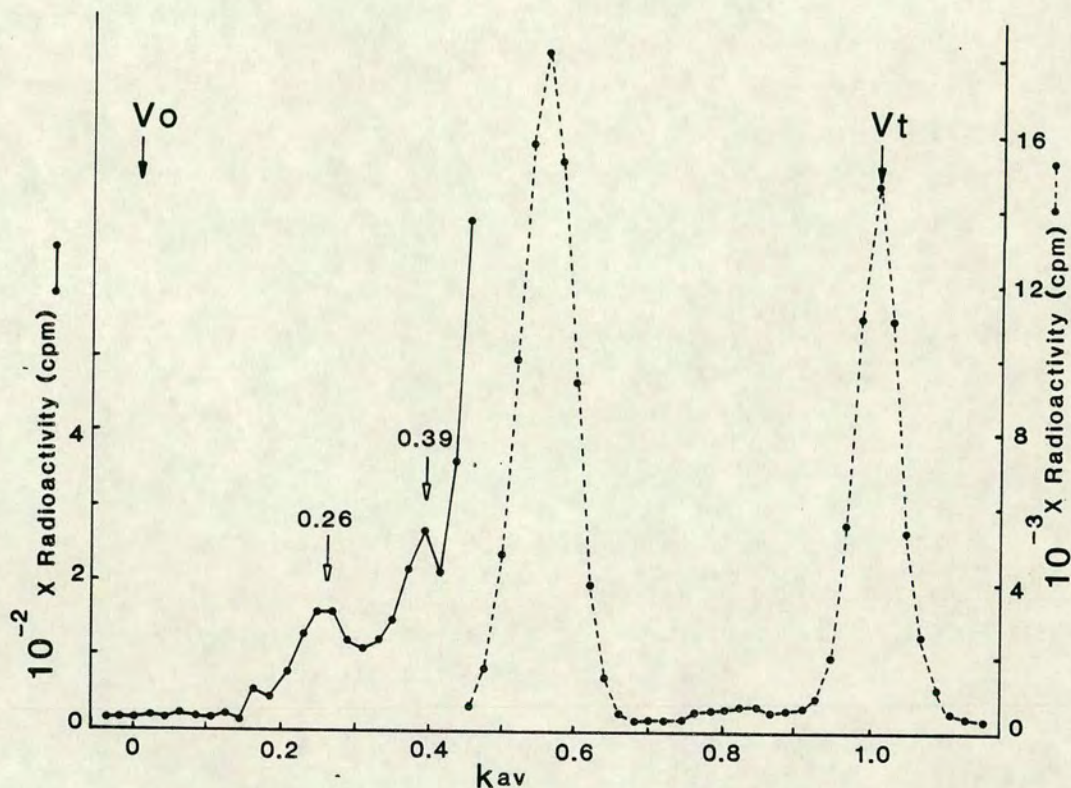


Fig. 31. Gel permeation chromatography (on a long Sephacryl S-400 column) of radioactive material leached from cell walls of Capsicum frutescens f_1 . The column of Sephacryl S-400 (1.5 cm x 158 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactive material was from cells fed with [^3H]proline and leached in 25 mM-lanthanum chloride and 10 mM-ascorbic acid. Radioactivity = cpm/0.1 ml. Extensin monomer = k_{av} 0.55. Putative extensin oligomers = k_{av} 0.26 and 0.39. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

out: (1) cation-exchange chromatography to test for binding to SP-50 Sephadex, as described earlier and (2) alkaline hydrolysis followed by HVPE (details in Methods) to look for the presence of hydroxyproline-oligoarabinosides.

Aliquots of the three high M_r peaks (k_{av} 0.26, 0.39 and 0.55) were applied to separate ion-exchange columns and the % binding was estimated; 90% of the [3H]extensin monomer bound to the column, and eluted at pH 9-9.5, but for the other two peaks only 25% of the radioactivity was bound. The low binding implied that these peaks did not contain extensin but it was possible that this test did not provide sufficient proof because it was difficult to predict whether extensin oligomers would bind to, and elute from, SP-50 Sephadex gel in the same way as extensin monomers. Discrepancy could arise from differences between monomers and oligomers as to how the polysaccharide sidechains are arranged to affect the positively charged lysine residues of the polypeptide backbone, and therefore the regions available for binding to the ion-exchange gel.

The results of alkaline hydrolysis and HVPE of further portions of the three peaks are shown in Fig. 32. Marker radioactive hydroxyproline-oligoarabinosides were obtained from a sample previously prepared from spinach suspension culture (Fry, 1983b). It can be seen that monomer extensin contained [3H]hydroxyproline-oligoarabinosides, as was expected, but neither of the other two samples appeared to contain any [3H]hydroxyproline-oligoarabinosides, again suggesting that they were not related to extensin. However, no other peaks of radioactive material were detected in either of these samples which suggests that the amounts of sample used were insufficient for detection by scintillation counting of paper

Fig. 32. Analysis of radioactive putative extensin oligomers for the presence of [³H]hydroxyproline-oligoarabinosides. Samples were alkaline hydrolysed and the hydrolysis products separated by HVPE at pH 2 in formic acid/acetic acid/water (1:4:45 by vol).

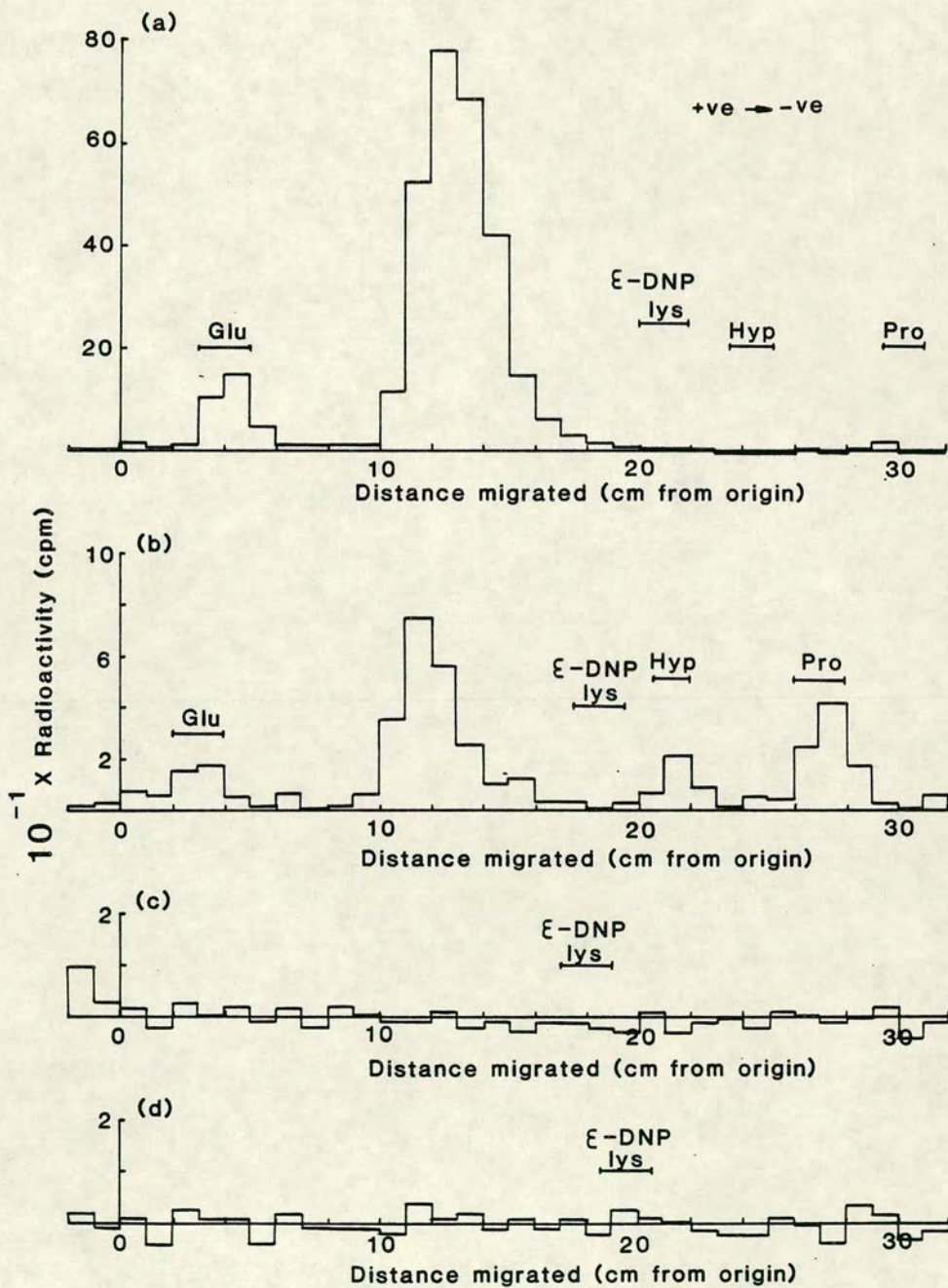
(a) marker hydroxyproline-oligoarabinoside.

(b) extensin monomer ($k_{av} = 0.55$).

(c) putative extensin oligomer ($k_{av} = 0.39$).

(d) putative extensin oligomer ($k_{av} = 0.26$).

Radioactivity = cpm/cm strip. Neutral marker = Glu. Yellow, +ve marker = ϵ -DNP lysine.



electrophoretograms.

In conclusion, it would appear that feeding an increased concentration of radioactive precursor to the suspension culture cells, and using a longer Sephacryl S-400 column to separate material in the resulting radioactive leachate, did result in the detection of small peaks of high M_r material not previously resolved or detected. However, these peaks did not appear to be extensin oligomers and because it did not seem possible to isolate salt-soluble extensin dimers from the cell walls of Capsicum frutescens f_1 suspension culture, the alternative approach of solubilising glycoprotein fragments from the wall extensin network was investigated.

Solubilisation of glycoprotein fragments by use of acidified chlorite treatments

In an attempt to solubilise intact extensin oligomers from the wall extensin network radioactive cell walls were suspended in a solution of B.S.A. and treated briefly with acidified chlorite. Cell walls were isolated from a Capsicum frutescens f_1 suspension culture that had been fed with [^3H]proline. A portion of the freeze dried walls was suspended in 0.6% (w/v) B.S.A. solution and aliquots of this suspension were pipetted into test tubes; a syringe pipette was used to ensure that all the cell walls were transferred. Aliquots of a 0.6% (w/v) sodium chlorite solution (pH 3.4) were added to the tubes, which were incubated at 60°C for 1, 2, 5, 10, 20 and 30 min. A control sample was treated for 10 min at pH 3.4 in the absence of acidified chlorite. The reactions were halted by addition of 1.8 M-ammonia solution to give a final pH of 8-9. All the samples were made to 1 M-sodium chloride/20 mM-Mes (pH 6.1) to (a) leach any solubilised

glycoprotein ionically bound to the cell walls and (b) prepare the samples for separation by gel permeation chromatography. The samples were centrifuged to pellet the cell walls and the supernatants collected; aliquots of the supernatant and the cell wall pellets were taken for scintillation counting in Triton scintillant. The cpm values for the wall pellets were adjusted by a factor of 2.3 (see Methods) so that the % of radioactivity solubilised at each time point could be calculated. The results (Fig. 33) showed that more glycoprotein was solubilised as acidified chlorite treatment time was increased; appearance of radioactivity in the control implied that not all of the non-covalently bound glycoprotein had been removed during cell wall isolation.

Analysis of the supernatants (control, 5 min and 30 min samples) by gel permeation chromatography on Sephacryl S-400 (Fig. 34) showed that in the control a small peak of material with $k_{av} = 0.55$ was present, suggesting that not all of the salt-soluble extensin had been removed from the walls. Radioactive material solubilised by acidified chlorite treatment appeared to contain a major peak of monomer extensin ($k_{av} = 0.56$) and a lower M_r peak ($k_{av} = 0.80$) which probably represented a breakdown product. No distinct peaks of extensin oligomer were present, although dimers could have been present as a high M_r shoulder on the major peak; in the following experiments the long Sephacryl S-400 column was used to improve the resolution of any dimers.

Continuous solubilisation of glycoprotein

The aim of this approach was to prevent the possible breakdown, resulting from prolonged exposure to acidified chlorite, of

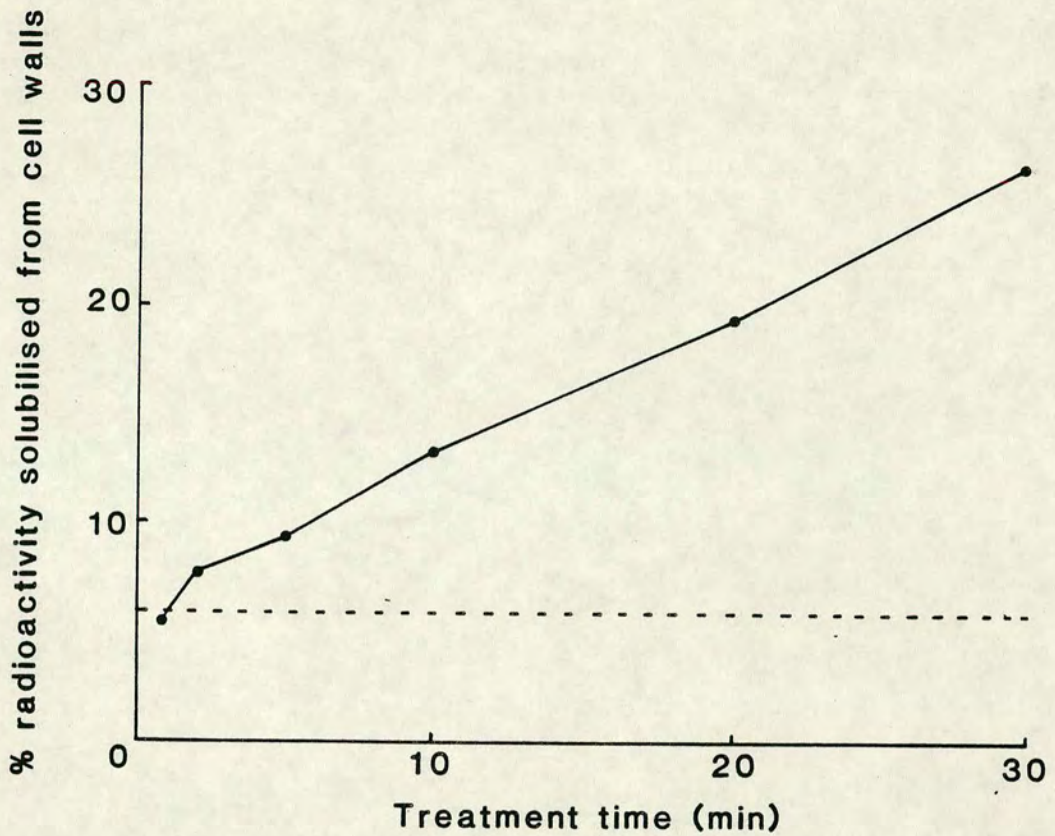


Fig. 33. Solubilisation of radioactive glycoprotein from cell walls by acidified chlorite treatment in the presence of B.S.A. Cell walls were incubated with acidified chlorite (pH 3.4) at 60°C for varying times and the reaction halted by addition of ammonia solution.

