UTILIZATION OF AUTOLYTICALLY ACTIVE CELL WALL FOR CHARACTERIZATION OF TOMATO FRUIT SOFTENING

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Cell walls from pericarp of normal tomato (Lycopersicon esculentum Mill. cv 'Rutgers') fruit released acid sugars in vitro in a reaction mediated by wall-bound polygalacturonase (PG; EC 3.2.1.15). Release was negligible in walls from normal green and the ripening mutant rin fruit, reflecting the absence of PG in these tissues. Pectin solubilization was most extensive at pH 2.5 with a less pronounced optimum at 5.5. Brief exposure of cell wall to low (1.5) or high (7.5) pH resulted in reduction of autolytic activity, which also was inhibited by high temperature, calcium, and treatments employed to dissociate wall-bound protein.

The rate and extent of acid sugar solubilization were dependent upon the techniques employed for handling the cell walls in vitro. Enzymically active cell walls subjected to either intermittent or continuous removal of the bathing medium released twice as much

polyuronide as walls incubated in a static system. Protocol that maximized acid sugar solubilization was employed to determine the neutral sugar content and size distribution of pectic polymers released during autolysis. Approximately 2% of the initial wall dry weight was released as neutral sugars. The predominant products were galactose (50%) and arabinose (30%). Minor quantities of rhamnose, xylose, glucose, and mannose were also detected. The acid:neutral sugar ratio of soluble autolysis products decreased from 10.8 to 2.9 as wall degradation proceeded. Pectin molecular size did not change appreciably during autolytic reactions.

Polyuronide solubilization was optimally enhanced by NaCl in the range of 200-300 mM. Pretreatment of enzymically active walls with 1 M NaCl in the pH range of 2.5-8.5 did not inhibit subsequent autolysis. The tenacity of PG-binding was further illustrated by the fact that 1 M NaCl treatments during wall extraction did not eliminate release of acid sugars. Interactions between pH, NaCl, and calcium were also investigated. The stimulation of wall-bound PG activity by 150-300 mM NaCl was most apparent at pH 2.5. Calcium (5 mM) did not inhibit autolysis at pH 2.5; however, at pH 4.5 calcium inhibition was apparent regardless of presence of NaCl. Polyuronide solubilization was negligible at pH 6.5 or 8.5.

CHAPTER 1
INTRODUCTION

Softening is one of the most dramatic events associated with tomato fruit ripening. A degree of control over the rate of texture change is desirable because consumer preferences for firmness vary widely, e.g., soft, fully ripe tomatoes are desired for processing, whereas firm fruit are preferred for shipping long distances or for slicing in the fast food industry.

The cell wall is the most significant structural entity of the plant cell (Bartnicki-Garcia, 1984). Fruit softening involves enzymic hydrolysis of specific cell wall polymers (Huber, 1983b). Cells with partially degraded walls yield to external pressures and slide against each other, i.e., they become soft. The enzyme most often implicated in tomato fruit softening is polygalacturonase (PG; EC 3.2.1.15) which is believed to be synthesized with the onset of ripening (Tucker and Grierson, 1982). Its role is to catalyze hydrolysis of pectic polymers of the middle lamella (Crookes and Grierson, 1983). The most common approach to investigating the action of PG and other cell wall hydrolases is to extract the enzyme(s) of interest and assay for activity with commercially prepared substrates (Wallner and Walker, 1975) or with a cell wall preparation (Hobson, 1964; Pressey and Avants, 1973). Such studies have provided much useful information. The interpretation of this

information in terms of *in situ* wall hydrolysis is limited by the fact that techniques used to prepare cell walls or commercially available carbohydrate polymers may remove factors that are crucial to the hydrolytic activity of the enzyme. An alternative to the above approach is to study cell wall autolysis, i.e., to extract walls under conditions which preserve the bond between PG and its substrate, then incubate the wall extract *in vitro* under conditions that permit enzymic activity (autolysis). Products of this reaction may constitute a more appropriate reflection of *in situ* wall hydrolysis associated with fruit softening.

Several workers have used cell wall autolysis to study wall metabolism associated with growth and development (Kivilaan et al., 1961; Lee et al., 1967; Huber and Nevins, 1979). This dissertation addresses the use of enzymically active cell walls isolated from tomato pericarp to investigate activity of wall-bound PG. Initial experiments compared the autolysis system to other *in vitro* cell wall studies of fruit softening. Variables such as salt stimulation or inhibition, pH, temperature, and inactivation treatments were investigated for their effect on autolysis. Several techniques for cell wall preparation and handling were evaluated. The rate and extent of acid sugar solubilization during *in vitro* autolysis were significantly affected by handling techniques employed. A system that maximized the release of autolysis products was chosen for characterization of the neutral sugar component and size distribution of solubilized pectic polymers. Further studies addressed the effects of NaCl, pH, and calcium on autolysis of isolated walls.

CHAPTER 2
LITERATURE REVIEW

Horticultural Significance of Tomato Fruit Softening

Tomato (Lycopersicon esculentum, Mill.) is the major fresh market vegetable produced in the state of Florida, where skyrocketing increases in production costs have emphasized the importance of preserving product quality from field to consumer (Taylor and Wilkowske, 1984). Climacteric fruits, such as tomato, may require marketing within a relatively short period in order to avoid the numerous pathological and physiological disorders that result in product deterioration (Conway, 1984). Texture is an important quality factor and change in texture (softening) often is measured in postharvest studies because it is closely associated with other features of ripening, such as color development (Hobson, 1964, 1965; Khudairi, 1972), climacteric behavior (Gertman and Fuchs, 1974; Tigchelaar et al., 1978; Yoshida et al., 1984) and catabolism of membrane constituents (Borochov and Faiman-Weinberg, 1984). The relationship between softening and ripening metabolism is so intimate that several workers addressed the idea that softening enzymes initiate ripening by solubilization of pectins. Wall-bound proteins released during pectin solubilization presumably start a cascade of metabolic reactions that collectively compose the phenomenon known as ripening (Strand et al., 1976; Tigchelaar et al., 1978; Poovaiah and

Nukaya, 1979). This idea has been somewhat refuted by evidence that the wall hydrolase primarily responsible for tomato fruit softening is synthesized de novo with the onset of ripening and its synthesis is preceded by the initiation of autocatalytic ethylene production (Tucker and Grierson, 1982; Brady et al., 1982). Ethylene, the "ripening hormone," is generally regarded as the initiator of ripening (Tigchelaar et al., 1978); however, the concept of tissue sensitivity to hormones has been proposed as a point of regulation of developmental processes (Trewavas, 1982). At present, the involvement of cell wall in initiation of ripening remains an enigma. The molecular biological approach to the study of ripening may provide the needed complement to physiological studies (Grierson et al., 1981a; 1981b).

Approaches to Study of Tomato Fruit Softening

The following is a brief review of approaches that formed the basis for this dissertation. Many of the investigations mentioned here are discussed in more detail later.

The simplest approach to the study of softening is direct measurement of fruit firmness. Although several techniques are available for quantifying texture changes (Rushing and Huber, 1983), electron microscopy (EM) provides a more direct way to observe the dissolution of cell wall during softening. Simpson et al. (1976) performed ultrastructural studies on ripening tomato fruit, utilizing the mutant rin for comparison to normal tomato. In a more recent investigation, Crookes and Grierson (1983) combined EM with enzymology to confirm that polygalacturonase (PG) causes dissolution

of middle lamella and cell wall in thin sections of mature-green pericarp in a manner indistinguishable from that caused by PG *in situ* in ripening pericarp.

Early investigations of cell wall modifications during softening involved unpurified extracts of wall hydrolases assayed *in vitro* with acid sugar substrates of varying degrees of polymerization and methylation (Jansen and MacDonnell, 1945; McCready et al., 1955; Patel and Phaff, 1960a; 1960b). Tomato was the first fruit found to contain PG, which had previously been isolated from fungi (McColloch and Kertesz, 1949). The action pattern of tomato PG was the focus of several early studies (McCready et al., 1955; Patel and Phaff, 1960a). Unpurified PG preparations degraded pectic acid extensively, leading to the erroneous proposal that tomato contains both endo- and exo-PG (Patel and Phaff, 1960a). The term "random cleavage" was introduced to describe PG action (Patel and Phaff, 1960a), but it seems unlikely the pattern of hydrolysis is left to chance in view of the finding of Pressey and Avants (1971) that substrate size may affect the rate of PG hydrolysis. Hobson (1964) introduced several new concepts with his work on PG activity in normal and blotchy tomato fruit. He reported that the appearance of PG during fruit ripening coincides with color development and softening. The enzyme is located primarily in pericarp and its extractability is enhanced by addition of NaCl or EDTA to the homogenization medium. Unripened (blotched) regions of pericarp did not develop PG nor did they soften (Hobson, 1964), and later work demonstrated that firm tomato varieties have less extractable PG than soft varieties (Hobson,

1965). The crude PG preparation had a bi-modal pH response which Hobson (1964) speculated was evidence for isozymes. Others have since employed purification schemes to characterize PG isozymes (Pressey and Avants, 1973; Ali and Brady, 1982; Tucker et al., 1980). Investigations of these isozymes are discussed later in this chapter.

The in vitro studies discussed above employed acid sugar polymers as substrate for PG. A disadvantage of this approach is that the structure of isolated polygalacturonan is drastically different from the structure of pectin. The following discussion addresses the use of isolated cell wall as substrate for tomato wall hydrolases.

Wallner and Walker (1975) performed studies to ascertain which hydrolases are present prior to and throughout tomato fruit softening. They reported that PG is the only hydrolase found in tomato that effectively degrades isolated cell walls. The authors also detected β-1,3-glucanase and β-galactosidase activity throughout ripening; however, they acknowledged that these enzymes may not be extracellular in origin, i.e., they may be artifacts of extraction. In a later study, Wallner and Bloom (1977) determined the composition of walls from green tomato pericarp before and after enzymic digestion in vitro and compared the results to similar analysis of walls from red pericarp that had undergone in situ hydrolysis during softening. The authors found similarities in polysaccharide degradation, e.g., polyuronide and neutral sugar solubilization, in both systems and suggested that cell walls are more appropriate

substrates than refined polymers for studies of PG activity *in vitro* if the objective is to approximate *in situ* conditions (Wallner and Bloom, 1977). Wall composition analyses were soon followed by more detailed characterization of the soluble products (Gross and Wallner, 1979). Crude enzyme hydrolysates of cell walls from green fruit were compared to water soluble pectins released *in vitro* from walls of ripening (pink) tomatoes. In addition, compositional changes (e.g, polyuronide and neutral sugar solubilization) in walls of the ripening mutant *rin*, which has no PG, were monitored over the period of development in which ripening would normally occur. Polyuronide solubilization from walls of *rin* fruit was not detected but was extensive from normal cell walls. Neutral sugars, primarily galactose and arabinose, were released from walls under all three circumstances, suggesting that cell wall neutral sugar solubilization in the latter stages of tomato fruit development is not dependent upon the presence of PG (Gross and Wallner, 1979). However, PG-mediated polyuronide solubilization without the loss of neutral sugars has not been demonstrated (Huber, 1983a). One aspect of the work of Gross and Wallner (1979) that is particularly pertinent to this dissertation is the *in vitro* release of water-soluble-pectin from cell walls of pink pericarp. The authors speculated that it was an artifact of the wall extraction procedure; however, it seems more likely to be a product of wall-bound PG activity (autolysis) since no steps were taken to inactivate or remove bound hydrolases.

Themmen et al. (1982) proposed that the unpurified enzyme preparations employed to investigate cell wall degradation may have

contained proteins other than PG that could enhance PG activity. The authors extracted and purified a single PG isozyme (PG II) from ripe tomatoes, then used it to hydrolyze walls from green fruit. They found that acid sugar solubilization was similar to that reported in previous work using unpurified enzyme (Hobson, 1964; Wallner and Bloom, 1977). However, Themmen et al. (1982) could not conclude that PG acts alone in wall solubilization because gel electrophoresis of a protein extract from the cell wall preparation revealed the presence of over 20 different polypeptides. Others have verified that a diverse population of proteins of unknown origin are associated with isolated cell walls (Kivilaan et al., 1961; Klis et al., 1974; Strand et al., 1976; Hobson et al., 1983). The preceding review deals specifically with investigations of cell wall hydrolases associated with tomato fruit softening. Similar studies have been performed with pears (Ahmed and Labavitch, 1980a; 1980b), apples (Knee, 1973; Bartley, 1974), papaya (Paull and Chen, 1983; Lourenco and Catutani, 1984), avocado (McCready et al., 1955; Awad and Lewis, 1980), strawberry (Huber, 1984a), and other fruit (Hinton and Pressey, 1980; Huber, 1983b). Nearly all of these studies were performed with extracted enzyme and employed commercially prepared carbohydrates or isolated cell walls as substrate.

An additional approach to the investigation of cell wall modifications during fruit softening is to extract the cell walls under the mildest possible conditions in order to preserve the enzyme-substrate complex. Incubation of the walls under conditions that allow activity of the wall-bound hydrolases (autolysis) may

constitute a more appropriate reflection of *in situ* wall hydrolysis. This concept was briefly introduced in the fruit softening literature by Knee (1973), who reported autolytic solubilization of neutral sugars from apple fruit cell walls *in vitro*. However, the amount of neutral sugar released was extremely low (< 0.1% wall dry wt) and polyuronide solubilization was only slightly greater than neutral sugar loss (Knee, 1973). It was later found that cell walls extracted from pink tomato pericarp released polyuronide if incubated in distilled water; the solubilized product was termed water-soluble-pectin and was reported to be an artifact of wall extraction (Gross and Wallner, 1979). It seems more likely to be a product of wall-bound PG activity. Gel filtration chromatography (Bio-Gel P-10) demonstrated that these water-soluble-pectins had mol wt greater than 20,000, whereas the acid sugars in a PG-hydrolysate of cell walls from green pericarp varied in size from mol wt greater than 20,000 down to oligosaccharides (Gross and Wallner, 1979). No further studies that utilize enzymically active walls have been reported in the fruit softening literature to date, although several advantages seem apparent in taking this approach. Perhaps the greatest advantage is that cell wall is presumably less altered when extraction is carried out under mild conditions, e.g., cold aqueous extraction with no exposure to buffers or other salts. It is especially desirable to preserve wall structure as much as possible because unidentified components may regulate the activity of wall hydrolases. Additionally, walls from ripening tissues may be

studied in vitro if autolysis is used to ascertain hydrolytic activity.

Although cell wall autolysis studies are virtually absent from the fruit softening literature, there are several reports of enzymic activity associated with isolated walls from vegetative tissue. Kivilaan et al. (1961) adopted, as a working hypothesis, the idea that the enzymes involved in cell wall synthesis are located in the walls. They performed assays using isolated cell walls as a source of enzyme and determined that inorganic pyrophosphatase, ATP-ase, uridine diphosphoglucose pyrophosphorylase, and invertase remain associated with isolated walls. These enzymes also were present in the cytoplasmic protein fraction but the specific activity was significantly lower, suggesting that the amount of non-specific enzyme binding relative to specific binding of protein to walls is very low (Kivilaan et al., 1961).

Lee et al. (1967) employed the wall extraction procedures of Kivilaan et al. (1961) to study the activity of wall-bound hydrolases associated with extension growth in corn coleoptiles. A 10% decrease in wall wt was attributed to autolysis of a non-cellulosic glucan accompanied by release of traces of arabinose and xylose, leading the authors to propose that both synthetic and hydrolytic enzymes are components of the primary cell wall (Lee et al., 1967). Later studies attributed the autolysis to endo- and exo-β-D-glucanases that exhibited strong cooperativity in hydrolyzing mixed-linkage glucans demonstrated to be a part of the hemicellulose B fraction (Huber and Nevins, 1979; 1981a). Galactose also was solubilized during

autolysis, suggesting that a wall-bound β-galactosidase may have been present. Autolytic activity increased with growth; however, this could not be attributed to the presence of more enzyme because of the simultaneous increase in the amount of substrate during growth (Huber and Nevins, 1981a). The suggestion that the hydrolytic activity of bound glucanases serves a wall-loosening role in extension growth is supported by the fact that antibodies raised against an extract of cell wall proteins inhibited growth of corn seedling sections as well as autolysis of the isolated walls. The precise mechanism of antibody inhibition in vivo is unresolved due to the assortment of proteins in the antigen. However, the in vitro effect was indicative of restricted enzyme mobility (Huber and Nevins, 1981b). One may conclude from this brief perspective that the use of cell-wall autolysis for softening studies was inevitable.

Cell Wall Hydrolases Associated with Tomato Fruit Softening

Considering the complexity of cell wall structure (Albersheim et al., 1984; Bartnicki-Garcia, 1984; Cooper et al., 1984), it is not surprising that so many cell wall hydrolases are found in softening fruit. The high specificity of enzyme action dictates a heterogeneous population of enzymes in order to effect wall modifications (Lamport, 1978). The involvement of cell wall hydrolases in fruit softening remains a timely topic and review articles have appeared regularly (Rexova-Benkova, 1976; Pressey, 1977; Hobson, 1981; Knee and Bartley, 1981; Huber, 1983b).