●—● , glycoprotein solubilised by acidified chlorite treatment;
 ----- , glycoprotein solubilised by incubation at pH 3.4 at 60°C for 30 min in the absence of sodium chlorite.

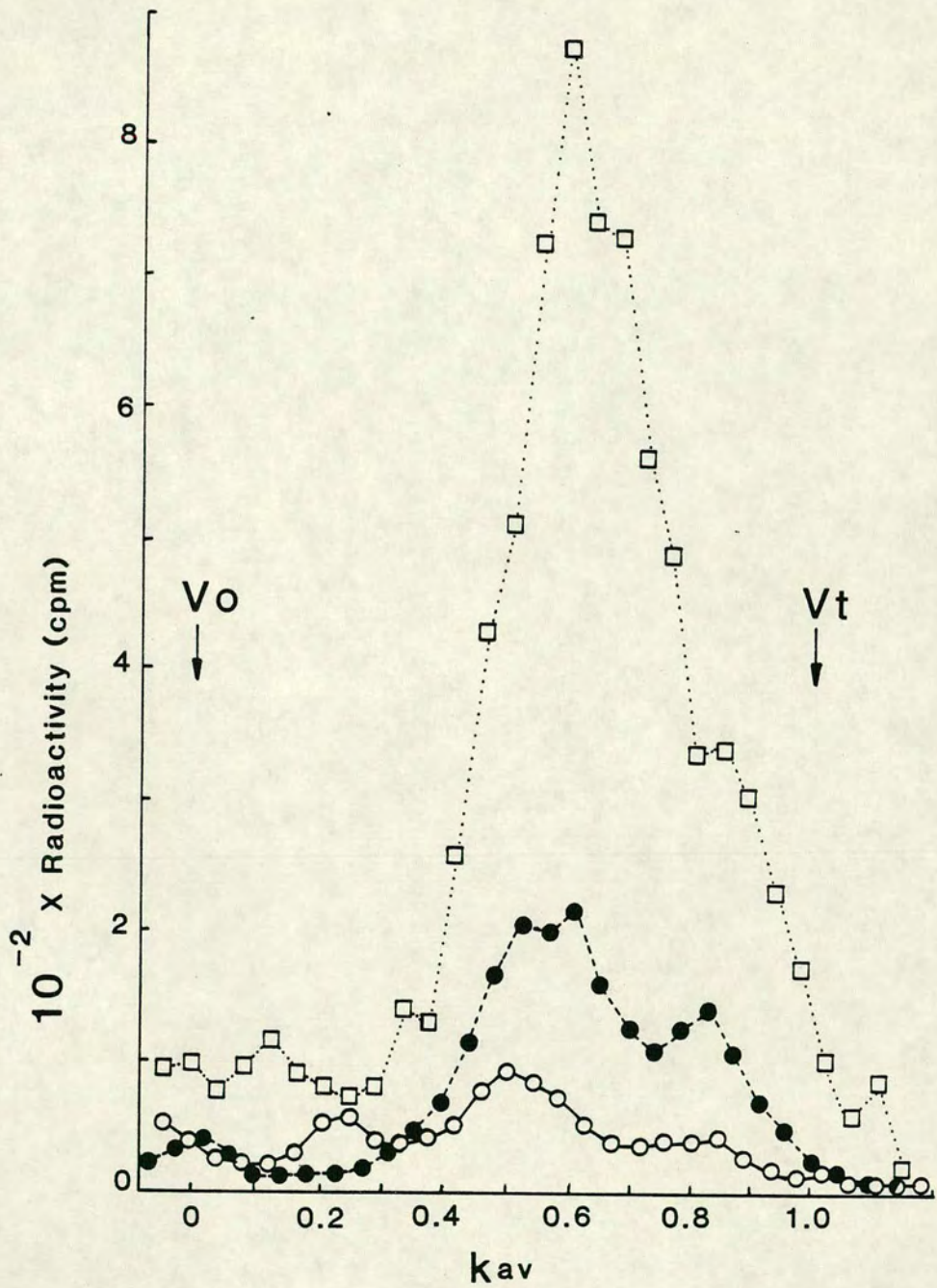
Fig. 34. Gel permeation chromatography (on Sephacryl S-400) of solubilised radioactive glycoprotein. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1).

○—○, control, glycoprotein solubilised by treatment at pH 3.4.

●—●, glycoprotein solubilised by 5 min acidified chlorite treatment.

□—□, glycoprotein solubilised by 30 min acidified chlorite treatment.

Radioactivity = cpm/0.2 ml. Extensin monomer = k_{av} 0.55. Breakdown product = k_{av} 0.80. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.



any solubilised extensin oligomers. Glycoprotein was continuously solubilised, collected and neutralised by passing a solution of B.S.A. and acidified chlorite through a column of cell walls and collecting the eluate into tubes containing ammonia solution (see Fig. 35 for a representation of the experimental apparatus). A column of cell walls, which had been isolated from a Capsicum frutescens f₁ suspension culture fed with [³H]proline, was trapped between glass wool plugs and maintained at 60°C by a heated water jacket. A solution of 0.3% (w/v) B.S.A., adjusted to pH 3.4 with acetic acid, was pumped through the column (flow rate = 2.5 ml min⁻¹) for 7 min to remove any soluble radioactive material. Fractions (2.5 ml) were collected into 1.8 M-ammonia solution and the samples were cooled on ice. After 7 min a solution of 0.3% (w/v) B.S.A. and 0.3% (w/v) sodium chlorite (pH 3.4) was pumped through the column for 25 min and further fractions (2.5 ml) were collected. Solubilised material was in the acidified chlorite environment for approx. 1 min, before it was neutralised in ammonia solution.

The experiment was repeated on another cell wall column with 0.2 M-sodium chloride present in each of the solutions in order to facilitate the leaching of any solubilised glycoprotein that was ionically bound to the cell wall column. Sodium chloride was used as the leaching salt rather than lanthanum chloride because it was found that lanthanum chloride and sodium chlorite, when mixed, formed a precipitate. Aliquots of each fraction, and the two residual cell wall columns, were scintillation counted in Triton scintillant and after correction of the cpm values for the wall columns (see Methods) the results were plotted as the % radioactivity solubilised in each fraction (Fig. 36). This allowed the two graphs to be compared. From

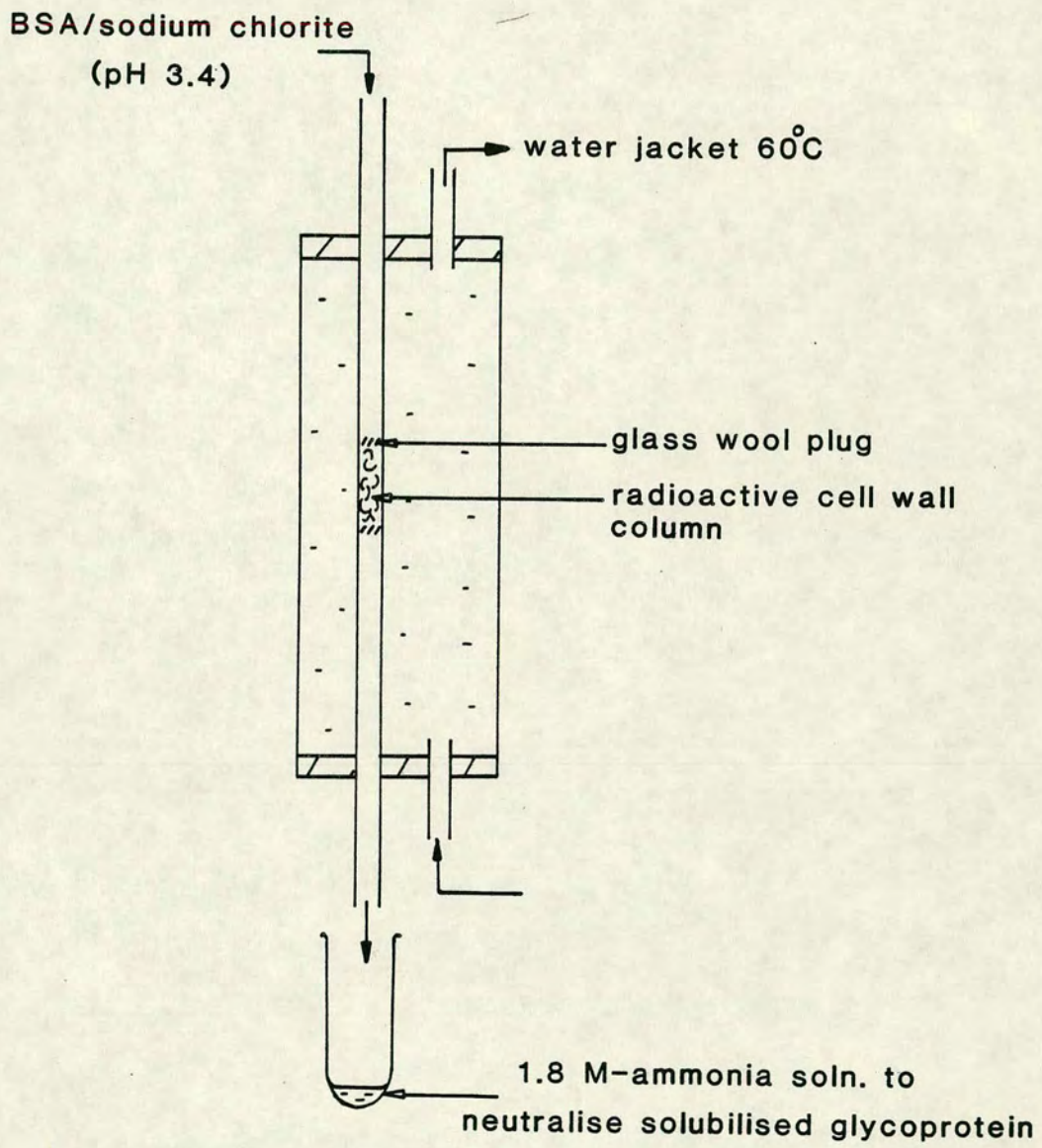


Fig. 35. Experimental apparatus for continuous solubilisation of radioactive glycoprotein from cell walls by acidified chlorite treatment. Flow rate = 2.5 ml min⁻¹. Cell wall column = 4 mm x 1 cm.

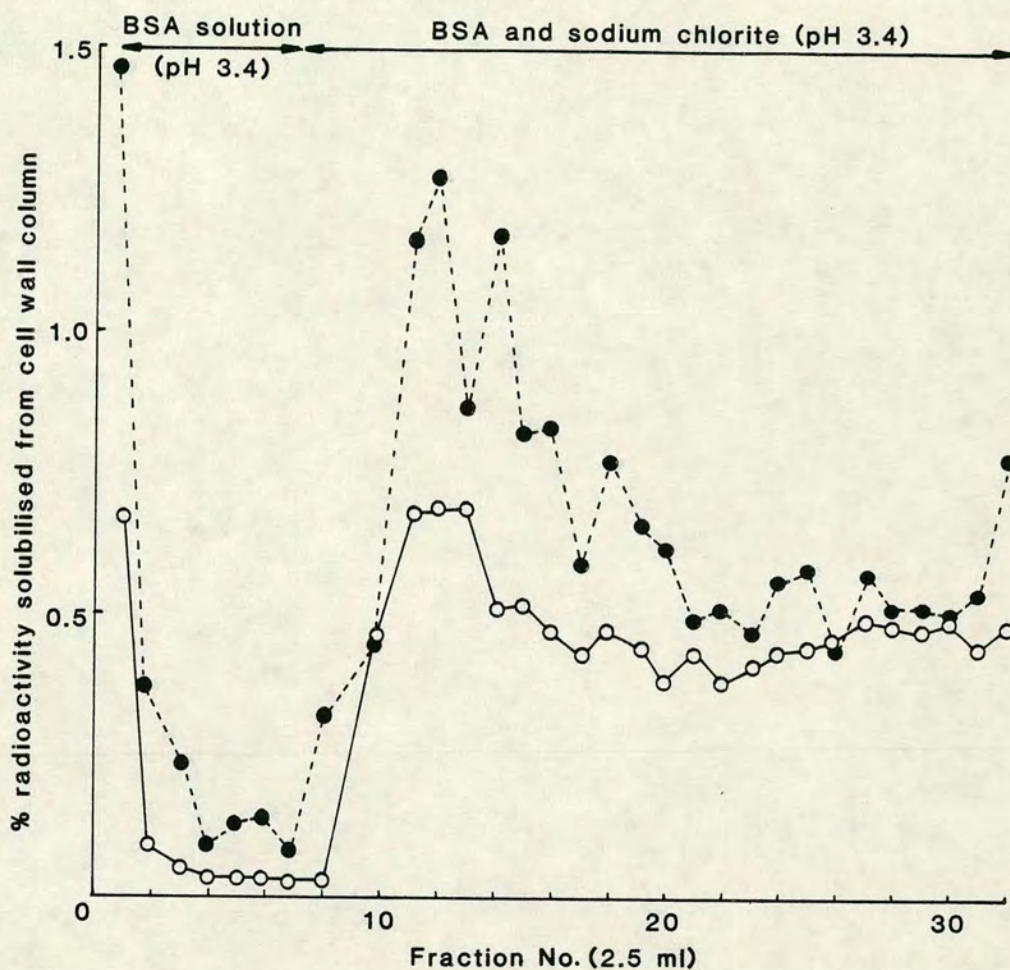


Fig. 36. Solubilisation of radioactive glycoprotein from cell wall columns by acidified chlorite treatment in the absence and presence of 1 M-sodium chloride. A solution of B.S.A. (pH 3.4) was pumped through initially to remove any soluble radioactive material and then B.S.A. plus acidified chlorite was pumped through the cell wall column. Flow rate = 2.5 ml min⁻¹. ○—○ , glycoprotein solubilised in the absence of 1 M-sodium chloride; ●---● , glycoprotein solubilised in the presence of 1 M-sodium chloride.

Fig. 36 it can be seen that the initial 'wash' in B.S.A. solution removed any soluble glycoprotein within the first few minutes and radioactivity in the solution dropped to a background value. The change to acidified chlorite solution resulted in very rapid solubilisation of covalently bound glycoprotein and the presence of sodium chloride did facilitate the release of more radioactive material.