Polygalacturonase (PG)

Several properties of PG (EC 3.2.1.15) were discussed in the previous section of this review because it is the enzyme having the greatest effect upon dissolution of middle lamella (Crookes and Grierson, 1983) and several researchers working independently have concluded that PG plays the most significant role in tomato fruit softening (Hobson, 1964; Wallner and Walker, 1975; Rexova-Benkova and Markovic, 1976). At least two isozymes, designated PG I and PG II, are known to be present in tomato fruit. They have mol wts of approximately 80,000 and 40,000 and thermostability limits of 78°C and 57°C, respectively, for PG I and PG II (Pressey and Avants, 1972; Tucker et al., 1980). More rigorous purification of PG II further resolved two isozymes, designated PG IIA and PG IIB having mol wts of 43,000 and 46,000, respectively (Ali and Brady, 1982). Antibodies raised against PG IIA reacted with all three isozymes, suggesting similar polypeptides. An additional distinct molecular form of PG was found in 'Longkeeper' tomato fruit (Pressey and Avants, 1982b). A heat-stable, non-dialyzable factor that apparently converts PG II to PG I was discovered in tomato but its role in softening metabolism has not been ascertained (Tucker et al., 1981). Tomato PG exhibits endo-activity (Hunter and Elkan, 1974) and its appearance in tomato fruit correlates with polyuronide depolymerization (Huber, 1983a) which also has been observed in ripening pear fruit (Bartley, 1982). There is no conclusive evidence for the presence of exo-PG in tomato but it is present in a number of other fruit types (Huber, 1983b). Products solubilized from cell wall during

softening, presumably by PG action, include polyuronide (Hobson, 1965; Pressey and Avants, 1971), neutral sugars (Gross and Wallner, 1979; Huber, 1983a; Gross and Sams, 1984), protein (Hobson, 1983), calcium (Rigney and Wills, 1981) and possibly other compounds. Additional characteristics of tomato PG are discussed in a later section on regulation of PG activity *in vitro*.

Pectinmethylesterase (PME)

Pectinmethylesterase (EC 3.1.1.11) has not been considered of primary importance in tomato fruit softening. However, the observation that PG does not hydrolyze highly methylated polygalacturonan *in vitro* led to the proposal that PME action is necessary prior to depolymerization by PG (Jansen and MacDonnell, 1945; Pressey and Avants, 1982b). Pectinmethylesterase is present throughout development of tomato fruit and there is no conclusive evidence that its activity may be limiting PG activity *in situ* during softening. Pectinmethylesterase is highly specific for galacturonan structure, attacking from the reducing end in a linear fashion to leave discreet regions of demethylated polygalacturonic acid. It does not proceed to complete deesterification and the precise degree of methylation at which its activity stops is unknown. Polygalacturonan with a degree of polymerization $\geq$10 is preferred substrate for PME (Rexova-Benkova and Markovic, 1976). Numerous isozymes of PME have been identified (Pressey and Avants, 1972; Delincee, 1976) which have pH optima near neutrality and are stimulated by calcium and sodium salts (Rexova-Benkova and Markovic, 1976).

Cellulase

Cellulase (EC 3.2.1.4) of the carboxymethylcellulase type is found in tomato fruit throughout development (Hobson, 1968; Sobotka and Stelzig, 1974). The level of activity in tomato fruit increases prior to appearance of PG, suggesting that cellulase may facilitate movement of PG to middle lamella (Babbit et al., 1973). Activity declines at about the pink stage of ripeness (Sobotka and Stelzig, 1974). A prominent role for cellulase in tomato fruit softening has not been established in spite of considerable research effort to do so. However, it does appear to be involved in formation of locular gel (Hall, 1964; Pharr and Dickinson, 1973; Huber, 1985). Avocado is the only fruit in which the primary softening mechanism is reported to be via cellulase activity (Awad and Lewis, 1980; Colinas-Leon and Young, 1981).

β-Galactosidase

The precise action of β-galactosidase (EC 3.2.1.23) is not well defined in the fruit softening literature, where its prominence may be due to the discovery that galactose is the neutral sugar solubilized from cell wall in greatest quantity during softening (Knee, 1973; Gross and Wallner, 1979). Pressey (1983) identified three isozymes in tomato pericarp. Although the significance of β-galactosidase in tomato fruit softening is not well defined, it is believed to be the enzyme responsible for softening in apple (Bartley, 1974).

β-1,3-Glucanase

The enzyme β-1,3-glucanase (EC 3.2.1.6) is present in tomato fruit throughout development (Wallner and Walker, 1975) but the level of activity increases in the latter stages of lycopene biosynthesis (Hinton and Pressey, 1980). Fruit had already softened considerably prior to the increase in glucanase activity, suggesting that it may be involved in the extensive wall degradation that occurs during senescence (Hinton and Pressey, 1980).

The preceding paragraphs briefly described the enzymes associated with cell wall degradation during tomato fruit softening. While it is clear that all could make some contribution to wall degradation, the enzyme PG is far more important at effecting tomato cell wall solubilization than other enzymes. At present, there is not a single study that correlates the activity of all of these hydrolases with softening in comparable tissue samples. Paull and Chen (1983) measured the activity of several wall hydrolases in ripening papaya fruit but provided no firmness data to correlate with enzyme activity.

Isolation of Plant Cell Walls

Problems Inherent to Wall Isolation Procedures

The plant cell wall may be regarded, for experimental purposes, as one vast macromolecule. Isolation of the cell wall without alteration is practically impossible; thus its disassembly is one of the first problems encountered by the cell wall researcher. The preferred method for cell wall disassembly is to employ degradative

enzymes, particularly carbohydrases, because of the specificity they afford (Lamport, 1978). Unfortunately, such a battery of purified enzymes is not available. A second, and more common, means of dismantling cell wall is to apply force to the matrix until it simply collapses. This may be accomplished by grinding with mortar and pestle (Bates and Ray, 1981), homogenization (Wallner and Bloom, 1977; Ahmed and Labavitch, 1980a; Buescher and Hobson, 1982), ball-milling (Lee et al., 1967; Selvendran, 1975), or utilization of a pressure cell (Talmadge et al., 1973). A problem inherent to these methods is how to retain an acceptable degree of structural integrity in the isolated walls while applying adequate force to disrupt the tissue (or cultured cells) so that no intact cells remain (Kivilaan et al., 1961). Selvendran (1975) emphasized that overmilling tissue produces powders that retain few of the characteristics of native cell walls.

An additional concern regarding cellular disruption is the medium in which the walls are to be isolated. If no liquid is added, the cytosol becomes the bathing medium and the possibility for bonding between cell wall and intracellular substances may be enhanced. Employment of non-aqueous solvents for initial homogenization particularly facilitates bonding of cytoplasmic proteins to cell wall (Selvendran, 1975). The specificity of this binding and the elution of proteins from cell wall are addressed later as separate topics. A serious consequence of cytoplasmic enzyme contamination is that the behavior of native wall-bound enzymes may be obscured (Kivilaan et al., 1961; Selvendran, 1975).

An additional problem is that binding of cytoplasmic components to a wall polymer may alter solubility of the carbohydrate (Selvendran, 1975). Solubility characteristics of wall constituents are important to the elucidation of wall structure (Talmadge et al., 1973).

The bathing medium for cell wall disassembly may be employed in subsequent washings to remove cytoplasmic contaminants and soluble wall components. Physical separation of walls from other cellular debris may be accomplished by either centrifugation (Bates and Ray, 1981), filtration (Buescher and Hobson, 1982) or both (Ahmed and Labavitch, 1980a; Talmadge et al., 1983). Filtration through materials such as Miracloth (Gross and Wallner, 1979; Buescher and Hobson, 1982) or coarse sintered glass (Talmadge et al., 1973) seems to offer the advantage over centrifugation of retaining only the wall fragments larger than the filter mesh, thus providing a degree of homogeneity to the wall preparation. The appropriate solvent for washing a centrifuged pellet or filtered residue depends on what needs to be solubilized and removed. Extracts that are exposed to strictly non-aqueous media, e.g., alcohol or acetone-insoluble-solids, should not be termed cell walls because they may contain other macromolecules such as proteins, starch, nucleic acids, or particulate cytoplasmic inclusions (Selvendran, 1975). Many of these contaminants are removed by aqueous wash (Klis et al., 1974).

Cell walls are commonly extracted in buffers because of the advantage of pH control (Talmadge et al., 1973; Gross, 1984). Chelating buffers should be avoided because they contribute to pectin solubilization (Jarvis, 1982; Buescher and Hobson, 1982). If minimal

wall modification is desired, the buffer pH should approximate that of the native matrix. Unfortunately, the cell wall pH in situ is not easily measured but is believed to be in the range of 4.0-5.0 (Lamport, 1978). Exposure to pH above neutrality may deesterify galacturonic acid residues, while exposure to low pH may hydrolyze methyl or acetyl groups (Ericson and Elbein, 1980). Manipulation of pH for elution of wall-bound pectins is discussed later.

Removal of lipophilic substances from cell walls may be accomplished by treatment with acetone or chloroform-methanol (Selvendran, 1975). Some plasma membrane constituents, e.g., glycolipids or glycoproteins, may be resistant to removal by these solvents if they are covalently bound to the cell wall (Kivilaan et al., 1961). Water-insoluble-minerals, particularly sodium and calcium, are often of concern in cell wall studies (Pressey and Avants, 1973; Jarvis, 1982; Demarty et al., 1984). These minerals may be removed with chelators but not without solubilization of pectins (Jarvis, 1982).