For each experiment, fractions 9-21 and 22-37 were pooled, dialysed to remove sodium chlorite, sodium chloride and ammonia and then trichloroacetic acid (TCA) was added to give a final concentration of 10% (w/v). The acid was added to precipitate the B.S.A. without affecting the soluble extensin; Heckman et al. (1988) have used this technique to precipitate contaminating proteins in preparations of soluble extensin. The samples were kept at 4°C for 1 h to precipitate B.S.A. and then centrifuged. Samples of the supernatant were taken for scintillation counting and, allowing for a 50% quenching effect by TCA, it was calculated that all of the radioactivity was recovered. The samples were dialysed, freeze dried and then analysed by gel permeation chromatography on the long Sephacryl S-400 column. A representative elution profile (Fig. 37) showed a broad peak of low M_r material which suggested that any extensin present had been broken down. This breakdown may be a result of the action of any residual traces of sodium chlorite present, or it may be caused by TCA, although the latter explanation is unlikely considering the results obtained by Heckman et al. (1988).

In conclusion, continuous neutralisation of solubilised glycoprotein did not result in isolation of extensin oligomers and the various procedures which had to be carried out in order to concentrate

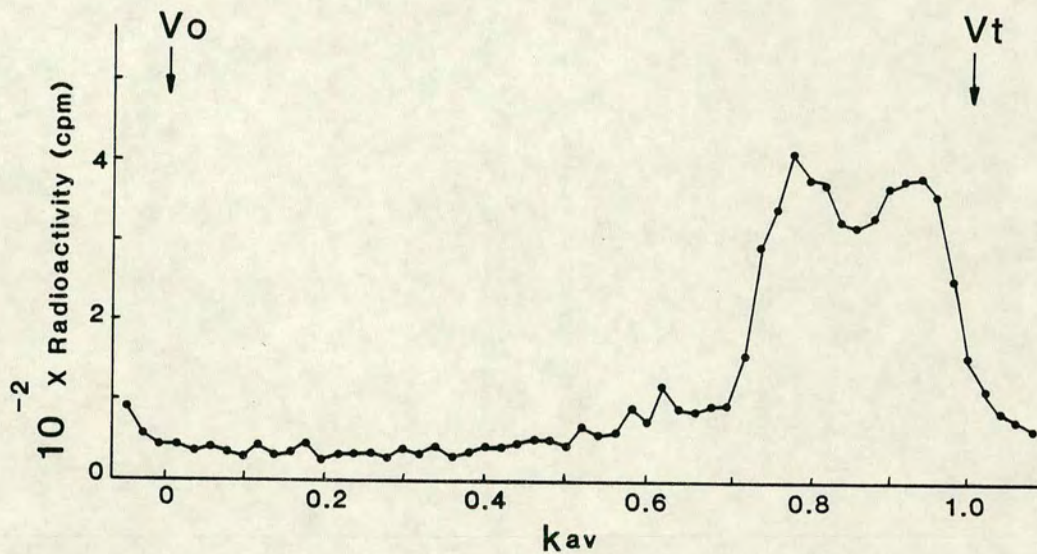


Fig. 37. Gel permeation chromatography (on a long Sephacryl S-400 column) of radioactive glycoprotein solubilised from a cell wall column. The column of Sephacryl S-400 (1.5 cm x 158 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). This sample represents pooled fractions 9-21 (+ 1 M-sodium chloride) from Fig. 36. Radioactivity = cpm/0.25 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

the radioactive material for gel permeation chromatography analysis led to breakdown of the material; in future investigations acidified chlorite treatments were carried out in test tubes.

Isolation of putative dimer

In this experiment glycoprotein was solubilised from cell walls by acidified chlorite treatment and the soluble fragments were analysed on the long (1.5 cm x 158 cm) Sephacryl S-400 column in an attempt to resolve and detect any dimers. Radioactive cell walls were suspended in B.S.A. solution (pH 3.4) for 5 min to remove any traces of non-covalently bound glycoprotein. After centrifugation the B.S.A. solution was removed, the wall pellet re-suspended in 0.6% (w/v) B.S.A. solution and treated with 0.6% (w/v) sodium chlorite (pH 3.4) at 60°C for 5 min. The reaction was halted with 1.8 M-ammonia solution and sodium chloride was added (final concentration 1 M) to leach any solubilised glycoprotein that remained ionically bound to the cell walls. The cell walls were pelleted by centrifugation; a sample of the supernatant and the wall pellet were monitored for radioactivity in Triton scintillant and it was calculated that 14% of the radioactive material was solubilised. An allowance was made for the difference in counting efficiency between the supernatant and wall pellet using the factor of 2.3 (see Methods for further details). The supernatant was analysed by gel permeation chromatography and the elution profile (Fig. 38) showed that a peak of monomer extensin ($k_{av} = 0.56$) and a broad peak of low M_r breakdown products had been solubilised; it was also estimated from the calibration graph of k_{av} against M_r (Fig. 8) that a high M_r shoulder ($k_{av} = 0.43$) on the monomer peak could contain extensin dimers.

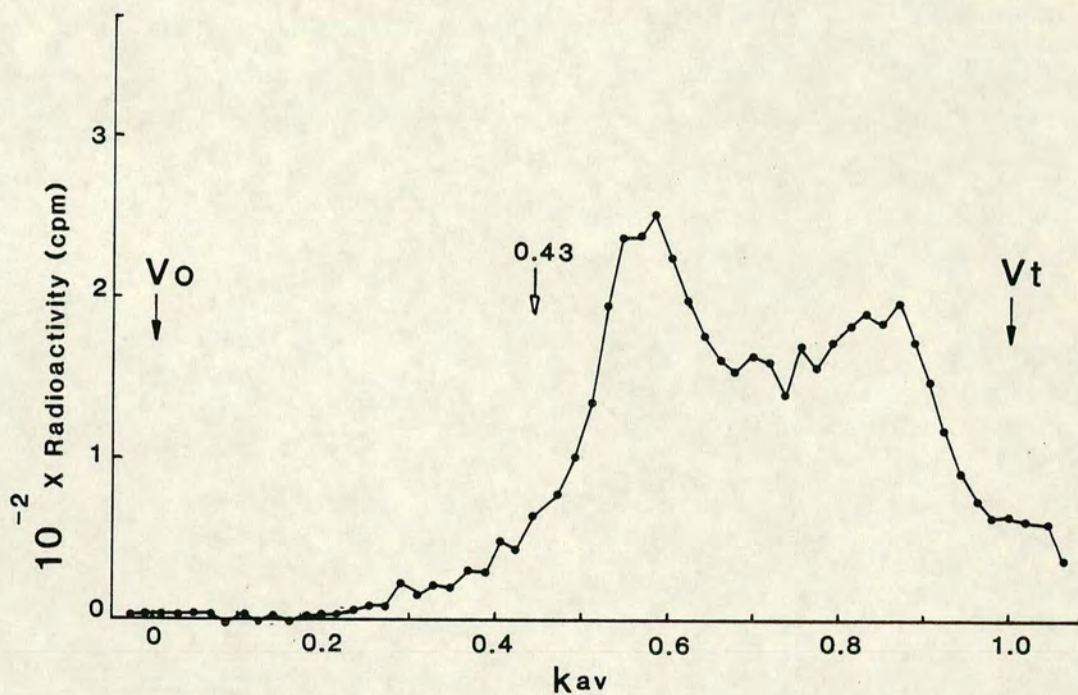


Fig. 38. Isolation of putative extensin dimer by gel permeation chromatography (on a long Sephacryl S-400 column) of glycoprotein solubilised from cell walls by acidified chlorite treatment. The column of Sephacryl S-400 (1.5 cm x 158 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Glycoprotein was solubilised by treating radioactive cell walls, in the presence of B.S.A., with acidified chlorite for 5 min at 60°C. Radioactivity = cpm/0.15 ml. Extensin monomer = k_{av} 0.56. Putative extensin dimer k_{av} 0.43. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

Fractions 47-55 were pooled, dialysed and dried under vacuum. A portion was re-analysed by gel permeation chromatography on the short (1.5 cm x 48 cm) Sephacryl S-400 column (Fig. 39) and the radioactivity eluted as a single peak with a k_{AV} value of 0.47; this value is slightly higher than the predicted $k_{AV} = 0.43$ for dimers but showed that the material had a higher M_r than monomer extensin. It was suggested that this peak could contain putative dimers. Alkaline hydrolysis and HVPE of a further portion of the pooled fractions (Fig. 40) confirmed that the material contained hydroxyproline-oligoarabinosides and was therefore extensin.

The remainder of the pooled material was dissolved in 0.6% (w/v) B.S.A. and treated with 0.6% (w/v) sodium chlorite (pH 3.4) at 60°C for 2 min. Following neutralisation with 1.8 M-ammonia solution the sample was analysed by gel permeation chromatography to see whether the putative dimer had been cleaved to produce a peak of monomer extensin. The results (Fig. 41) showed that only low M_r products were present and this implied that the acidified chlorite treatment had caused breakdown of the material; this was surprising because similar analysis of the solubilised monomer extensin peak (pooled fractions 60-75), before and after acidified chlorite treatment in the presence of B.S.A., did not show any shift in position of the peak (Fig. 42). This latter result was in agreement with previous results where it had been found that B.S.A. protected against peptidyl cleavage during acidified chlorite treatment. From this investigation it had not been possible to show whether extensin dimers had been isolated.

A final attempt was made to solubilise extensin oligomers by acidified chlorite treatment of cell walls that had been isolated from

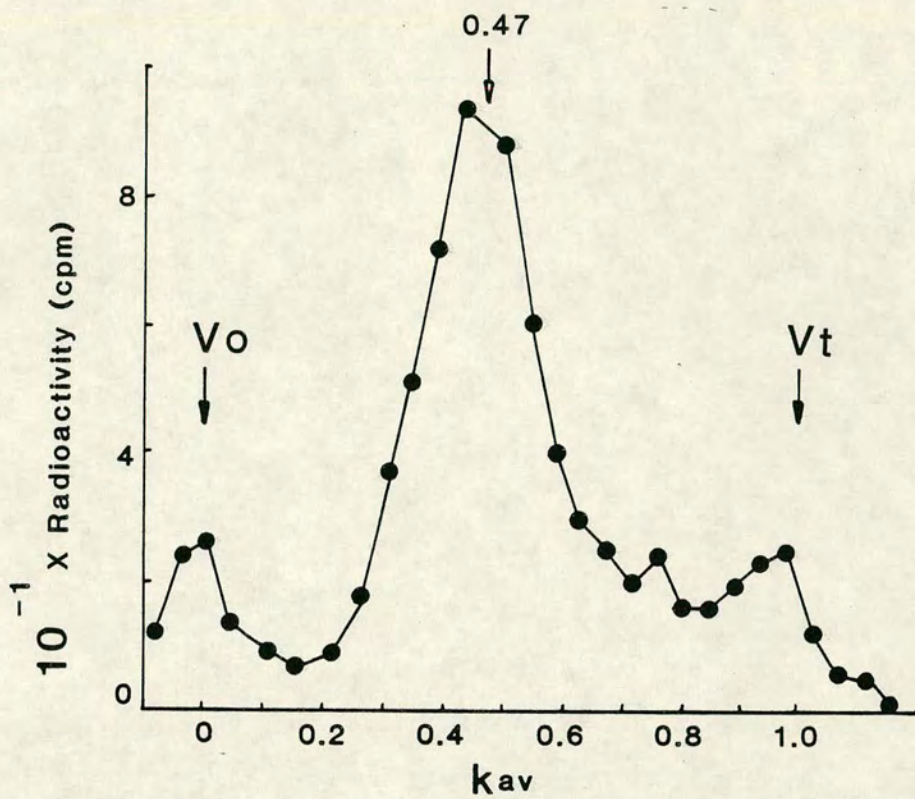


Fig. 39. Further analysis of putative extensin dimer by gel permeation chromatography (on Sephacryl S-400). The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactivity = cpm/0.25 ml. Putative extensin dimer = k_{av} 0.47. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

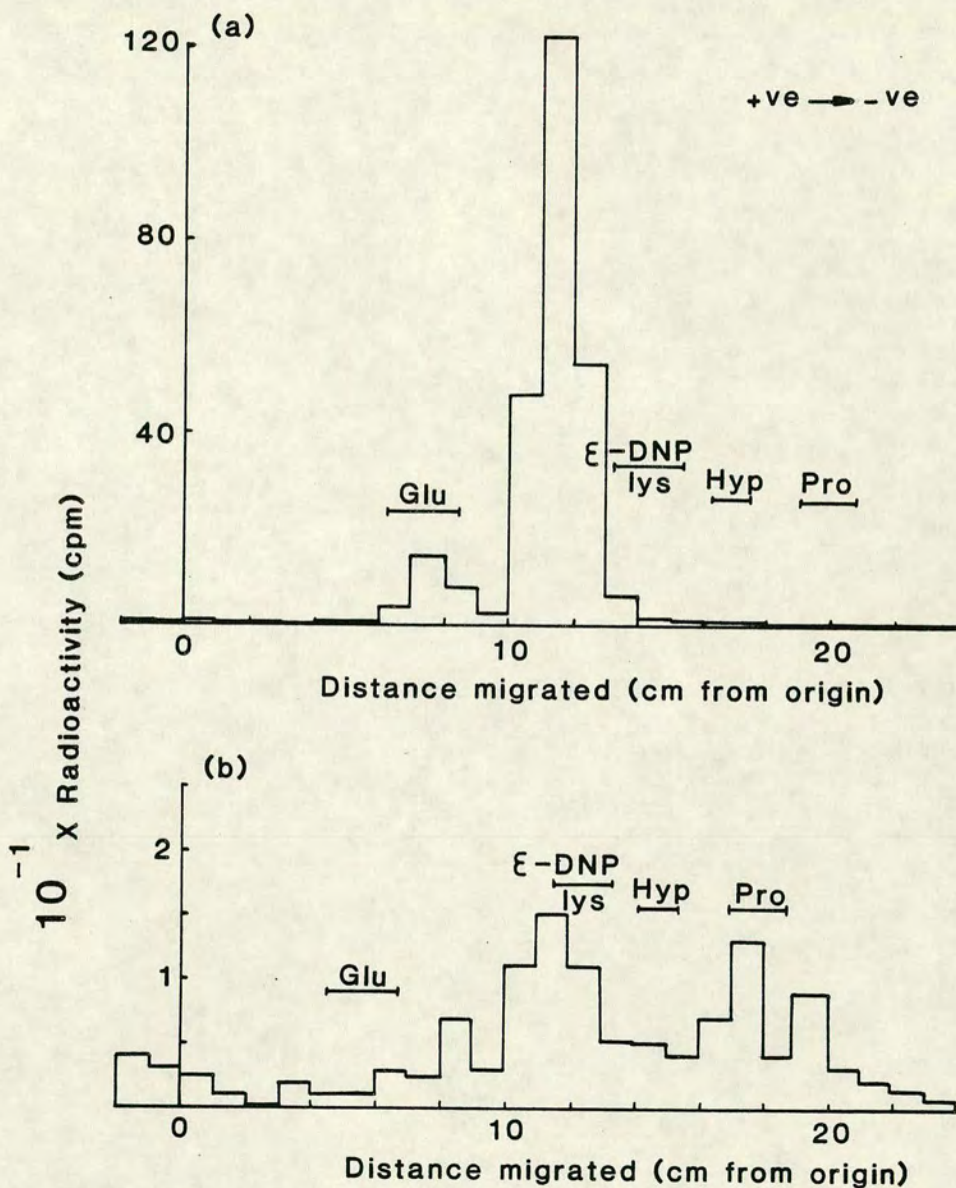


Fig. 40. Analysis of radioactive putative extensin dimer for the presence of $[^3\text{H}]$ hydroxyproline-oligoarabinosides. Samples were alkaline hydrolysed and the hydrolysis products separated by HVPE at pH 2 in formic acid/acetic acid/water (1:4:45 by vol).

(a) marker hydroxyproline-oligoarabinoside.

(b) putative extensin dimer ($k_{av} = 0.47$).

Radioactivity = cpm/strip. Glu = neutral marker. $\epsilon\text{-DNP lys}$ = yellow, +ve marker.

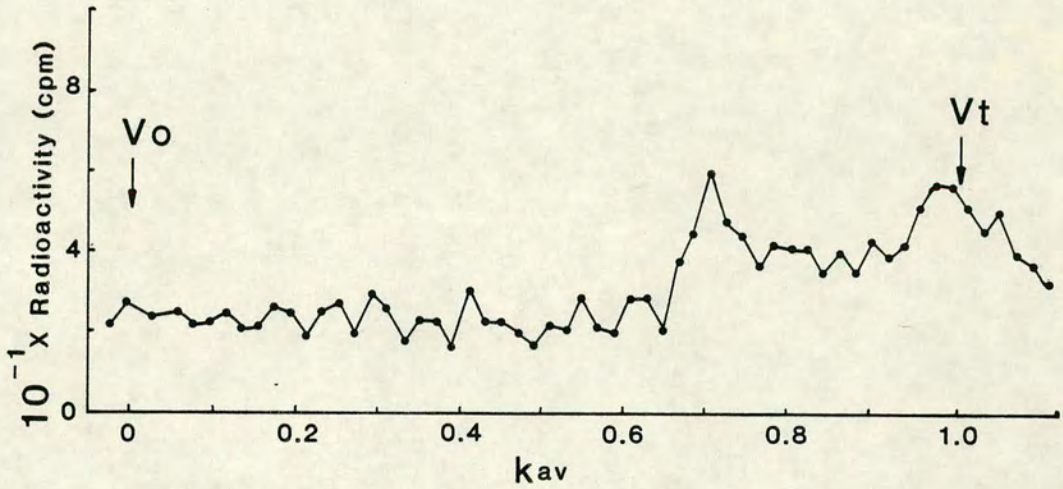


Fig. 41. Gel permeation chromatography (on a long Sephacryl S-400 column) of acidified chlorite treated, radioactive putative extensin dimer. The column of Sephacryl S-400 (1.5 cm x 158 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). The sample was treated, in the presence of B.S.A., with acidified chlorite for 2 min at 60°C. Radioactivity = cpm/1.0 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

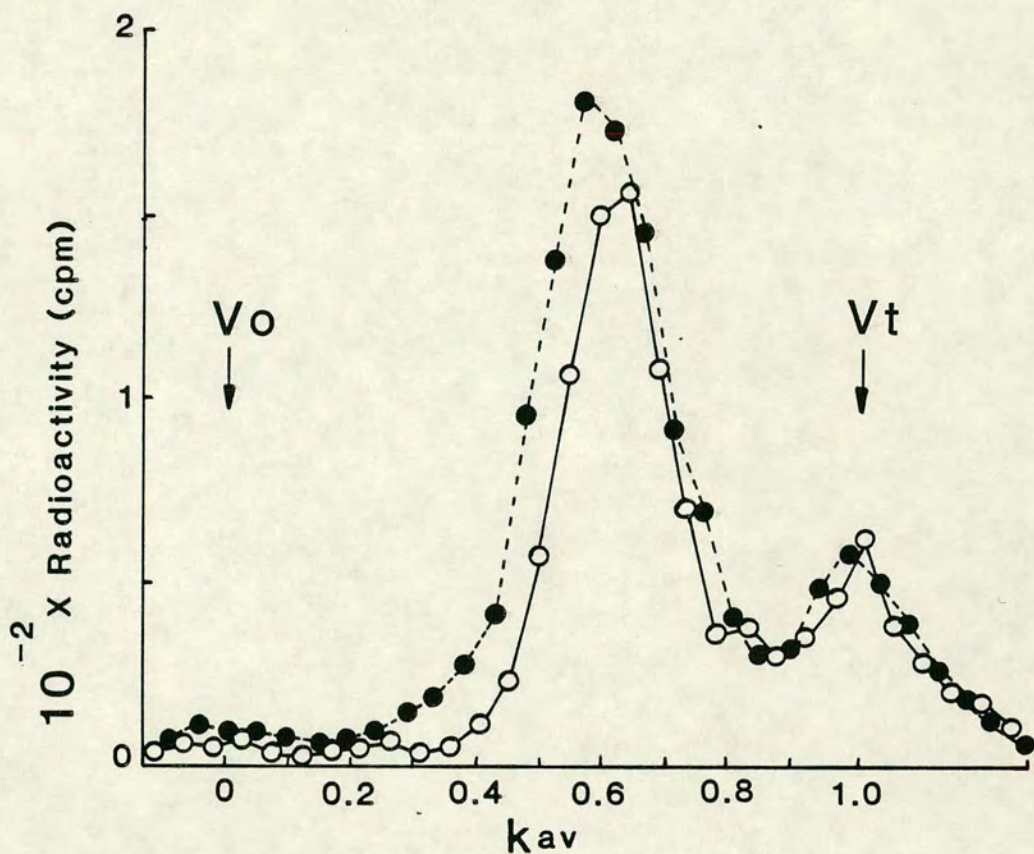


Fig. 42. Gel permeation chromatography (on Sephacryl S-400) of solubilised radioactive extensin monomer before and after acidified chlorite treatment in the presence of B.S.A. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). ○—○, extensin monomer (untreated); ●---●, extensin monomer (dissolved in 0.6% (w/v) B.S.A. and treated with 0.6% (w/v) sodium chlorite (pH 3.4) at 60°C for 2 min). Radioactivity = cpm/0.5 ml. V₀, elution volume of Blue Dextran; V_t, elution volume of sucrose.

Capsicum frutescens f₁ suspension culture fed with [³H]arabinose. [³H]arabinose was used as the radioactive precursor because it contained more radioactivity than the [³H]proline; therefore any oligomers present in small amounts should be easier to detect because they would also contain comparatively more radioactivity. The cell walls were suspended in a solution of 0.6% (w/v) B.S.A. and 0.2 M-sodium chloride; sodium chloride was present throughout the treatment so that glycoprotein would be leached from the cell walls as soon as it was solubilised and could, therefore, be afforded protection against any further breakdown from acidified chlorite by the presence of the B.S.A. in the solution. The sample was treated with acidified chlorite, neutralised with ammonia and analysed by gel permeation chromatography as in previous experiments. The elution profile (Fig. 43) again showed a peak of monomer extensin ($k_{av} = 0.56$) with a high M_r shoulder of putative dimer molecules ($k_{av} = 0.45$). Alkaline hydrolysis and HVPE confirmed that hydroxyproline-oligoarabinosides were present in the peak of monomer extensin and in pooled fractions (49-56) of the shoulder. A portion of the dialysed, dried material was re-analysed by gel permeation chromatography and a second portion was further treated with acidified chlorite in the presence of B.S.A. before re-analysis. Comparison of the two elution profiles (Fig. 44) showed that the material eluted in the same position before and after acidified chlorite treatment. As found in the above experiment the putative dimer re-eluted with an intermediate k_{av} of 0.49, although the width and shape of the peak made it difficult to determine an accurate value. Similarity of the two elution profiles before and after acidified chlorite treatment showed that B.S.A. had protected the molecules against peptidyl cleavage but it did not provide firm

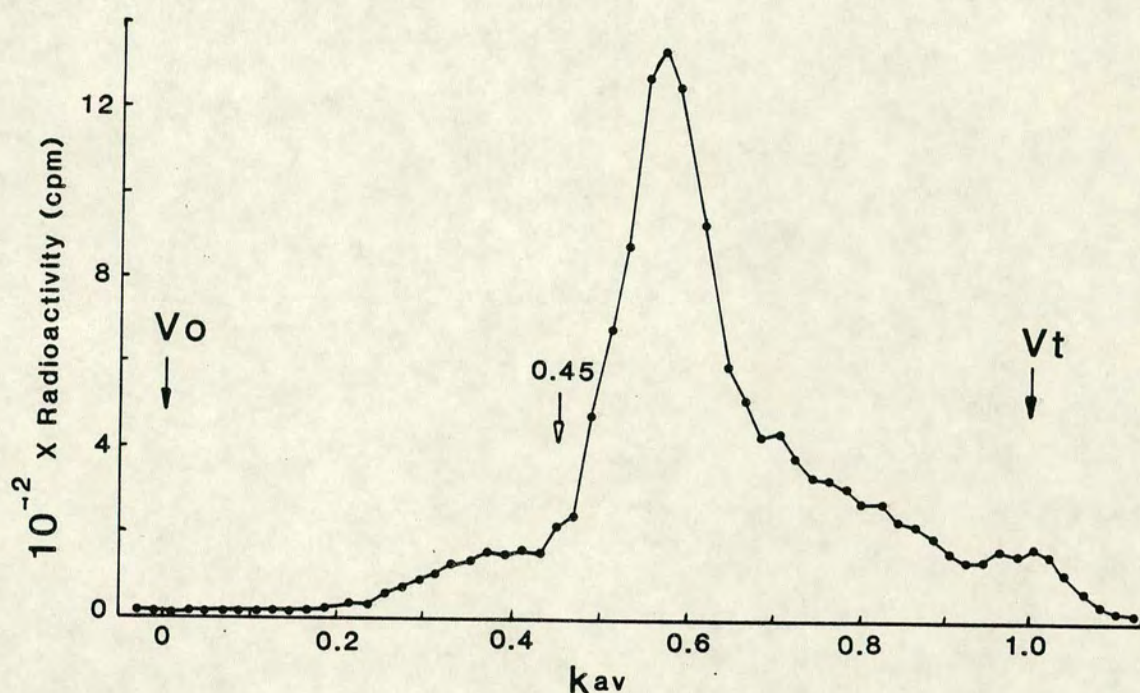


Fig. 43. Gel permeation chromatography (on a long Sephacryl S-400 column) of [arabinosyl-³H]glycoprotein solubilised from cell walls by acidified chlorite treatment. The column of Sephacryl S-400 (1.5 cm x 158 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactivity = cpm/0.1 ml. Extensin monomer = k_{av} 0.56. Putative dimer = k_{av} 0.45. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

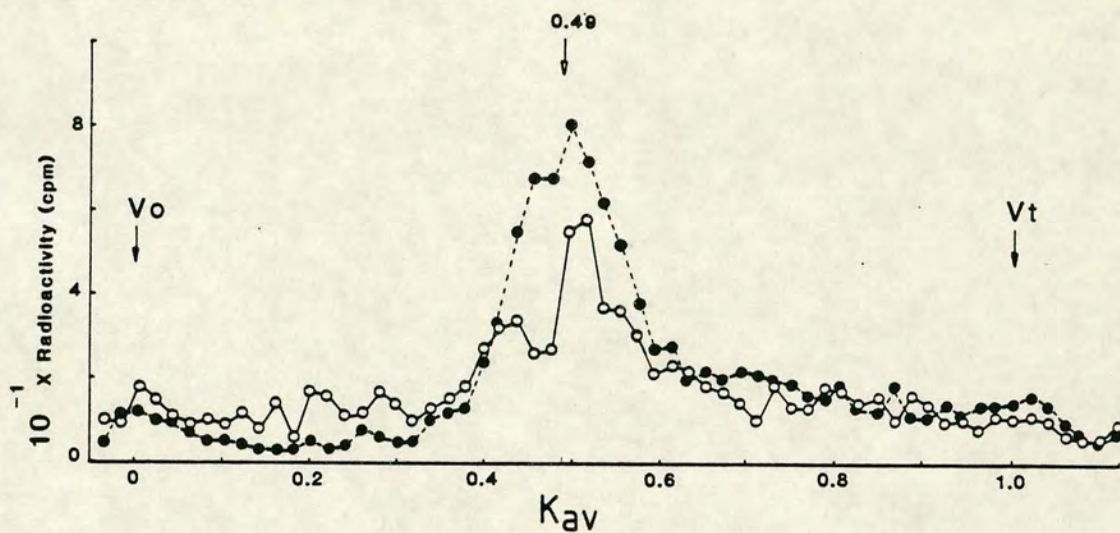


Fig. 44. Further analysis of putative [arabinosyl-³H]extensin dimer before and after acidified chlorite treatment in the presence of B.S.A., by gel permeation chromatography (on a long Sephacryl S-400 column). The column of Sephacryl S-400 (1.5 cm x 158 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). ○—○, putative extensin dimer (untreated); ●---●, putative extensin dimer (dissolved in 0.6% (w/v) B.S.A. and treated with 0.6% (w/v) sodium chlorite (pH 3.4) at 60°C for 5 min). Putative dimer = k_{av} 0.49. Radioactivity = cpm/0.4 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

evidence that putative dimers had been cleaved to monomer extensin. Several interpretations were possible from this result: (a) both peaks contained monomer extensin, which eluted with an apparent M_r slightly higher than that usually found, (b) the material solubilised from the cell walls contained 1.5 cross linked molecules of monomer extensin, thus giving an intermediate k_{av} value or (c) the peaks contained dimer extensin that was cross linked by an intermolecular bridge not susceptible to cleavage by acidified chlorite.

Conclusion

It did not seem possible to isolate extensin oligomers by either of the two approaches investigated. Although small peaks of high M_r radioactive material were isolated by salt elution of cell suspension cultures these did not appear to contain extensin and it is therefore suggested that monomer extensin is cross linked directly into the cell wall extensin network in Capsicum frutescens f₁. This result was surprising considering the successful isolation of oligomers by Heckman et al. (1988) from other cell suspension cultures. It is possible that dimers may be formed as intermediate cross linking products that are trapped around the molecules of other cell wall components and so cannot be leached from the cell wall by salt elution; experiments to investigate this possibility are described in the next chapter.