An additional consideration in selecting an isolation procedure is whether or not it facilitates determination of wall yield. Direct measurement of yield (weighing) is the method of choice but mandates that the walls be dried. Air drying of walls is accelerated by removal of water with ethanol or acetone (Talmadge et al., 1973). Freeze drying is sometimes employed (Klis et al., 1974); however, the possibility of low temperature inactivation of wall enzymes should be considered (Pressey, 1983). A difficulty that may arise during drying is the formation of aggregates that subsequently require

further mechanical disruption to facilitate uniform rehydration (Buescher and Hobson, 1982).

Wall-isolation protocol is typically tailored to preserve the wall component of greatest interest. For example, in studies that required preservation of protein-cell wall interactions, 80% glycerol was employed as the extraction medium. The glycerol was then removed from walls by washing first with ethanol, then acetone (Kivilaan et al., 1961; Lee et al., 1967). In a study directed to structural analysis of the wall, Talmadge et al. (1973) required a homogenous wall preparation and employed rather extensive protocol to obtain same. They utilized cultured sycamore cells that had only primary wall; these were ruptured in a pressure cell in neutral buffer, centrifuged, and the pellet washed with buffer, H_2O, and chloroform-methanol before filtering through coarse sintered glass. This procedure apparently was adequate for sycamore wall isolation but the structural analyses may have limited application to fruit cell wall, which may contain up to 6 times as much pectin as found in sycamore (Talmadge et al., 1973).

In an attempt to obtain a homogenous tomato pericarp cell wall preparation, several solvents (e.g., H_2O, ethanol, acetone, and chloroform-methanol) were utilized for repeated homogenization, filtration, and washing of the walls (Buescher and Hobson, 1982).

In view of the variability found in wall isolation procedures, it may be worthwhile to study several methods before choosing a specific protocol.

Binding of Polygalacturonase to Cell Wall

Little is known of the specific nature of PG-cell wall binding and virtually no data are available to characterize the catalytic site of PG. Speculation that PG binding sites *in situ* are associated with specific neutral sugar cross-links (Gross and Wallner, 1979) is not supported by the fact that PG will hydrolyze linear galacturonan *in vitro* in the absence of neutral sugars other than those attached to the PG protein itself (Ali and Brady, 1982). Although specific information is limited, there are considerable data of a more general nature on protein-cell wall binding.

Kivilaan et al. (1961) suggested that binding of non-wall enzymes to cell wall during isolation is minimal based on observations that specific activity of wall synthesizing enzymes was much greater in cell wall than in the cytoplasmic protein fraction. Also, oven-heated cell walls did not adsorb more enzyme (UDPG phosphorylase) when exposed to purified enzyme and the isolation procedure repeated. These results do not conclusively rule out contamination by cytoplasmic enzymes because binding sites could have been saturated during cellular disruption. It does, however, seem intuitively correct that cell wall enzymes bind to wall with greater specificity than non-wall enzymes. Bartley (1974) utilized walls from apple fruit as a source of ß-galactosidase and found 100% more activity associated with walls than was found in the soluble fraction.

These observations bring up the question of percentage saturation of protein-cell wall binding sites. Jansen et al. (1960)

reported that walls isolated from Avena coleoptiles could bind up to 200 times the amount of PME that would normally be found in the unmodified matrix. There are no existing data on the frequency of PG binding sites in tomato fruit cell wall so the percentage saturation of these sites is not calculatable.

Polygalacturonase is a glycoprotein (Ali and Brady, 1982) so there is the potential for covalent binding, however transient, between PG and the cell wall (Lamport, 1980). Strand et al. (1976) reported that a fungal endo-PG released detectable quantities of protein from 2 M NaCl washed cell walls of three plant species. Similar release by exo-PG was not observed. The authors suggested that proteins are covalently bound to polygalacturonan and are solubilized along with pectic polymers by the action of endo-PG. In a study of protein solubilization from tomato fruit walls by fungal PG, Hobson (1983) characterized protein-cell wall interactions as 1) those broken by salts, 2) those released by PG, and 3) structural glycoprotein resistant to salts and PG. Glycoproteins are sometimes difficult to purify and characterize because of microheterogeneity, i.e., subtle changes in the glycosyl moiety that may dramatically alter properties of the protein, such as its solubility, thermal stability, resistance to proteases, and extent of detergent binding (Lamport, 1980).

Other evidence strongly suggests that PG-cell wall binding is not exclusively covalent. Hobson (1964) reported that NaCl up to 1.7 M enhanced extraction of PG from homogenates of tomato pericarp. He could not exclude the possibility that pectic polymers, which might

be covalently bound to PG, also were solubilized by NaCl. Whether or not pectins are degraded by salts is not resolved. Knee (1973) reported that salt did not enhance removal of polyuronide from apple cell walls. In contrast, Gross (1984) reported that up to 20% of the polyuronide in tomato cell walls was ionically associated.

Isozymes of PG apparently have differential NaCl solubilites; PG II was extracted from tomato homogenate with 125 mM NaCl whereas 1 M NaCl was required to dissociate PG I from the pellet (Pressey and Avants, 1982b). The assumption that salt soluble PG represents 100% of the enzyme (Hunter and Elkan, 1974; Ali and Brady, 1982; Buescher and Hobson, 1982; Pressey and Avants, 1982; Paul and Chen, 1983) is not valid unless residual wall-bound PG is accounted for.

Wall-bound enzymes other than PG may be quite resistant to salt treatments. Huber and Nevins (1979; 1981) demonstrated that 3 M LiCl was required for dissociation of glucanases from autolytically active corn coleoptile cell wall. Substrate was not removed by the salt treatment. In addition, tomato cellulase extraction was not enhanced by up to 2 M NaCl although PG yield was doubled by the presence of salt (Babbit et al., 1973). The authors interpreted these results in terms of differential binding characteristics of cellulase and PG to the cell wall.

Binding of PG to cell wall apparently is perturbed by pH above neutrality. Numerous workers have reported that PG extraction from tomato fruit is enhanced by pH in the range of 7.0-10.0 (Babbit et al., 1973; Pressey and Avants, 1973; Ali and Brady, 1982). Polygalacturonase is a basic protein having an isoelectric point of

9.2-9.4 (Ali and Brady, 1982). Exposure to pH > 7.0 for prolonged periods may cause an irreversible decrease in PG activity (Ali and Brady, 1982).

Removal of PG or other proteins from cell wall is less of a problem if retention of enzyme activity is not a consideration. General protein extractants such as phenol:acetic acid:water (PAW) or sodium dodecyl sulfate (SDS) effectively strip many proteins from isolated walls (Selvendran, 1975). Non-ionic detergents such as Triton X-100 or Tween-60 are generally less effective in protein removal (Klis et al., 1974).

Bound enzymes may be inactivated *in situ* by oven-heating (Kivilaan et al., 1961), boiling 80% EtOH (Knee, 1973), or boiling water (Pressey and Avants, 1982b). Boiling cell walls in aqueous medium results in pectin degradation (Albersheim et al., 1960).

Regulation of Polygalacturonase In Vitro

No other factor affects apparent PG activity *in vitro* as dramatically as the nature of the substrate employed to characterize its activity. The reason for this is found in the diversity of D-galacturonan structure, which is a function of the degrees of polymerization and esterification, the ratio and distribution of neutral sugars, and the presence of acetyl groups (Huber, 1983b). These factors affect the extent of interchain association in the presence of calcium, causing considerable difficulty in ascertaining the structural configuration of substrate at the moment of hydrolysis by PG. Substrates that have been utilized to characterize PG

activity *in vitro* vary in complexity from pure galacturonan, seldom found in nature (Aspinall, 1980), to isolated pectic polymers or cell wall preparations (Wallner and Bloom, 1977). The following discussion provides an overview of factors that affect PG activity *in vitro* with consideration given to the substrates employed.

A logical approach to investigating the regulation of an enzyme is to search for factors that influence its activity. Unfortunately, naturally occurring, specific inhibitors of tomato PG are not well characterized. Benzyl isothiocyanate, found in papaya (Tang, 1971; 1974) and dextran sulfate both inhibited PG activity *in vitro* against a simple galacturonan substrate (Ali and Brady, 1982). Calcium inhibits *in vitro* PG activity to varying degrees (Ali and Brady, 1982; Buescher and Hobson, 1982). Its predominance in middle lamella, plus the observation that PME is activated by calcium (Ali and Brady, 1982) suggest that this divalent cation may have a prominent role in the regulation of softening.