Acidified chlorite treatment of radioactive cell walls under conditions shown not to cleave peptide bonds did allow solubilisation of glycoprotein and intact monomer extensin was isolated. This is the first report of such solubilisation of extensin from cell walls and shows that extensin is cross linked into the wall network by bonds

that are susceptible to acidified chlorite. Although such acidified chlorite treatments have also been shown to cleave isodityrosine, it was not possible to conclude that isodityrosine was the cross linking molecule which had been cleaved in extensin in order to solubilise monomers.



Release of trapped dimers

Introduction

In the last chapter it was concluded that extensin dimers cannot be detected in salt leachates from cell walls of Capsicum frutescens f₁ suspension cultures and it was suggested that dimers may be trapped by forming cross links around the molecules of other cell wall components, such as cellulose microfibrils. One way of investigating this possibility would be to remove the other wall components in order to leave an extensin network and, at the same time, release any trapped extensin oligomers. Driselase (Sigma Chemical Co.), a mixture of fungal endo- and exo-enzymes, has been shown to digest various cell wall polysaccharides including cellulose, whilst it has been suggested that the polypeptide backbone of extensin may be resistant to Driselase digestion (Fry, 1988), and so treatment of radioactive Capsicum frutescens f₁ cell walls with Driselase could facilitate the release of any trapped dimers.

In this chapter, experiments to investigate the action of Driselase upon extensin are described; first it was necessary to determine whether Driselase contains β -arabinosidase activity and/or protease activity. It is known that Driselase has α -arabinosidase activity which will cause the terminal arabinose units to be cleaved from the extensin tetra-arabinoside side chains, but because the other arabinose units are β -linked (see Introduction) they will only be removed if Driselase possesses β -arabinosidase activity.

Driselase digestion of [arabinosyl-³H]extensin

Two samples of freeze dried [arabinosyl-³H]extensin monomer

were dissolved in pyridine/acetic acid/water (1:1:23 by vol) (pH 4.7) and treated with Driselase, dissolved in the same solution, to give final concentrations of 1% and 2% (w/v) Driselase. The samples were incubated at 37°C and aliquots of each sample were removed at 0, 5, 10, 20, 30, 45, 60, 120, 240 and 360 min and loaded onto Whatman 3MM paper for analysis by paper chromatography (see Methods). At time 0 min all of the radioactivity was present as polymer (Fig. 45) but incubation with Driselase resulted in the release of arabinose from the side chains of extensin molecules and the reaction occurred at a slightly faster rate with 2% Driselase present. After 360 min incubation 38-46% of the radioactive polymer had been broken down, which suggested that β -arabinosidase activity was present because if only the terminal arabinose residues of hydroxyproline-tetra-arabinosides had been removed, by α -arabinosidase activity, a maximum of 25% of the radioactive polymer could be lost.

An additional aliquot of the 1% Driselase incubation mixture, removed after 60 min, was frozen in liquid nitrogen to prevent further enzyme activity. The sample was later thawed and analysed by gel permeation chromatography on a Sephacryl S-400 column to see whether there was a shift in the elution position of monomer extensin, and therefore a change in apparent M_r . The elution profile (Fig. 46) showed that no peak of monomer extensin ($k_{av} = 0.55$) was present, although a peak of material of low M_r was detected. This could represent (i) partially deglycosylated extensin, which would appear smaller to the gel permeation medium if extensin folded up more tightly when the arabinoside sidechains were partially removed or (ii) extensin which had been broken down to low M_r products by proteolytic activity. It was therefore decided to investigate next the activity

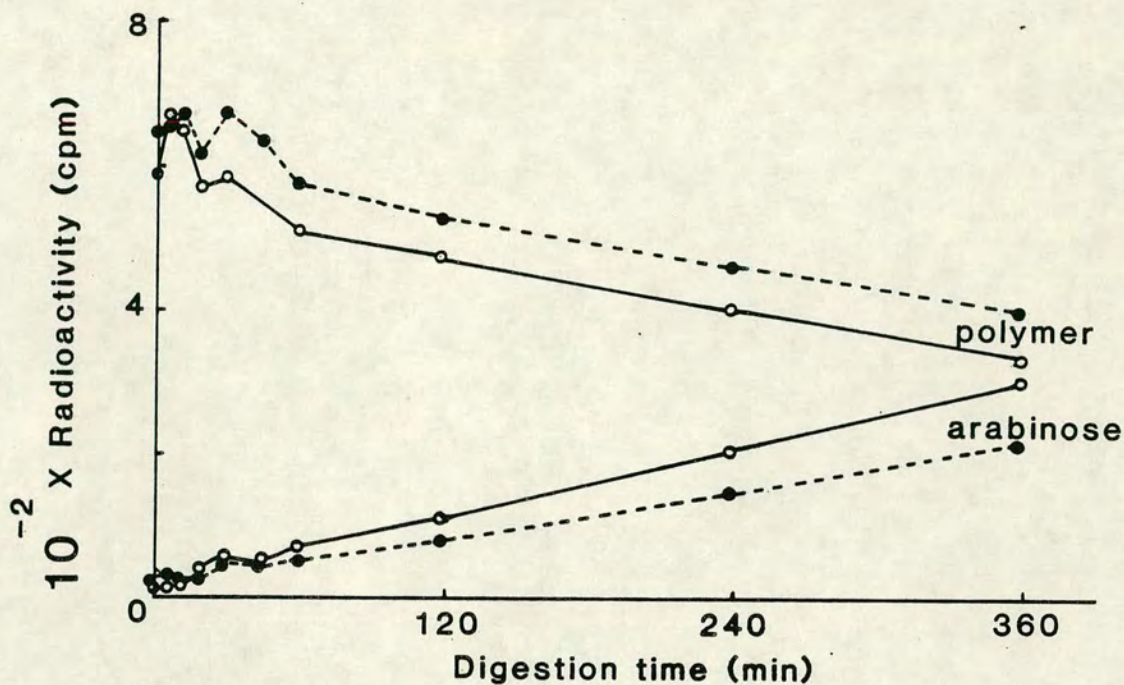


Fig. 45. Partitioning of radioactivity between polymer and free arabinose in Driselase digested [arabinosyl-³H]extensin monomer. Samples of [arabinosyl-³H]extensin were digested with 1% and 2% (w/v) Driselase in pyridine/acetic acid/water (1:1:23 by vol) (pH 4.7) at 37°C for varying times. The digests were analysed by paper chromatography in butan-1-ol/acetic acid/water (12:3:5 by vol).

●—●, [arabinosyl-³H]extensin digested with 1% (w/v) Driselase;

○—○, [arabinosyl-³H]extensin digested with 2% (w/v) Driselase.

Radioactivity = cpm/0.025 ml.

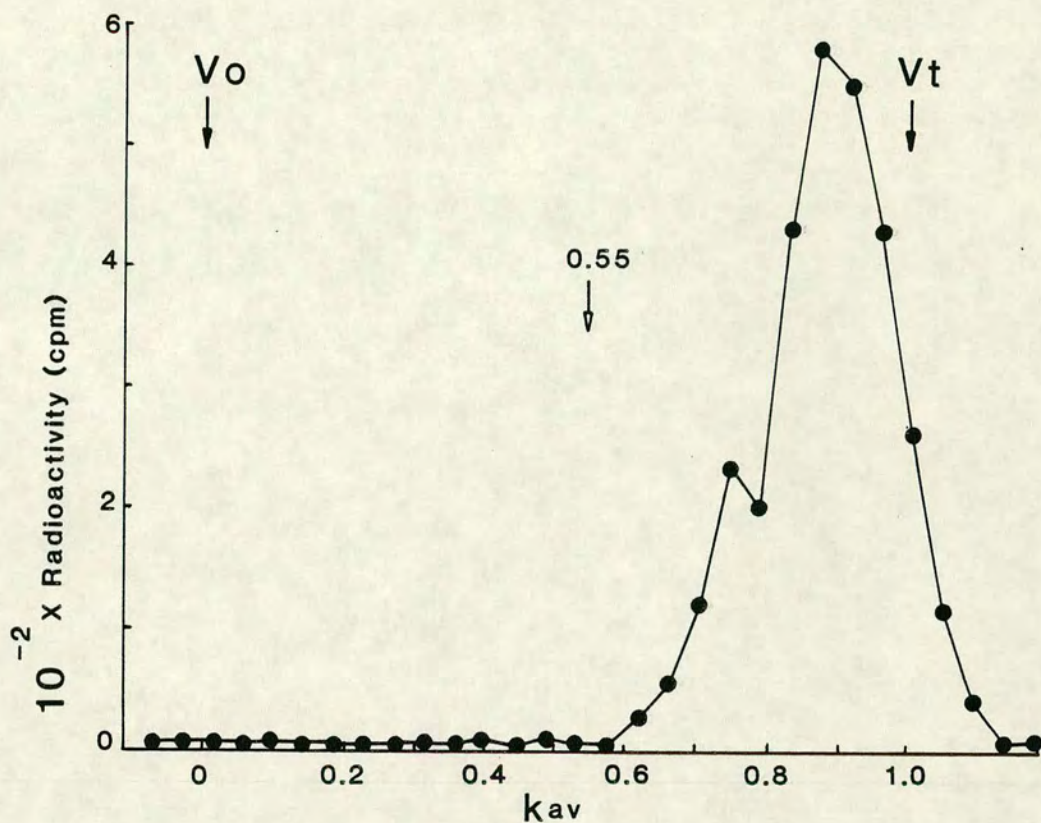


Fig. 46. Gel permeation chromatography (on Sephacryl S-400) of Driselase digested [arabinosyl-³H]extensin monomer. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). The sample of [arabinosyl-³H]extensin monomer was digested with 1% (w/v) Driselase at 37°C for 60 min. Elution position of extensin monomer = k_{av} 0.55. Radioactivity = cpm/0.45 ml. V₀, elution volume of Blue Dextran; V_t, elution volume of sucrose.

of Driselase upon [(hydroxy)prolyl-³H]extensin monomer to see whether Driselase did contain any proteolytic enzymes.

Driselase digestion of [(hydroxy)prolyl-³H]extensin

Freeze dried [(hydroxy)prolyl-³H]extensin monomer was dissolved in pyridinium acetate buffer (pH 4.7), incubated with Driselase [final concentration = 1% (w/v)] at 37°C and a time course carried out as in the previous experiment. Results of paper chromatography (Fig. 47) showed a decrease in radioactive polymer during the first 60 min of incubation with Driselase but after that the cpm values reached a plateau. No radioactivity was detected as proline or hydroxyproline which showed that extensin was not broken down to these amino acids; however the results did suggest that some cleavage of peptide bonds had occurred. The elution profile of a sample (60 min incubation) analysed by gel permeation chromatography (Fig. 48) revealed the presence of a peak with low apparent M_r , as found in the previous experiment, but it was not possible to conclude whether this shift in peak position was the result of cleavage of the polypeptide backbone or removal of the oligoarabinoside side chains. A solution to this problem would be to deglycosylate extensin monomer and see where the resulting material eluted on a gel permeation chromatography column.

Deglycosylation of extensin monomer

Anhydrous hydrogen fluoride has been used to deglycosylate extensin (Mort & Lamport, 1977) but this reagent is extremely corrosive and so, trifluoromethanesulphonic acid (TFMS) which has also been shown to deglycosylate various glycoproteins (Desai et al., 1983;

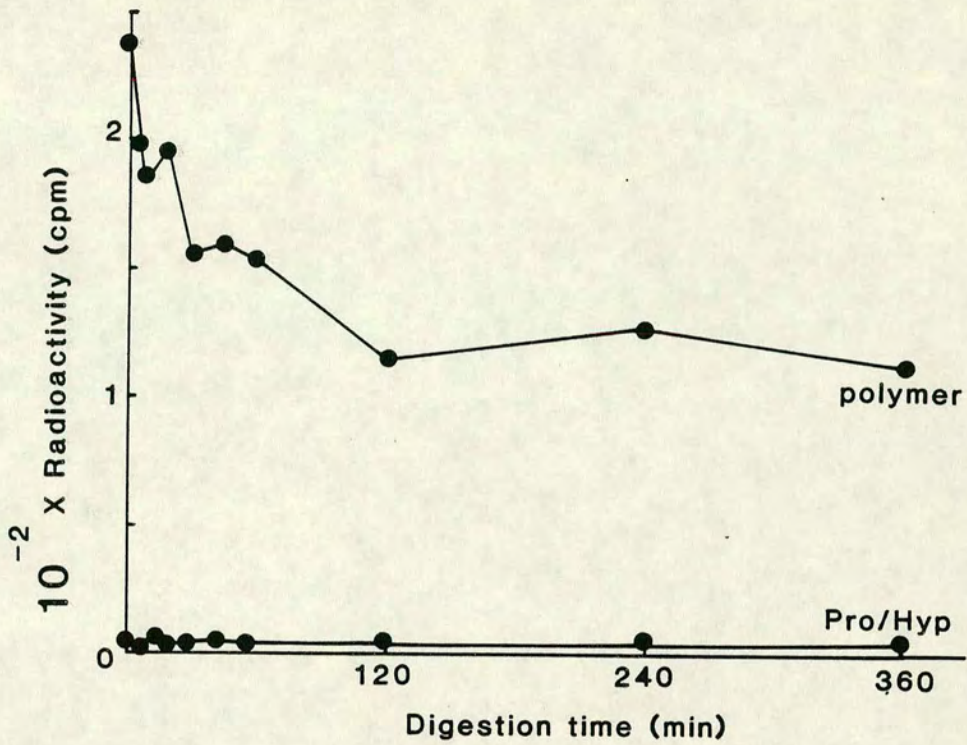


Fig. 47. Partitioning of radioactivity between polymer and free proline/hydroxyproline in Driselase digested [(hydroxy)prolyl- ^3H]-extensin monomer. The sample was digested with 1% (w/v) Driselase in pyridine/acetic acid/water (1:1:23 by vol) at 37°C for varying times. The digests were analysed by paper chromatography in butan-1-ol/acetic acid/water (12:3:5 by vol). Radioactivity = cpm/0.025 ml.