Review articles on the role of calcium in the cell wall (Demarty et al., 1984) and its importance in ripening and senescence (Poovaiah, 1979; Ferguson et al., 1984) are currently available. Plant tissues generally contain 1-3% (dry wt) calcium, 60% of which is associated with cell wall, 7% is membrane-bound, and 33% is soluble (Demarty et al., 1984). Cell wall calcium is found in the pectin-rich middle lamella where it serves as a cross-link between adjacent polymers of rhamnogalacturonan (Aspinall, 1980; Demarty et al., 1984). This binding is strongly cooperative and is affected by pH; protons and calcium ions have approximately equal affinity for

carboxyl groups (Gidley et al., 1980). Thus the cell wall, particularly the middle lamella, behaves as an ion-exchange system for cationic molecular species. This probably accounts for the slow apoplastic movement of calcium in plant tissues (Demarty et al., 1984). The extent of calcium binding *in situ* is a function of the degree of esterification and rhamnose substitution of galacturonan as well as the presence of neutral sugar side chains (Huber, 1983b). There is some evidence that calcium is solubilized from middle lamella *in situ* during ripening, presumably by the action of PG (Rigney and Wills, 1981). The fate of this solubilized calcium is unknown. If transported across the plasmalemma, it could have many effects on intracellular metabolism (Wills and Rigney, 1979; Williamson, 1981; Marme, 1982).

The magnitude of calcium inhibition of *in vitro* PG activity is quite variable and may be reversed by addition of chelators (Ali and Brady, 1982; Buescher and Hobson, 1982). The variability may be attributed to differences in the assay conditions employed by different authors since there is no single, widely accepted technique for determining PG activity. Assay conditions may vary in terms of pH, ionic strength of bathing medium, choice of reaction buffer, nature of the substrate, and presence of PG isozymes. The effects of salts and pH have been studied in some detail and are discussed next with regard to regulation of PG *in vitro*.

Sodium chloride within the 200-300 mM range significantly stimulated *in vitro* activity of tomato PG (Pressey and Avants, 1973) as well as PME (Pressey and Avants, 1972; Lourenco and Catutani,

1984). The stimulation of PG was most pronounced at low pH when substrate was relatively large (Pressey and Avants, 1971). Under these circumstances hydrogen bonding may cause aggregation of pectic polymers and limit the access of PG to its site(s) of hydrolysis (Pressey and Avants, 1971; Gidley et al., 1980). Concentrations of NaCl higher than 300 mM caused a reduction in PG activity (Pressey and Avants, 1973). Chloride salts other than NaCl generally do not stimulate PG to the same extent, suggesting that the requirement for Na^+ may be rather specific (Pressey and Avants, 1973).

The pH optima for tomato PG isozymes *in vitro* is generally in the range of 4.0-5.0 if simple polygalacturonan is employed as substrate (Patel and Phaff, 1960a; Hobson, 1964; Pressey and Avants, 1973). The degree of polymerization of polygalacturonan influences the pH optimum, which shifts to the acid side if substrate is relatively small (Pressey and Avants, 1971). There is clearly an interaction between pH, calcium, and NaCl in regulation of PG activity *in vitro*. Any interpretation of these observations in terms of *in situ* PG regulation must take into consideration the effects of pH, calcium, and NaCl on properties of cell wall. Therefore, it seems pertinent to discuss the physical state of polysaccharides as a feature of regulation of PG *in vitro*.

Degradation of cell wall by PG *in vitro* is measured by the solubilization of wall fragments. Solubilized products within each fragment must diffuse into the bathing medium before they are detected, by which time more subtle, and perhaps more meaningful, events may have occurred. The rate of diffusion of solubilized

polygalacturonans is a function of several factors that affect their conformation and behavior in solution. High performance gel exclusion chromatography was used to ascertain the radii of gyration (R_g), i.e., rodlike vs. random coil behavior, of soluble pectins. The R_g was dependent upon the polymer size, its degree of methylation (Brant, 1980; Fishman et al., 1984), and the presence of calcium, which increased the R_g by cross-linking pectins to form larger aggregates (Kawabata et al., 1981). There is no conclusive evidence that softening in situ requires the removal of polymers from their structural site.

Other workers have approached cell wall rheology in terms of sol:gel transitions (Gidley et al., 1980; Morris et al., 1982; Powell et al., 1982; Oakenfull and Scott, 1984). These in vitro studies with simple pectic polymers are extremely difficult to interpret in terms of PG regulation in situ, where physiological restrictions are imposed upon the variables in question. An important consideration in cell wall sol:gel studies is the presence of wall-bound PME which, in the presence of calcium, caused gelatinization of pectins in vitro (Yamoaka, 1982; Yamoaka et al., 1983). The hypothesis that this gelatinization might be due to direct protein-pectin interaction rather than by calcium bridges (Bates and Ray, 1981) has been discredited to some extent (Pressey, 1984). Non-specific pectin-protein binding in processed tomato products is of interest because it affects viscosity of the product (Takada and Nelson, 1983).

The preceding discussion of the regulation of PG activity in vitro hopefully has drawn attention to the number of variables

involved. There is no reason to assume the process is less complicated *in situ*.

Implications of Microbial Contamination in Cell Wall Studies

Cell wall is not merely a passive physical barrier to microorganisms, but rather a dynamic entity having some capacity to interact with its surroundings, particularly when invaded by pathogens (Lamport, 1978). Pathogens show remarkable adaptability to new hosts by secreting wall-degrading enzymes that facilitate the spread of infection (Kolattukudy, 1984; Forster and Rasched, 1985). The products of pathogen destruction are termed elicitors if the plant cell responds by producing metabolites that aid in the suppression of further infection. Pectic fragments may act as elicitors (West et al., 1984; Neff and Binns, 1985). Pathogen-induced wall degradation is similar to that which occurs during fruit softening; therefore, it is imperative that consideration be given to the problems of microbes and their enzymes. Tomatoes intended for use in cell wall studies should be cultivated under the most sanitary conditions possible and injuries during harvesting and handling should be minimized. Surface sterilization of fruit prior to dissection further reduces the possibility of contamination. An additional precaution for *in vitro* studies is to employ an antibiotic; several have been evaluated for their interference of carbohydrate assays (Lindner and Shomer, 1984).

CHAPTER 3
ENZYMICALLY ACTIVE CELL WALL AS A TOOL FOR INVESTIGATION OF FRUIT SOFTENING

Fruit softening is generally attributed to degradation of the cell wall matrix by a series of enzymic, and perhaps some non-enzymic, mechanisms. Hydrolytic enzymes that have been investigated include polygalacturonases (Hobson, 1964), pectinmethylesterases (Jansen and MacDonnell, 1945), cellulase (Awad and Lewis, 1980), β-1,3-glucanase (Hinton and Pressey, 1980), β-galactosidase (Pressey, 1983) and others that are discussed in a recent review article (Huber, 1983b).

The traditional approach to studying the wall metabolism of softening has been to extract the enzymes of interest and assay with a commercially prepared substrate or with some type of cell wall preparation (Hobson, 1964; Wallner and Bloom, 1977). Such work has been informative but is subject to certain limitations. For example, the use of buffers containing citrate in experiments with cell wall preparations should be avoided bcause the chelation properties of citrate may enhance both enzymic and nonenzymic pectin solubilization (Buescher and Hobson, 1982; Carr and Ng, 1959), thus introducing uncertainty into both the quantitative and qualitative properties of the reaction in question. Secondly, the carbazole method (Dische, 1947) for determination of uronic acid degradation products, still

widely employed, is subject to interference by neutral sugars (Blumenkrantz and Asboe-Hansen, 1973). A more specific method for total uronic acid determination employs m-hydroxydiphenyl in chromagen formation and is not affected by the presence of other sugars (Blumenkrantz and Asboe-Hansen, 1973). Finally, the use of a reducing group assay specific for hexuronic acids (Milner and Avigad, 1967) has been generally ignored.

Perhaps the major criticism of cell wall softening studies is that extraction procedures tend to disturb the intimate relationship between the polysaccharide matrix and its bound protein. This problem may be circumvented by isolating cell walls under conditions which minimize the disruption of protein-cell wall interactions, then incubating that preparation under conditions favorable for continued enzymic activity. Several workers have used in vitro autolysis as a tool to characterize the wall metabolism of coleoptile tissue (Huber and Nevins, 1979; Kivilaan et al., 1961; Lee et al., 1967). Such an in vitro system seems ideal for study of the enzymic interactions that occur in cell walls of ripening fruits. Gross and Wallner (1979) reported a solubilization of uronic acids from cell walls of tomato incubated in distilled water. They described this product as water soluble pectin and assumed it was an artifact of extraction, but it seems more likely that they were observing an autolytic solubilization of wall pectins. Knee (1973) reported autolytic activity in acetone insoluble residue from apple fruit tissue but the analyses employed were for neutral sugars rather than the acid sugars that predominate in pectins. The objective of this study is to

characterize autolytic activity in enzymatically active cell wall preparations from tomato fruit through the course of their development.

Materials and Methods

Plant Material

Normal tomato (Lycopersicon esculentum Mill. cv. 'Rutgers') plants and the ripening mutant rin plants were grown at the University of Florida IFAS Horticultural Unit near Gainesville. Fruit were harvested at the following developmental stages: immature-green, mature-green, breaker, turning, pink, red (ripe), and overripe, based on a U.S.D.A. visual aid for color classification of tomatoes (Anon., 1975). Fruit were surface sterilized with 100 ppm NaOCl and sectioned, seed and placental tissue were removed and pericarp tissue stored at -20°C in sealed polyethylene bags.