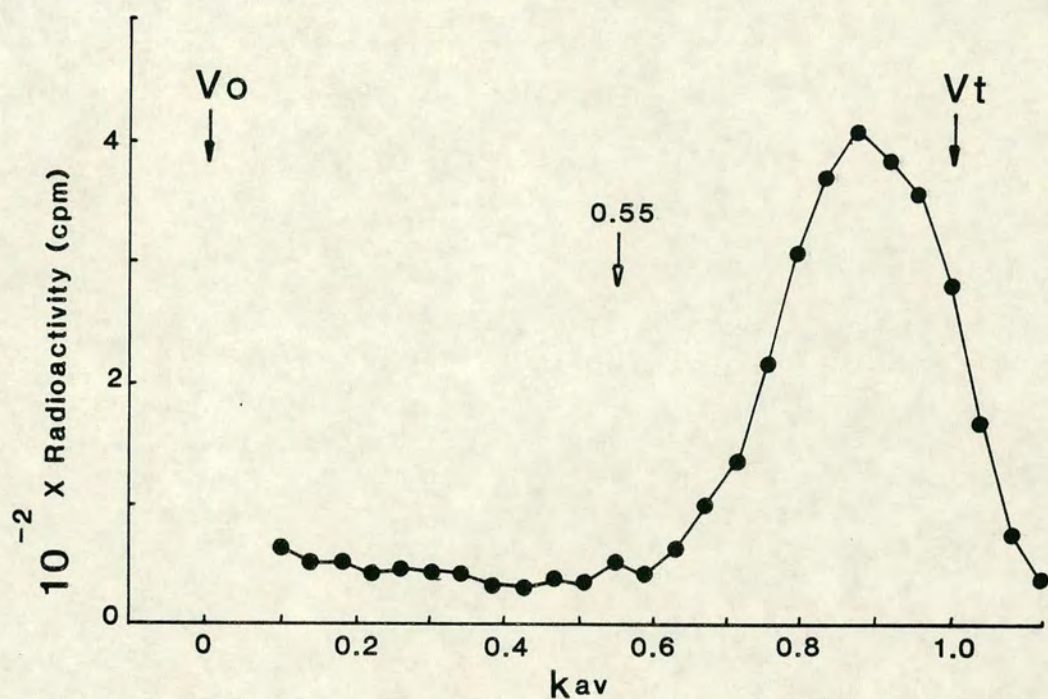


Fig. 48. Gel permeation chromatography (on Sephacryl S-400) of Driselase digested [(hydroxy)prolyl- ^3H]extensin monomer. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). The sample of [(hydroxy)prolyl- ^3H]extensin monomer was digested with 1% (w/v) Driselase at 37°C for 60 min. Elution position of extensin monomer = k_{av} 0.55. Radioactivity = cpm/0.75 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

Sojar & Bahl, 1987) was tested upon [(hydroxy)prolyl-³H]extensin monomer and [arabinosyl-³H]extensin monomer. Successful deglycosylation would result in the removal of radioactive arabinose residues which would be detected in the dialysis medium, but radioactivity should not be lost from the extensin containing [³H]hydroxyproline residues, and so the elution position of deglycosylated extensin could be determined.

Samples of [arabinosyl-³H]extensin monomer and [(hydroxy)prolyl-³H]extensin monomer were treated with TFMS acid as described in the Methods, and the acid was neutralised by procedure B (addition of diethyl ether and 50% pyridine in water). The neutralised, aqueous samples were dialysed against 2 mM-pyridine, adjusted to pH 5.5 with acetic acid, and aliquots of the sample and dialysis medium were scintillation counted. From the cpm values it was calculated that 10% of the [³H]arabinose was present in the sample and 90% had diffused into the dialysis medium, whilst 50% of the [³H](hydroxy)proline remained in the sample. This suggested that extensin had been deglycosylated; however it would appear that some breakdown of the peptide backbone had also occurred because [³H]proline or [³H]hydroxyproline residues were present in the dialysis medium. The dialysed [(hydroxy)prolyl-³H]extensin sample was dried under vacuum and analysed by gel permeation chromatography on Sephacryl S-400 and the elution profile (Fig. 49) showed that the radioactive material eluted as a single, broad peak with a $k_{av} = 0.69$. The material therefore had a lower M_r than monomer extensin but did not elute at V_t , which suggested that this could be the elution position of deglycosylated extensin; however, the possibility that some cleavage of the peptide backbone had also occurred could not be

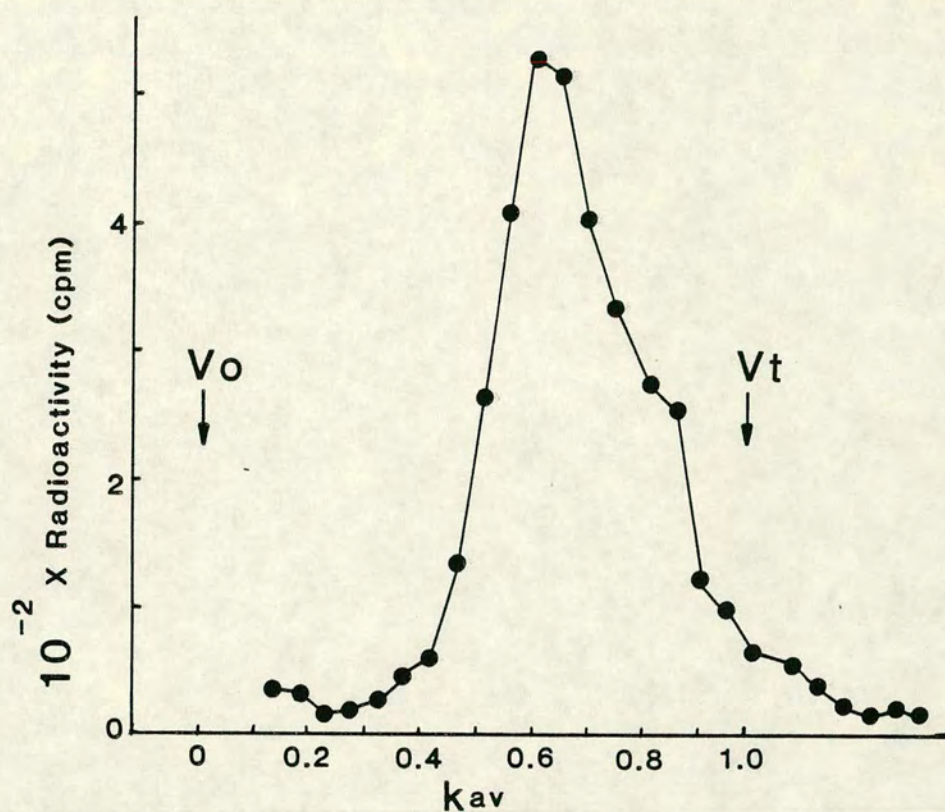


Fig. 49. Gel permeation chromatography (on Sephacryl S-400) of [(hydroxy)prolyl-³H]extensin monomer deglycosylated by treatment with TFMS acid. The column of Sephacryl S-400 (1.5 cm x 48 cm) was eluted with 1 M-sodium chloride/20 mM-Mes (pH 6.1). Radioactivity = cpm/0.75 ml. V_0 , elution volume of Blue Dextran; V_t , elution volume of sucrose.

excluded. The important point is that the apparent M_r of this material was higher than that of Driselase-treated extensin which implied that Driselase does contain a protease capable of causing peptide cleavage in monomer extensin.

Driselase digestion in the presence of protease inhibitors

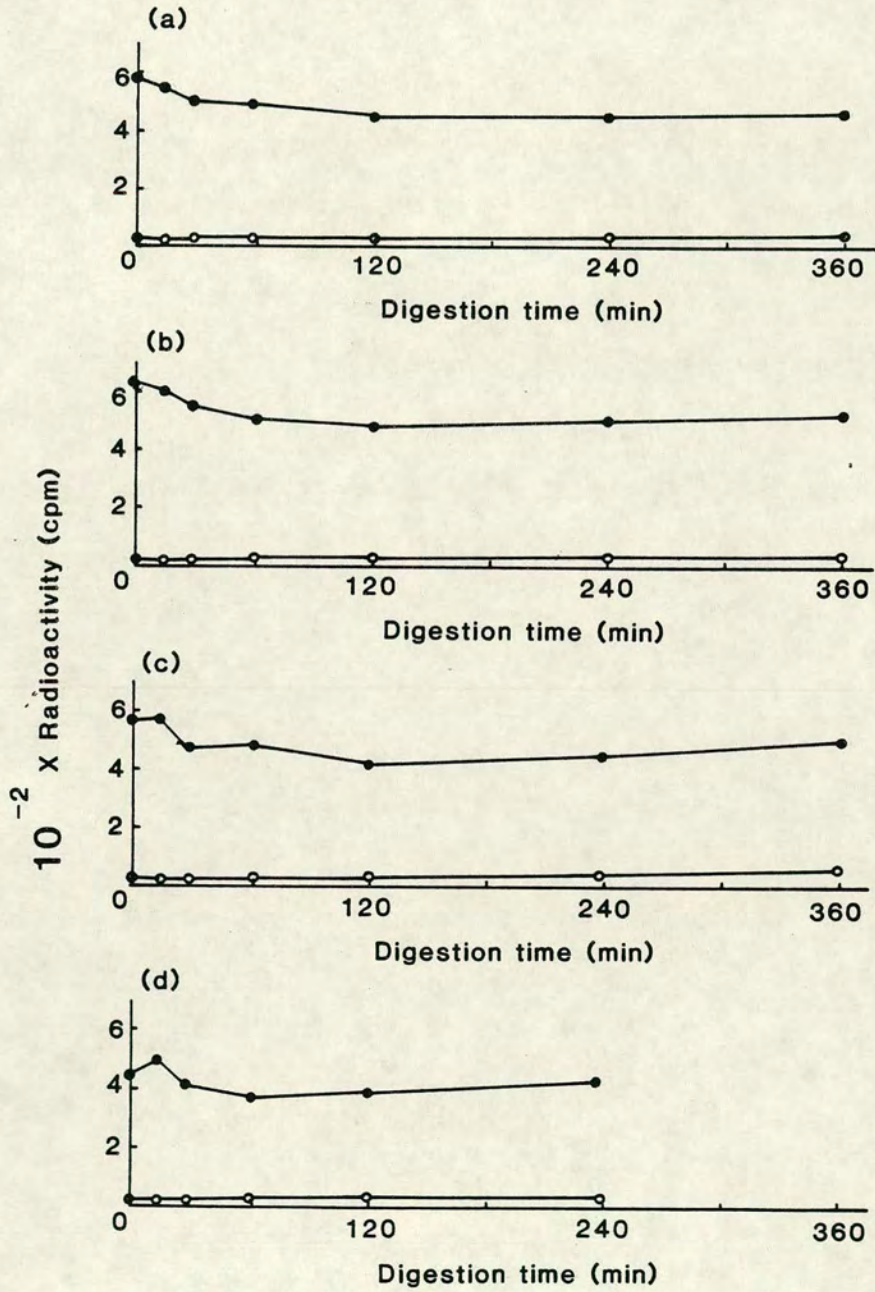
An attempt was made to block the action of the apparent protease in Driselase by use of (a) phenylmethylsulphonylfluoride (PMSF) which is a serine protease inhibitor active at a concentration of 1 mM (Means & Feeney, 1971), (b) non-radioactive arabinose (final concentration = 40 mM) which could block arabinosidase activity (Fry, 1983b) and, by leaving extensin in a fully glycosylated state, may exclude proteases from acting upon the peptide backbone and (c) B.S.A. at a final concentration of 1% (w/v) which could block protease activity by providing an alternative, more abundant, substrate for enzyme digestion.

Freeze dried [(hydroxy)prolyl- ^3H]extensin monomer was divided into four samples and incubated with Driselase [final concentration = 1% (w/v)], alone and in the presence of each of the above potential inhibitors. Driselase and PMSF were pre-incubated together at 37°C for 10 min prior to addition to the sample. All of the samples were incubated at 37°C and a time course was carried out in which aliquots were removed from each sample, loaded onto Whatman 3MM and analysed by paper chromatography (see Methods). The results (Fig. 50) showed that for the control (minus inhibitor) there was a decrease in radioactivity at the origin during the first 60 min of incubation and then the cpm values reached a plateau; this was accompanied by detection of free proline/hydroxyproline, which

Fig. 50. Partitioning of radioactivity between polymer and free proline/hydroxyproline in [(hydroxy)prolyl-³H]extensin monomer digested with Driselase in the presence of potential protease inhibitors. The samples were digested with 1% (w/v) Driselase in pyridine/acetic acid/water (1:1:23 by vol) at 37°C for varying times in

- (a) the absence of an inhibitor
- (b) the presence of 1 mM-PMSF
- (c) the presence of 40 mM-arabinose
- (d) the presence of 1% (w/v) B.S.A.

The digests were analysed by paper chromatography in butan-1-ol/acetic acid/water (12:3:5 by vol). ●—● , radioactive polymer; ○—○ , proline/hydroxyproline. Radioactivity = cpm/0.025 ml.



confirmed that an active protease was present in Driselase. Similar results were observed for each of the other samples which would imply that none of the potential inhibitors had inhibited the protease activity.

Conclusion

Investigations into Driselase digestion of [arabinosyl]-³H]-extensin monomer and [(hydroxy)prolyl]-³H]extensin monomer suggested that Driselase contained β -arabinosidase and protease activity. It was too difficult to interpret from gel permeation chromatography results whether a shift in peak position was due to removal of the arabinose side chains or to cleavage of the peptide backbone; an attempt to solve this problem by deglycosylation with TFMS acid, which would demonstrate the elution profile of deglycosylated extensin, was not completely successful because there also appeared to be some peptidyl cleavage during this treatment. Further experiments with monomer extensin and TFMS acid would be necessary to confirm whether the peptide backbone was cleaved, and to assess the extent of such cleavage. However, the decrease in apparent M_r following Driselase digestion of extensin ($k_{av} = 0.88$) was much greater than the decrease resulting from TFMS acid treatment ($k_{av} = 0.69$), where 90% of the [³H]arabinose was removed; this provided further evidence that Driselase contained protease activity.

Tests with a variety of possible protease inhibitors, intended to block enzymic digestion of the peptide backbone of extensin, did not prove successful and it was concluded that Driselase could not be satisfactorily used in an attempt to isolate any intact, trapped extensin dimers. The experiments showed, however, that

Driselase would appear to contain more enzymes active upon cell wall components than was previously thought. No further investigations into methods for releasing trapped dimers were conducted but it is suggested that stepwise degradation of cell walls with purified enzymes, such as cellulase, would form a better approach.

DISCUSSION

It is generally agreed (Lampert, 1985; Roberts et al., 1985) that extensin is a structural component of the primary cell wall but the problem of how it is held in the cell wall remains unsolved. As discussed in the Introduction there is evidence to show that extensin exists as a network, which suggests that there must be cross-links between extensin molecules. Various pieces of indirect evidence point to isodityrosine as the prime candidate for this cross-link; however, conclusive proof remains elusive. In the Introduction various questions were asked that need to be answered in order to demonstrate the existence of intermolecular isodityrosine cross-links in extensin and, having described experiments undertaken to attempt to find the answers, we are now in a position to consider the results and discover what can be learned from them.