Cell Wall Preparation

Modifications of cell wall during fractionation may result from mechanical stress, heat, pH extremes, chelators, and wall-bound enzymes. Since our objective was to preserve the integrity of polyuronides and their bound proteins, exposure to heat, chelating buffers, and strong ionic environment, was avoided. Approximately 100 g frozen pericarp tissue was partially thawed, peeled, and homogenized in 250 ml distilled water (4°C) or in acetate buffer (40 mM, pH 4.5) for 1 minute in an Osterizer blender set at maximum speed. The homogenate was transferred to Miracloth and washed with 3 liters distilled water (4°C) with continuous stirring. Excess water

was squeezed through the Miracloth and the remaining wall material used for autolysis experiments.

Autolysis Experiments

Approximately 100 mg (dry wt) of freshly prepared wall material were placed in 15 ml acetate buffer (40 mM, pH 4.5 unless otherwise stated) and incubated 22 hours in a shaking water bath at 22°C except in experiments involving temperature as a variable. An antibiotic (Thimerosal, 0.02%) was included to suppress possible microbial intereference. Sodium azide is often employed but was inappropriate here because of its strong interference with the Blumenkrantz assay. In other experiments, buffer contained 150 mM NaCl and 1, 10, or 100 mM $CaCl_2$. Some wall preparations were pre-treated by various methods designed to inhibit the autolytic reaction, including boiling 80% EtOH (20), boiling H_2O (24), and phenol:acetic acid:water (2:1:1, w/v/v). The latter technique was employed by Jarvis (1982) to inactivate cell walls and is based on a report by Selvendren (1975) that such treatment effectively removes protein from extracted cell walls.

Aliquots of 0.5 ml were removed from the reaction mixture at the start of incubation and at intervals throughout the trial. These aliquots were filtered through Whatman G/C glass fiber discs and analyzed for total uronic acids (Blumenkrantz and Asboe-Hansen, 1973) and for hexuronic acid reducing groups (Milner and Avigad, 1967). Some samples were analyzed for ß-eliminative breakdown of pectins as described by Albersheim (1960) and modified by Ayers et al. (1966). At the end of the incubation period (usually 22 hrs) remaining walls

were transferred to Miracloth, washed with distilled water, then placed in 30 ml 100% acetone. This mixture was then filtered through tared glass fibre filters and dried to a constant weight for cell wall dry weight determination. Acid sugars (µg equivalents) released during autolysis were added to obtain an estimate of initial dry weight. All experiments were repeated at least twice with two or three replications in each trial.

Results and Discussion

Homogenization of tomato pericarp in distilled H_2O yielded an extract with a pH of 4.3 for green fruit and about 4.2 for ripe fruit. There was no apparent advantage to extracting in acetate buffer in this pH range so the initial grinding was in distilled H_2O. All carbohydrate assays at time 0 were negative, indicating that soluble polysaccharides had been removed from the wall preparation.

There was a dramatic increase in the *in vitro* cell wall autolytic activity as ripening proceeded (Fig. 3-1). There was virtually no autolytic activity in walls extracted from immature-green fruit, with insignificant increases through the mature-green, breaker, and turning stages. In contrast, walls from pink fruit released up to 35 µg galacturonic acid equivalents/mg wall dry wt/hr for the first two hours, eventually solubilizing acid sugars constituting from 15 to 20% of the total wall weight in the 22 hr incubation period. Slight increases in initial rate were observed in red and overripe tissue, which is consistent with activity values

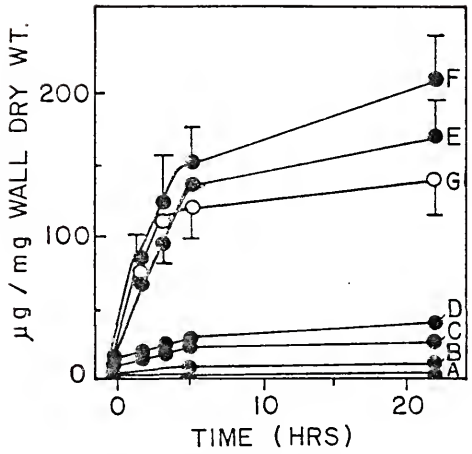


Fig. 3-1. Release of polyuronide from cell wall of tomato pericarp at different stages of development. Autolytic reactions were run at pH 4.5 in presence of 150 mM NaCl and 0.02% Thimerosal at 22°C. A = immature-green; B = mature-green; C = breaker; D = turning; E = pink; F = red (ripe); G = overripe (senescent). Bars indicate standard error and, when absent, fall under the symbol.

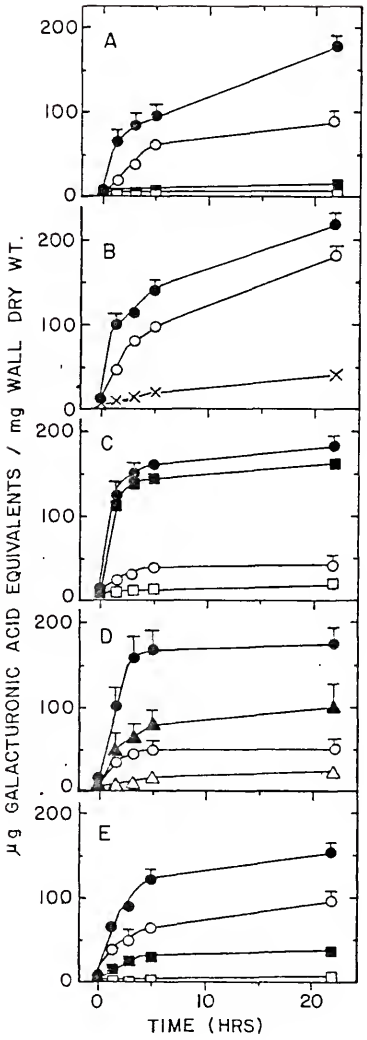
reported by Hobson (1964) for polygalacturonase (PG) extracts prepared from fruit at these stages of ripeness. He reported a dramatic rise in PG activity as fruit approached the orange-red (pink) stage with a gradual increase through overripeness. However, we observed a decrease in total uronic acid released from walls from overripe tissue (Fig. 3-1), which may be due to extensive solubilization of polygalacturonans *in situ* and their removal during the wall isolation procedure. Cell wall extracted from *rin* tissue did not autolyze (data not shown), an observation consistent with reports that PG is not present in *rin* (Buescher and Tigchelaar, 1975). To more fully characterize the features of enzymatically active cell wall, pink tissue was selected for most experiments because its initial rate of cell wall autolysis was rapid and it probably contained more unsolubilized polyuronide than ripe or senescent tissue.

Polygalacturonase activity *in vitro* is optimally enhanced by 150 mM NaCl (Pressey and Avants, 1982). We observed approximately a 100% increase in both the initial rate of autolysis and in total product solubilized when 150 mM NaCl was added to the reaction mixture (Figure 3-2A). Similar treatment had no such effect on walls from mature-green fruit, which is consistent with reports that PG is absent at this stage of tomato development (Hobson, 1964).

Temperature within the physiological range affected autolytic behavior in a manner consistent with the proposal that the activity is enzymic (Fig. 3-2B). One might anticipate that exposure to higher temperature could serve as a means of inactivating the walls.

Fig. 3-2. Characterization of the autolytic reaction in cell wall from pericarp of pink tomato fruit. Reactions were run at 22°C (except in 3-2B) and in the presence of 150 mM NaCl and 0.02% Thimerosal. Bars indicate standard error and, when absent, fall under the symbol.

A. Effect of 150 mM NaCl. (● = pink + 150 mM NaCl, ○ = pink without NaCl, ■ = mature-green + 150 mM NaCl, □ = mature-green without NaCl)
B. Effect of temperature. (× = 4°C, ○ = 24°C, ● = 34°C)
C. Effect of boiling 80% EtOH and hot (90°C) H_2O pre-treatments. (● = cold H_2O, ○ = hot H_2O, ■ = cold EtOH, □ = hot EtOH)
D. Effect of phenol:acetic acid:H_2O pretreatment. (● = water pre-treatment control, ▲ = reducing groups for water control, ○ = phenol:acetic acid:H_2O pre-treatment; △ = reducing groups for pre-treatment samples)
E. Effect of $CaCl_2$. (● = no $CaCl_2$, ○ = 1 mM, ■ = 10 mM, □ = 100 mM)


A
B
C
D
E
200
100
0
0
10
20
TIME (HRS)
µg GALACTURONIC ACID EQUIVALENTS / mg WALL DRY WT.

However, hot (90°C) water results in solubilization of pectin, and possibly depolymerization via β-elimination (Albersheim et al., 1960) and, therefore, is not an appropriate means of inactivating autolytic activity. Knee (1973) proposed boiling in 80% EtOH as a means of enzyme inactivation without pectin degradation. In the present study, both boiling H_2O and 80% EtOH inactivated the wall preparation (Fig. 3-2C); however, the boiling EtOH treatment did not result in release of detectable amounts of wall material. Another method of wall inactivation (Selvendren, 1975) involves exposure to phenol:acetic acid:H_2O (2:1:1, w/v/v) that presumably removes wall protein. Jarvis (1982) used this technique in experiments that presumed the absence of any wall-bound enzyme activity. However, our wall preparations were not fully inactivated by this method, which reduced initial rates and total product solubilized by about 75% (Fig. 3-2D). The amount of reducing groups relative to total uronic acid, shown in Figure 3-2D and Table 3-1, is typical of results obtained throughout all experiments, with reducing groups generally amounting from one-half to two-thirds of the total acid sugars solubilized. Gel filtration analysis of autolytic products confirmed the absence of monomer (data not shown), which is consistent with the concept that the primary mechanism for polyuronide solubilization in tomato fruit involves only endo-PG activity (Pressey, 1977).

Polygalacturonase activity *in vitro* is dramatically inhibited by 10^{-7} M calcium (Wills and Rigney, 1979). Figure 3-2E illustrates the effect of $CaCl_2$ on release of pectins from wall from pink tissue. A minimum of 10^{-3} M $CaCl_2$ was required to achieve 50% inhibition of

Table 3-1. Effect of pH on total polyuronides released from cell wall of pink tissue during a 22 hr incubation at 22°C period in presence of 150 mM NaCl and 0.02% Thimerosal.

	μg galacturonic acid equivalents/mg wall dry wt	
pH	total uronic acid	hexuronic acid reducing groups
1.5	23	8
2.5	292	66
3.5	202	89
4.5	180	74
5.5	232	66
6.5	83	22
7.5	42	19

autolysis, a concentration several orders of magnitude higher than levels required to inhibit the activity of purified enzyme preparations assayed using commercial pectin preparations. We considered the possibility that this discrepancy may be due to the presence of membrane fragments in our wall preparation that have calcium binding sites, but pretreatment with 100% acetone did not significantly alter the inhibitory effect of calcium. However, cell walls have many such binding sites (Jarvis, 1982) that may have sequestered the excess calcium and the inhibition may be due to an interaction with substrate rather than to direct effects on the

protein. Similar results were obtained when $CaSO_4$ was substituted for $CaCl_2$ (data not shown). Tomato PG activity *in vitro* is also inhibited by 0.001% dextran sulfate (Ali and Brady, 1982) but such effects were not observed in our system. This may be explained by the inability of the high molecular weight dextran to diffuse through the wall matrix and bind to the enzyme.

Experiments to determine the pH optimum for autolytic activity revealed a peak at 5.5 that may represent the optimum for enzyme-mediated solubilization. Exposure to low (1.5) or high (7.5) pH extremes resulted in substantial reduction of activity (Table 3-1). Other workers have reported pH optima for PG activity *in vitro* between 4.0 and 5.0 (Hobson, 1964; Pressey and Avants, 1982b; Themmen et al., 1982), but we observed at least a 30% increase in polyuronide solubilization when pH was lowered to 2.5. This may be non-enzymic degradation since reducing groups do not appear in comparable quantity. This observation seems reasonable when one considers the involvement of calcium in cross-linking of galacturonic acid residues between adjacent pectic polymers. Presuming that increasing $[H^+]$ could result in displacement of calcium, one might predict an increase in polyuronic solubilization by at least two mechanisms. The first is non-enzymic, whereby Ca^{++}-stabilized regions of polygalacturonan are spontaneously solubilized with removal of Ca^{++}, thus accounting for fewer reducing groups at pH 2.5. Secondly, loosening of wall matrix via Ca^{2+} removal could reduce the physical resistance encountered by enzymes as they migrate to potential sites of hydrolysis, thus increasing the efficiency of enzymic activity. A

range of activity was observed up to pH 5.5 and we could not rule out the possibility of β-eliminative degradation. However, all assays for the presence of β-elimination products were negative. One interpretation of the pH effect is that slight changes in cell wall matrix pH may provide an elegant means of regulation of enzyme-mediated fruit softening.

Several distinct advantages are obvious in using enzymatically active cell walls for study of softening metabolism. First, it more closely resembles the situation *in situ* and thus is a more realistic system with which to study the enzymology of softening. Secondly, it may prove to provide a system for observation of sequential processes during softening, e.g. pectin degradation accompanied by loss of neutral sugars and hemicellulose modifications that, until recently, had not been studied in tomato (Huber, 1983a). In addition, it is ideal for study of binding characteristics of protein to the polysaccharide matrix. No single *in vitro* system reported to date has this potential for characterizing patterns of wall degradation in ripening fruit. Interesting relationships have been observed between pH and ionic strength of the extraction media and subsequent autolytic activity. There seems to be a complex arrangement of ionic and covalent bonds that are disrupted by various means to ultimately lead to softer fruit. Work is continuing on analysis of solubilized carbohydrate and protein products in the autolysis system.

CHAPTER 4
RELATIONSHIP BETWEEN HANDLING TECHNIQUES AND SOLUBILIZATION OF CELL-WALL CONSTITUENTS DURING AUTOLYSIS OF ISOLATED TOMATO CELL WALLS

The employment of enzymically active cell walls to investigate fruit softening was described in Chapter 3. These experiments confirmed that polygalacturonase-mediated softening metabolism may be elucidated by examination of autolytically active wall. In order to maximize its usefulness, several aspects of the *in vitro* autolysis system need refinement.

Previous experiments (Chapter 3) were performed using freshly prepared, hydrated cell-walls which precluded the possibility of taking accurate preautolysis dry weight measurement. Efficiency of postautolysis cell-wall recovery also could not be determined under those circumstances. A technique for cell-wall preparation and handling is needed that allows accurate dry weight determination prior to autolysis and ensures recovery of insoluble wall material postautolysis. Additionally, earlier experiments addressed only the acid sugars released by autolysis. Soluble neutral sugars also should be defined because they are covalently bound to rhamnogalacturonan and thus are important structural features of pectin (Albersheim, 1976; Aspinall, 1980). Aliquot removal as a technique for sampling solubilized acid sugars (Chapter 3) has limitations when employed for neutral sugar determination. First,

neutral sugar levels are too low for detection in a small aliquot of reaction buffer. Secondly, sequential release of products may be masked unless complete removal of soluble constituents is conducted at each sampling. A means of sampling must be employed that maximizes the amount of soluble product available for analysis and reduces the possibilities of product overlap in time-course experiments.

An additional parameter requiring further investigation is the size distribution of pectic polymers released during autolysis. Examination of this property may offer insight into the substrate specificity of wall-bound polygalacturonase (PG). Gross and Wallner (1979) provided some groundwork for these analyses in studies of water and enzyme-soluble pectins released from tomato fruit cell walls. Water soluble pectins (presumably autolysis products) consisted exclusively of large molecular weight polymers, while enzyme soluble pectins ranged considerably in size. The present study addresses the possibility of a temporal relationship between cell-wall autolysis and the size distribution of solubilized acid sugar.

The primary objective of this study is to examine various methods of cell-wall preparation and handling available for further characterizing the autolytic system. The conditions that appear most satisfactory in circumventing the problems discussed above will be employed to determine the neutral sugar content and relative size distribution of pectins released during autolysis of tomato cell walls.

Materials and Methods

Plant Material

Tomato (Lycopersicon esculentum Mill. cv 'Rutgers') plants were grown at the University of Florida Horticultural Unit near Gainesville. Fruit were harvested at mature-green, pink, and red (ripe) developmental stages based on a United States Department of Agriculture visual aid for color classifications of tomatoes (Anon., 1975). Fruit were surface sterilized with 100 ppm NaOCl and sectioned; seed and placental tissue were removed and pericarp tissue stored at -20°C in sealed polyethylene bags.

Cell Wall Preparation

Approximately 100 g of frozen pericarp were partially thawed, peeled, and homogenized in 400 ml of distilled H_2O (4°C) for 1 min in a Sears blender set at maximum speed. The homogenate was transferred to Miracloth (Calbiochem) and washed with 3 liters of distilled H_2O (4°C) with continuous stirring. Excess water was squeezed through the Miracloth and the remaining wall material used immediately for autolysis experiments.

In other experiments, walls were washed with 1 liter of acetone (1°C) prior to removal from Miracloth. The acetone-washed material (cell-wall) was suspended in 100 ml of acetone for 1 hr. This suspension was filtered through Whatman GF/C filters and rinsed with acetone (approximately 500 ml) until pigment was removed, then the cell walls were transferred to a beaker and dried at 30°C with occasional stirring to reduce formation of aggregates. Dry

cell walls were stored at 22°C in sealed containers and were used for autolysis experiments within 2 weeks after preparation.