It was first necessary to identify a cell suspension culture that was rich in extensin and this was investigated by feeding radioactive precursors to a variety of suspension cultures. It was found that Capsicum frutescens suspension culture was most efficient at incorporating radioactivity, in the form of [³H]proline, into insoluble wall glycoprotein, particularly into hydroxyproline residues. Capsicum frutescens m culture, of compact cells, had a higher hydroxyproline content than other, finer cell suspension cultures, although Capsicum frutescens f₁, a more friable culture than Capsicum frutescens m, had an even higher hydroxyproline content. It seems probable that the amount of extensin present in cell walls varies between species (Fry, 1982) but that within a species more friable cultures have a higher hydroxyproline content; this is in

agreement with Halmer & Thorpe (1976), who observed that, in tobacco callus tissue, friable tissue had a higher hydroxyproline content than compact tissue.

Having determined that Capsicum frutescens f₁ suspension culture was a suitable source of extensin the complete amino acid profile of the cell wall glycoprotein was obtained. Cell wall glycoprotein was also prepared from natural plant tissues in order to monitor the variation of amino acids present (particularly hydroxyproline), and also to examine for the presence of isodityrosine. O'Neill & Selvendran (1980), in their study of parenchyma tissue from runner beans, pointed out the relevance of examining natural tissues which may differ from the experimental systems of cell suspension cultures and isolated carrot root discs upon which most work has been done. The results of amino acid analysis showed that hydroxyproline was present at low levels in cell walls of all the tissues tested and the hydroxyproline content of the cell suspension culture was higher; this is in agreement with the findings of Lamport (1965). Hydroxyproline content of the natural plant tissues examined was less than that reported in runner beans but variation between plant species and tissues is, perhaps, to be expected. Of the plant tissues examined all were dicotyledonous except for onion; as mentioned in the Introduction monocotyledonous plants typically have a lower HRGP content.

Isodityrosine was detected in all of the samples, indicating that this phenolic amino acid is also present in natural plant tissues, although it was not always possible to quantify the amount. Capsicum fruit and Capsicum frutescens f₁ suspension culture both contained significant amounts of isodityrosine; this further suggests

that Capsicum frutescens f₁ culture is a good system to study in order to demonstrate the existence of intermolecular isodityrosine. However, the results of amino acid analysis do not allow any distinctions between intra- and intermolecular isodityrosine to be made.

The potential of sodium chlorite and N-bromosuccinimide as reagents to solubilise glycoprotein from Capsicum frutescens f₁ cell walls was investigated, and it was shown that sodium chlorite was the more suitable reagent, solubilising 85-90% glycoprotein compared to 5-6% solubilised by N-bromosuccinimide. N-Bromosuccinimide also proved to be an unsuitable reagent because it cleaved peptide bonds, in agreement with earlier observations (Ramachandran & Witkop, 1967). In order to discover whether sodium chlorite solubilises glycoprotein by specifically cleaving phenolic cross-links, a sample of salt-soluble monomer extensin, leached from spinach suspension culture cell walls, was treated with acidified sodium chlorite. O'Neill & Selvendran (1980) reported that the only side-effect of sodium chlorite treatment was the modification of lysine and tyrosine residues but gel permeation chromatography of acidified chlorite treated monomer extensin clearly showed that acidified chlorite had a more drastic effect upon extensin. Two interpretations of this result were considered: (i) acidified chlorite can cleave peptide bonds (ii) spinach monomer extensin was not really monomeric and was, in fact, already cross-linked by isodityrosine. It was shown that the former suggestion was correct because purification and amino acid analysis of spinach monomer extensin revealed that no isodityrosine was detectable. Amino acid analysis confirmed that the material leached from spinach cell walls was extensin and the amino acid profile was

very similar to that of the tomato suspension culture precursor P1 (Smith et al., 1986). The finding that acidified chlorite was able to cleave peptide bonds meant that glycoprotein solubilisation could have occurred by peptidyl cleavage and was not necessarily by cleavage of phenolic bonds; this therefore suggested that it might not be possible to use sodium chlorite as a specific cleavage reagent.

Cleavage of peptide bonds by acidified sodium chlorite was investigated further by treating a standard protein, B.S.A., with acidified chlorite. The results of this showed that B.S.A. could be cleaved and that the ratio of B.S.A.:sodium chlorite was an important factor in predicting peptide cleavage; with an excess of sodium chlorite present peptidyl cleavage of B.S.A. was observed but at molar ratios of 0.75:1 B.S.A.(amino acyl residues):sodium chlorite, or higher, no peptide cleavage was detected. It is not known how this effect works, nor whether acidified chlorite breaks specific peptide bonds or causes random cleavage. The possibility that a trace impurity present in the sodium chlorite could be responsible was considered. The purity of the sodium chlorite was checked by ion chromatography but analysis did not reveal the presence of any anionic contaminants, other than those known to be present (sodium chloride, sodium carbonate and sodium chlorate). Furthermore, a sample of purified sodium chlorite, recrystallised to remove any contaminants, and then used in an acidified chlorite treatment of monomer extensin, caused the same cleavage (as shown by gel permeation chromatography) as that caused by treatment with crude sodium chlorite.

The study of acidified chlorite action upon proteins was extended to consider lysozyme. Under the limited conditions tested no cleavage was detected, but problems with protein precipitation caused

this study to be abandoned. To understand the effects that sodium chlorite treatments have on proteins and glycoproteins further experiments need to be undertaken.

The possibility of using B.S.A. to protect the peptide backbone of extensin molecules from cleavage during acidified chlorite treatments was investigated, because in experiments with salt-soluble extensin there was always an excess of sodium chlorite present. Treatment of spinach monomer extensin with acidified chlorite in the absence and presence of B.S.A. demonstrated that B.S.A. could protect the peptide bonds in extensin; the most probable explanation for this effect was that the excess of B.S.A. peptide bonds would be more likely to be cleaved by sodium chlorite. Having now ascertained that cleavage of peptide bonds by sodium chlorite can be avoided in the presence of B.S.A., it is equally important to determine whether cleavage of isodityrosine is also affected. Experiments with free isodityrosine treated with acidified chlorite in the absence and presence of B.S.A. demonstrated that isodityrosine cleavage still occurred, although at a slower rate. This would suggest that the reaction mechanism for action of acidified chlorite upon isodityrosine differs from that upon peptide bonds. If it is assumed that the likelihood of a chlorite molecule reacting with isodityrosine or protein is equal, then a slower rate of breakdown of isodityrosine would be expected in the presence of B.S.A.. It should be noted that free isodityrosine may react differently from isodityrosine cross-links in cell wall glycoprotein. In conclusion, conditions have been defined where peptidyl cleavage can be avoided and sodium chlorite can be used to specifically cleave isodityrosine.

In order to demonstrate the existence of intermolecular

isodityrosine by this specific chemical treatment it was also necessary to isolate extensin fragments (for example, dimers) that could then be cleaved to monomers. A gel permeation chromatography system was devised to optimise separation of monomers from oligomers and the column was calibrated with dextran markers in order to help predict the elution position of extensin dimers. It was assumed that dimers would elute with an apparent M_r of approximately twice that of monomers but the exact elution position of dimers would depend upon their shape, which would in turn be affected by the location of cross-links. Electron microscopy visualisation of extensin oligomers (Stafstrom & Staehelin, 1986a; Heckman et al., 1988) may help to determine the true appearance of these molecules. Heckman et al. (1988) have suggested that extensin molecules may reptate (end on migration of a linear polymer); this would lead to their retardation in the gel matrix and thus give a different elution position from that predicted by the M_r of the molecule. By the use of dextran markers it was hoped to partially overcome this problem because linear dextran molecules might also be expected to migrate through the gel matrix by reptation.

Salt-soluble extensin oligomers have been isolated from the walls of suspension culture cells (Heckman et al., 1988) and would therefore appear to be a good system in which to look for intermolecular isodityrosine in extensin. Several experiments were carried out in an attempt to isolate extensin oligomers from Capsicum frutescens f₁ suspension cultures. Peaks of high M_r radioactive material were detected following separation by gel permeation chromatography but these did not appear to contain extensin, as determined by alkaline hydrolysis to test for the presence of

characteristic hydroxyproline-tetra-arabinosides. The inability to isolate salt-soluble oligomers would suggest that either extensin monomers are inserted directly into the wall extensin network in Capsicum frutescens f₁ suspension cultures, or that extensin dimers do exist but they are present at levels too low to be detected, or are trapped around other components of the cell wall, such as cellulose microfibrils. The possibility of releasing trapped extensin oligomers was investigated by Driselase digestion of cell walls; if the fungal enzyme mixture, Driselase, did not contain any protease activity then it could be used to digest the other wall components and release extensin oligomers. However, it was shown that Driselase did appear to contain some protease activity which could not be easily inhibited, and so this approach proved unsuitable. The use of purified enzymes to digest specific wall components would provide a more controlled way of further investigating the possible release of trapped dimers.

An alternative way to isolate extensin dimers was to solubilise glycoprotein from cell walls by the use of brief acidified chlorite treatments, in the presence of B.S.A. to prevent peptidyl cleavage of extensin, and then to separate the solubilised fragments. The results of such experiments showed that monomer extensin could be isolated by this method; this is the first report of isolation of intact monomer extensin from the covalently bound cell wall extensin network and demonstrates (a) that B.S.A. does protect against extensin backbone cleavage during acidified chlorite treatment and (b) that extensin must be held in the wall network by a phenolic cross-link that is susceptible to cleavage by acidified chlorite. A peak of high M_r material, a putative extensin dimer, was also isolated and shown to contain hydroxyproline-tetra-arabinosides but it was not possible to

demonstrate conversion of this material to monomer extensin by a further acidified chlorite treatment in the presence of B.S.A..

The findings of this study are in agreement with, and provide further indirect evidence for, the hypothesis that extensin monomers are cross-linked into an insoluble network within the cell wall, but they did not show that isodityrosine was the cross-link formed. Recently, it has been suggested that other unidentified cross-links (Cooper et al., 1987), possibly involving lysine or histidine residues, may be responsible for cross-link formation but no such amino acid complexes have yet been identified. Although it must be concluded that the identity of the intermolecular cross-link remains elusive, isodityrosine is still the strongest candidate for this role. Conclusive proof might be obtained from an experiment where radioactive isodityrosine residues in extensin, prepared by feeding the radioactive precursor [¹⁴C]tyrosine to suspension culture cells, are shown to be broken down as extensin oligomers are converted to monomers by acidified chlorite treatment.

It has also been suggested that a trimer corresponding to isodityrosine could exist in cell walls of mature suspension cultures (Fry, 1983a; Biggs & Fry, 1986) and it would be interesting to identify and further characterise this molecule, which could form intermolecular bridges or large intramolecular loops in extensin. A trimer could not, however, form tight intramolecular loops of the type reported for isodityrosine (Epstein & Lamport, 1984).

Identification of the cross-link in extensin is an important step towards our understanding of how extensin is held in the wall and how it functions as a structural component. The contribution of an extensin network to cell wall models could then be determined, and we

can extrapolate from working models of the cell wall to consider how processes such as extension growth, wall morphology and defence against pathogen invasion may operate and be regulated. The role of a cross-linked extensin network in determining the properties, such as crunchiness, tissue coherency, resilience to processing, and digestibility, of food plant cell walls is also important in that it may ultimately allow food scientists to modify food processing in order to obtain pre-determined characteristics in processed plant foodstuffs.

REFERENCES

- Akiyama Y. & Kato K. (1976) Hydroxyproline arabinosides from suspension-cultured tobacco cell wall. *Agric. Biol. Chem.* **40**, 2343-2348.
- Akiyama Y. & Kato K. (1977) Structure of hydroxyproline arabinosides from tobacco cells. *Agric. Biol. Chem.* **41**, 79-81.
- Akiyama Y., Mori M. & Kato K. (1980) ^{13}C -NMR analysis of hydroxyproline arabinosides from Nicotiana tabacum. *Agric. Biol. Chem.* **44**, 2487-2489.
- Albersheim P. (1975) The walls of growing plant cells. *Scientific American* **232**, 80-95.
- Albersheim P. (1978) Concerning the structure and biosynthesis of the primary cell walls of plants. In *Biochemistry of Carbohydrates, II*, Vol 16, (Manners D.J. ed.), 127-151, University Park Press, U.S.A.
- Allen A. & Neuberger A. (1973) The purification and properties of the lectin from potato tubers, a hydroxyproline-containing glycoprotein. *Biochem. J.* **135**, 307-314.
- Allen A.K., Desai N.N., Neuberger A. & Creeth J.M. (1978) Properties of potato lectin and the nature of its glycoprotein linkages. *Biochem. J.* **171**, 665-674.

- Andersen S.O. (1964) The cross-links in resilin identified as dityrosine and trityrosine. *Biochim. Biophys. Acta* **93**, 213-215.
- Ashford D. & Neuberger A. (1980) 4-Hydroxy-L-proline in plant glycoprotein. *TIBS* **5**, 245-248.
- Biggs K.J. & Fry S.C. (1987) Phenolic cross-linking in the plant cell wall. In *Physiology of Cell Expansion during Plant Growth* (Cosgrove D.J. & Knievel D.P., eds.), 46-57, American Society of Plant Physiologists, U.S.A.
- Burton B.A. & Brant D.A. (1983) Comparative flexibility, extension and conformation of some simple polysaccharide chains. *Biopolymers* **22**, 1769-1792.
- Cassab G.I. & Varner J.E. (1988) Cell wall proteins. *Ann. Rev. Plant Physiol. & Plant Molecular Biol.* **39**, 321-353.
- Cassab G.I., Nieto-Sotelo J., Cooper J.B., van Holst G.J. & Varner J.E. (1985) A developmentally regulated hydroxyproline-rich glycoprotein from the cell walls of soybean seed coats. *Plant Physiol.* **77**, 532-535.
- Catt J.W., Hills G.J. & Roberts K. (1976) A structural glycoprotein, containing hydroxyproline, isolated from the cell wall of *Chlamydomonas reinhardtii*. *Planta* **131**, 165-171.

- Chen J. & Varner J.E. (1985a) An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin. *EMBO J.* **4**, 2145-2155.
- Chen J. & Varner J.E. (1985b) Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4399-4403.
- Clarke A.E., Anderson R.L. & Stone B.A. (1979) Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochem.* **18**, 521-540.
- Conrad T.A., Lamport D.T.A. & Hammerschmidt R. (1987) Detection of glycosylated and deglycosylated extensin precursors by indirect competitive ELISA. *Plant Physiol.* **83**, 1-3.
- Cooper J.B. (1984) Hydroxyproline synthesis is required for cell wall regeneration. In *Structure, Function & Biosynthesis of Plant Cell Walls* (Dugger W.M. & Bartnicki-Garcia S., eds.), American Society of Plant Physiologists, U.S.A.
- Cooper J.B. & Varner J.E. (1983) Insolubilization of hydroxyproline-rich cell wall glycoprotein in aerated carrot root slices. *Biochem. Biophys. Res. Comm.* **112**, 161-167.
- Cooper J.B. & Varner J.E. (1984) Cross-linking of soluble extensin in isolated cell walls. *Plant Physiol.* **76**, 414-417.

- Cooper J.B., Chen J.A. & Varner J.E. (1984) The glycoprotein component of plant cell walls. In *Structure, Function & Biosynthesis of Plant Cell Walls* (Dugger W.M. & Bartnicki-Garcia S., eds.), American Society of Plant Physiologists, U.S.A.
- Cooper J.B., Chen J.A., van Holst G.J. & Varner J.E. (1987) Hydroxyproline-rich glycoprotein of plant cell walls. *TIBS* **12**, 24-27.
- Dawson R.M.C., Elliott D.C., Elliott W.H. & Jones K.M. (1986) *Data for Biochemical Research*, Oxford.
- Dence C.W., Gupta M.K. & Sarkanen K.V. (1962) Studies on oxidative delignification mechanisms. Part II: Reactions of vanillyl alcohol with chlorine dioxide and sodium chlorite. *TAPPI* **45**, 29-38.
- Desai N.N., Allen A.K. & Neuberger A. (1983) The properties of tomato lectin after deglycosylation by trifluoromethanesulphonic acid. *Biochem. J.* **211**, 273-276.
- Dische Z. (1962) Color reactions of carbohydrates. In *Methods in Carbohydrate Chemistry*, (Whistler R.L. & Wolfrom M.L., eds.), vol.1, 475-514, Academic Press, N.Y.
- Edge A.S.B., Faltynek C.R., Hof L., Reichert L.E. & Weber P. (1981) Deglycosylation of glycoprotein by trifluoromethanesulfonic acid. *Anal. Biochem.* **118**, 131-137.

- Epstein L. & Lamport D.T.A. (1984) An intramolecular linkage involving isodityrosine in extensin. *Phytochem.* **23**, 1241-1246.
- Esquerré-Tugayé M.T., Lafitte C., Mazau D., Toppan A. & Touzé A. (1979) Cell surfaces in plant-micro-organism interactions. II Evidence for the accumulation of hydroxyproline-rich glycoprotein in the cell wall of diseased plants as a defense mechanism. *Plant Physiol.* **64**, 320-326.
- Everdeen D.S., Kiefer S., Willard J.J., Muldoon E.P., Dey P.M., Li X. B. & Lamport D.T.A. (1988) Enzymic cross-linkage of monomeric extensin precursors in vitro. *Plant Physiol.* **87**, 616-621.
- Fry S.C. (1982) Isodityrosine, a new cross-linking amino acid from plant cell wall glycoprotein. *Biochem. J.* **204**, 449-455.
- Fry S.C. (1983a) Oxidative phenolic coupling reactions cross-link hydroxyproline-rich glycoprotein molecules in plant cell. *Current Topics in Plant Biochem. & Physiol.* **2**, 59-72.
- Fry S.C. (1983b) Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta* **157**, 111-123.
- Fry S.C. (1984) Isodityrosine, a diphenyl ether cross-link in plant cell wall glycoprotein: identification, assay and chemical synthesis. *Methods in Enzymol.* **107**, 388-397.
- Fry S.C. (1985) Primary cell wall metabolism. *Oxford Surveys of Plant*

- Molecular & Cell Biology **2**, 1-42.
- Fry S.C. (1986a) Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Ann. Rev. Plant Physiol.* **37**, 165-186.
- Fry S.C. (1986b) Polymer-bound phenols as natural substrates of peroxidases. In *Molecular & Physiological Aspects of Plant Peroxidases* (Greppin H., Penel C. & Gaspar Th., eds.), 169-182, Geneva University Press.
- Fry S.C. (1987) Formation of isodityrosine by peroxidase isozymes. *J. Exp. Bot.* **38**, 853-862.
- Fry S.C. (1988) *The Growing Plant Cell Wall: chemical and metabolic analysis*, Longman Scientific, Essex.
- Halmer P. & Thorpe T.A. (1976) Kinetin induced changes in cell wall composition of tobacco callus. *Phytochem.* **15**, 1585-1588.
- Heckman J.W., Terhune B.T., Lamport D.T.A. (1988) Characterization of native and modified extensin monomers and oligomers by electron microscopy and gel filtration. *Plant Physiol.* **86**, 848-856.
- Hood E.E., Shen Q.X. & Varner J.E. (1988) A developmentally regulated hydroxyproline rich glycoprotein in maize pericarp cell walls. *Plant Physiol.* **87**, 138-142.
- Keegstra K., Talmadge K.W., Bauer W.D. & Albersheim P. (1973) The

- structure of plant cell walls. III A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.* **51**, 188-197.
- Kieliszewski M. & Lamport D.T.A. (1986) Cross-reactivities of polyclonal antibodies against extensin precursors determined by ELISA techniques. *Phytochem.* **25**, 673-677.
- Kieliszewski M. & Lamport D.T.A. (1987) Purification and partial characterization of a hydroxyproline rich glycoprotein in a graminaceous monocot, Zea mays. *Plant Physiol.* **85**, 823-827.
- Lamport D.T.A. (1965) The protein component of primary cell walls. *Adv. in Bot. Res.* **2**, 151-218.
- Lamport D.T.A. (1967) Hydroxyproline-O-glycosidic linkage of the plant cell wall glycoprotein extensin. *Nature* **216**, 1322-1324.
- Lamport D.T.A. (1969) The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymic degradation of primary cell walls. *Biochem.* **8**, 1155-1163.
- Lamport D.T.A. (1977) Structure, biosynthesis and significance of cell wall glycoprotein. In *Recent Advances in Phytochemistry*, Vol II, (Loewus F.A. & Runeckles V.C., eds.), Plenum Press, New York.
- Lamport D.T.A. (1980) Structure and function of plant glycoproteins. In *Biochemistry of Plants* (Preiss J., ed.), Vol 3, 501-541, Academic

Press, New York.

- Lamport D.T.A. (1985) The primary cell wall: a new model. In Cellulose: Structure, Modification & Hydrolysis, (Young R. & Rowell R., eds.), Wiley-Interscience, New York.
- Lamport D.T.A. (1986) Roles for peroxidases in cell wall genesis. In Molecular & Physiological Aspects of Plant Peroxidases, (Greppin H., Penel C. & Gaspar Th., eds.), 199-208, Geneva University Press.
- Lamport D.T.A. & Epstein L. (1983) A new model for the primary cell wall: A concatenated extensin-cellulose network. Current Topics in Plant Biochem. & Physiol. **2**, 73-83.
- Lamport D.T.A., Katona L. & Roerig S. (1973) Galactosylserine in extensin. Biochem. J. **133**, 125-131.
- Lamport D.T.A. & Northcote D.H. (1960) Hydroxyproline in the cell walls of higher plants. Nature **188**, 665-666.
- McNeil M., Darvill A.G., Fry S.C. & Albersheim P. (1984) Structure and function of the primary cell walls of plants. Ann. Rev. Biochem. **53**, 625-663.
- Means G.E. & Feeney R.E. (1971) Chemical modification of proteins, Holden-Day Inc., London.
- Memelink J. (1988) PhD Thesis, University of Leiden, The Netherlands.

- Mort A.J. & Lamport D.T.A. (1977) Anhydrous hydrogen fluoride deglycosylates glycoproteins. *Anal. Biochem.* **82**, 289-309.
- Murray R.H.A. & Northcote D.H. (1978) Oligoarabinosides of hydroxyproline isolated from potato lectin. *Phytochem.* **17**, 623-629.
- Neville A.C. (1967) Factors affecting the tertiary structure of resilin in locusts. *J. Cell Science* **2**, 273-280.
- O'Neill M.A. & Selvendran R.R. (1980) Glycoproteins from the cell walls of Phaseolus coccineus. *Biochem. J.* **187**, 53-63.
- Owens R.J. & Northcote D.H. (1980) The purification of potato lectin by affinity chromatography on a fetuin-Sepharose matrix. *Phytochem.* **19**, 1861-1862.
- Pharmacia Handbooks: Gel filtration theory and practice. Ion exchange chromatography principles and methods.
- Preston R.D. (1979) Polysaccharide conformation and cell wall function. *Ann. Rev. Plant Physiol.* **30**, 55-78.
- Ramachandran L.K. & Witkop B. (1967) N-Bromosuccinimide cleavage of peptides. *Methods in Enzymol.* **11**, 283-299.
- Roberts K., Gurney-Smith M. & Shaw P.J. (1972) Structure, composition and morphogenesis of the cell wall of Chlamydomonas reinhardtii. *J. Ultrastruc. Res.* **40**, 599-613.

- Roberts K., Grief C., Hills G.J. & Shaw P.J. (1985) Cell wall glycoproteins: structure and function. *J. Cell Sci. Suppl.* 2, 105-127.
- Robinson D.G., Andrae M. & Sauer A. (1985) Hydroxyproline-rich glycoprotein biosynthesis: a comparison with that of collagen. In *Biochemistry of Plant Cell Walls* (Brett C.T. & Hillman J.R., eds.), pp.155-176, Cambridge Univ. Press.
- Sadava D. & Chrispeels M.J. (1973) Hydroxyproline-rich cell wall protein (extensin): role in the cessation of elongation in excised pea epicotyls. *Dev. Biol.* **30**, 49-55.
- Sadava D., Walker F. & Chrispeels M.J. (1973) Hydroxyproline-rich cell wall protein (extensin): biosynthesis and accumulation in growing pea epicotyls. *Dev. Biol.* **30**, 42-48.
- Sarkanen K.V., Kakehi K., Murphy R.A. & White H. (1962) Studies on oxidative delignification mechanisms. Part I Oxidation of vanillin with chlorine dioxide. *TAPPI* **45**, 24-29.
- Schenk R.U. & Hildebrandt A.C. (1972) Medium techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* **50**, 199-204.
- Selvendran R.R. (1975) Cell wall glycoproteins and polysaccharides of parenchyma of Phaseolus coccineus. *Phytochem.* **14**, 2175-2180.
- Selvendran R.R., Davies A.M.C. & Tidder E. (1975) Cell wall glyco-

- proteins and polysaccharides of mature runner beans. *Phytochem.* **14**, 2169-2174.
- Showalter A.M., Bell J.N., Cramer C.L., Bailey J.A., Varner J.E. & Lamb C.J. (1985) Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6551-6555.
- Smith J.J., Muldoon E.P. & Lamport D.T.A. (1984) Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. *Phytochem.* **23**, 1233-1239.
- Smith J.J., Muldoon E.P., Willard J.J. & Lamport D.T.A. (1986) Tomato extensin precursors P1 and P2 are highly periodic structures. *Phytochem.* **25**, 1021-1030.
- Soila R., Lehtikoski O. & Virkola N.E. (1962) On the reactions taking place during the chlorine dioxide bleaching stage. *Svensk. Papperstid.* **65**, 632-639.
- Sojar H.T. & Bahl O.P. (1987) Chemical deglycosylation of glycoproteins. *Methods in Enzymol.* **138**, 341-350.
- Stafstrom J.P. & Staehelin L.A. (1986a) Cross-linking patterns in salt-extractable extensin from carrot cell walls. *Plant Physiol.* **81**, 234-241.
- Stafstrom J.P. & Staehelin L.A. (1986b) Role of carbohydrate in

- extensin structure. *Plant Physiol.* **81**, 242-246.
- Stafstrom J.P. & Staehelin L.A. (1987) A second extensin-like hydroxyproline-rich glycoprotein from carrot cell walls. *Plant Physiol.* **84**, 820-825.
- Stafstrom J.P. & Staehelin L.A. (1988) Antibody localization of extensin in cell walls of carrot storage roots. *Planta* **174**, 321-332.
- Stermer B.A. & Hammerschmidt R. (1984) Heat shock induces resistance to Cladosporium cucumerinum and enhances peroxidase activity in cucumbers. *Physiol. Plant Pathology* **25**, 239-249.
- Stuart D.A. & Varner J.E. (1980) Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. *Plant Physiol.* **66**, 787-792.
- Taiz L. (1984) Plant cell expansion. *Ann. Rev. Plant Physiol.* **35**, 585-657.
- Tierney M.L. & Varner J.E. (1987) The extensins. *Plant Physiol.* **84**, 1-2.
- Tierney M.L., Wiechert J. & Pluymer D. (1988) Analysis of the expression of extensin and p33-related cell wall proteins in carrot and soybean. *Mol. Gen. Genetics* **211**, 393-399.

- van Holst G.J. & Varner J.E. (1984) Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. *Plant Physiol.* **74**, 247-251.
- von Sachs J. (1865) *Handbuch der Experimentalphysiologie der Pflanzen*. Engelmann, Leipzig.
- White J.A., Hart R.J. & Fry J.C. (1986) An evaluation of the Waters Pico-Tag system for the amino acid analysis of food materials. *J. Automatic Chem.* **8**, 170-177.
- Wilson K. & Goulding K.H. (1986) *A Biologist's Guide to Principles & Techniques of Practical Biochemistry*, 3rd. edn., Edward Arnold Ltd., London.
- Wilson L.G. (1987) PhD Thesis, University of Surrey.
- Wilson L.G. & Fry J.C. (1986) Extensin — a major cell wall glycoprotein. *Plant, Cell & Environment* **9**, 239-260.
- Wise L.E., Murphy M. & D'Addieco A.A. (1946) Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *TAPPI* **122**, 11-19.
- Yang J.M. & Goring D.A.I. (1978) The topochemistry of the reaction of chlorine dioxide with lignin in black spruce wood. *Holzforschung* **32**, 185-188.

Appendix 1: Composition of suspension culture medium for Capsicum frutescens (Schenk & Hildebrandt, 1972).

Macroelements	mg l ⁻¹	
KNO ₃	2500	
MgSO ₄ .7H ₂ O	400	
NH ₄ H ₂ PO ₄	300	
CaCl ₂ .2H ₂ O	200	
Microelements		
MnSO ₄ .H ₂ O	10	
H ₃ BO ₃	5	
ZnSO ₄ .7H ₂ O	1	
KI	1	
CuSO ₄ .5H ₂ O	0.2	
Na ₂ MoO ₄ .2H ₂ O	0.1	
CoCl ₂ .6H ₂ O	0.1	
FeSO ₄ .7H ₂ O	15	
Na ₂ EDTA	20	
Organics		
Inositol	1000	
Thyamine HCl	5	
Nicotinic acid	5	
Pyridoxine	0.5	Sucrose 30 gl ⁻¹
Hormones		
2,4-D	0.5	
Kinetin	0.1	

The medium was adjusted to pH 5.6-5.8 with 1M NaOH.