Cell-Wall Rehydration

Cell walls (100 mg dry wt) were placed in a 50 ml centrifuge tube in an ice bath and rehydrated by dropwise addition of 15 ml cold (1°C) reaction buffer (50 mM Na acetate, pH 4.5, 150 mM NaCl, 0.02% Thimerosal). Samples were held at 1°C for 30 min with occasional stirring, then centrifuged (10 min at 10,000 RPM) with a Beckman Model J2-21 centrifuge equipped with JA-20 rotor. The supernatant was filtered through Whatman GF/C filters to obtain a time-zero sample. Cell walls were resuspended in 15.0 ml reaction buffer and incubated at 34°C.

Sampling Techniques for Soluble Products

Aliquots (0.5 ml) of reaction buffer were removed at intervals as previously described (Chapter 3) or, in other experiments, reaction mixtures were centrifuged (10 min at 10,000 RPM) at each interval and supernatant filtered through Whatman GF/C filters. Walls were then resuspended in 15.0 ml reaction buffer and the incubation resumed.

An additional technique was designed that allowed continuous sampling of soluble products throughout each experiment. Cell walls (100 mg dry wt) were rehydrated and packed into a water-jacketed column (1.6 x 6.0 cm) maintained at 34°C and continuously eluted with reaction buffer to remove solubilized products. Fractions (5 ml/fraction) were collected with an ISCO Model 1850 fraction collector.

Postautolysis Wall Recovery

Insoluble walls were recovered postautolysis either by Miracloth filtration as previously described (Chapter 3) or by centrifugation (5 min at 10,000 RPM), supernatant removal, and resuspension of walls in 30 ml acetone. This suspension was filtered through tared Whatman GF/C filters, dried at 30°C, and weighed.

Soluble Product Analysis

Neutral sugars were analyzed as alditol acetate derivatives (Jones and Albersheim, 1972) using a Hewlett-Packard Model 5710A gas chromatograph equipped with flame ionization detectors and a Hewlett-Packard Model 3390A integrator. Total acid sugars and acid reducing sugars were assayed using the techniques of Blumenkrantz and Asboe-Hansen (1973) and Milner and Avigad (1967) respectively. Soluble protein was determined with a Bio-Rad (Bio-Rad Laboratories, Richmond, CA) assay after the method of Bradford (1976). Gel filtration analysis of 80% ethanol insoluble galacturonan was performed on a bed (1.5 x 4.5 cm) of either Fractogel HW-50 (EM Science, Gibbstown, NJ) or Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, CA). Samples were eluted with Na-acetate buffer (50 mM, pH 5.0) containing 100 mM NaCl and 5 mM Na_2-EDTA. Fractions of 2 ml (Fractogel) or 1.5 ml (Bio-Gel) were collected using an LKB (Model 2070) fraction collector.

Wall Compositional Analysis

Dry cell walls were analyzed for total pectin (Ahmed and Labavitch, 1977), lipids (Harborne, 1973), nitrogen (Nelson and

Sommers, 1973) and calcium (Soil Science Dept. Analytical Laboratory, Univ. of Florida).

Autolysis Experiments

The following experiments were conducted at 34°C and were repeated at least once with two or three replications each time.

Experiment 1. Two variables (1. length of incubation time, and 2. method of postautolysis cell-wall recovery) were examined for their effect on the estimation of acid-sugars released during autolysis. Freshly prepared, hydrated cell walls from pink pericarp were incubated for either 5 hrs or 22 hrs and recovered postautolysis either by centrifugation or Miracloth filtration as described earlier in this section.

Experiment 2. Two techniques of cell-wall preparation were examined for their effect on autolysis of cell walls isolated from pink or red pericarp. Freshly prepared, hydrated cell walls were compared to acetone-washed and dried cell-wall material. Sampling for soluble products was done by aliquot (0.5 ml) removal and postautolysis cell wall recovery accomplished by centrifugation as described.

Experiment 3. This is the first of two experiments that address sampling techniques for soluble autolysis products. Acetone-washed and dried cell walls from pink pericarp were rehydrated and packed into a column that was eluted with reaction buffer as described. Solubilization of cell walls pretreated with boiling 80% EtOH to inactivate bound enzymes also was examined on the column. For comparison to earlier studies, additional samples were incubated in

centrifuge tubes with soluble product sampling by aliquot removal and postautolysis cell-wall recovery by centrifugation.

Experiment 4. This experiment compares two methods of sampling for soluble autolysis products (1. aliquot removal, and 2. centrifugation and supernatant removal) from cell walls incubated in centrifuge tubes. Acetone-washed and dried cell walls isolated from either pink or mature-green pericarp were used and in some experiments cell walls were exposed to boiling 80% EtOH prior to incubation.

Experiment 5. A system of cell wall handling was chosen from preceding experiments for use in further characterization of soluble autolysis products. Cell walls extracted from pink pericarp were acetone-washed, dried, and rehydrated in centrifuge tubes to observe the autolysis reaction. Centrifugation and supernatant removal were used for soluble product sampling and postautolysis cell wall recovery. Analyses of soluble products included acid sugars, neutral sugars, protein, and gel filtration chromatography of pectin polymers.

Results

Experiment 1

Postautolysis dry wt of cell walls was considerably lower in samples recovered by Miracloth filtration compared to dry wt of pellets recovered in centrifuged samples. Dry wts of pellets recovered after 5 hrs incubation were 2.5 times greater than residue retained by Miracloth and this weight difference increased to 3.5

fold when incubation was extended to 22 hrs (Table 4-1). All samples were approximately identical in wt when autolysis was initiated and the substantial difference in weights of cell walls recovered postautolysis reflects a difference in methods used to recover the insoluble material.

Postautolysis dry weight is used to calculate the preautolysis cell-wall weight and thus is a key factor in estimating the release of solubilized products as a percentage of cell-wall weight. When dry weight is low the corresponding value for soluble product may be high. This is most exemplified in cell walls incubated 22 hrs and recovered on Miracloth, which have a postautolysis dry weight recovery less than one-third of those recovered using centrifugation (Table 4-1). The calculated value for solubilized acid sugar is 4 times greater for samples recovered on Miracloth compared to centrifuged samples after 22 hrs incubation (Fig. 4-1). This difference is less pronounced (2.5 fold) in samples incubated for 5 hrs. Quantities of acid sugar released were similar in all samples at intervals throughout the experiment (data not shown) therefore the large differences observed in estimates of soluble acid sugars as a percentage of wall dry weight (Fig. 4-1) are directly related to the reduced amount of wall material recovered on Miracloth compared to centrifugation (Table 4-1).

Experiment 2

Cell walls prepared from pink pericarp were acetone-washed and dried (see Methods) and, upon rehydration, released acid sugars at

Table 4-1. Estimates of wall dry wts (mg) of samples described in Fig. 4-1 below.

Reaction time (hrs)	Postautolysis Recovery Method	
	Miracloth filtration	Centrifugation
5	51.9	124.2
22	30.9	112.7

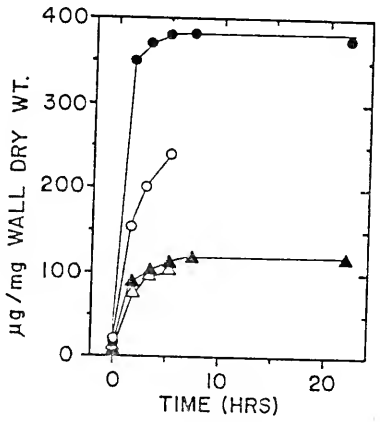


Figure 4-1. Effect of incubation time and postautolysis recovery method on estimate of solubilized acid sugars during autolysis of freshly prepared cell walls from 'Rutgers' pink pericarp at 34°C (●,○ = Miracloth filtration;▲,△= centrifugation). The experiment was repeated with similar results each time. These data are from a single experiment with duplicate samples and standard errors are smaller than the symbols.

rates comparable to freshly prepared cell walls (Fig. 4-2). However, walls from red tissue that were acetone-washed and dried lost approximately 25% autolytic capacity compared to the fresh extract (Fig. 4-2). Dry walls from both pink and red pericarp were stored up to 2 weeks in sealed containers with no loss of autolytic activity (Fig. 4-2).

Yield (dry wt) of cell walls prepared in this manner decreased over 50% during ripening (Table 4-2) but pectin content of the extracts decreased only slightly from 34% in mature-green to 32% in overripe. Compositional analyses revealed that approximately 1% of wall dry weight is calcium, 6-8% is protein, and there was no detectable lipid.

Table 4-2. Yield of cell-walls extracted from 'Rutgers' pericarp at different stages of development.

Stage of Development	Wall yield (mg/g fresh wt.)
Mature-green	1217.3
Pink	950.7
Red (ripe)	790.8
Overripe (senescent)	546.8

Experiment 3

A technical difficulty encountered in designing the column was the selection of an appropriate supportive material to retain the cell walls. Small pore materials such as fine sintered glass,

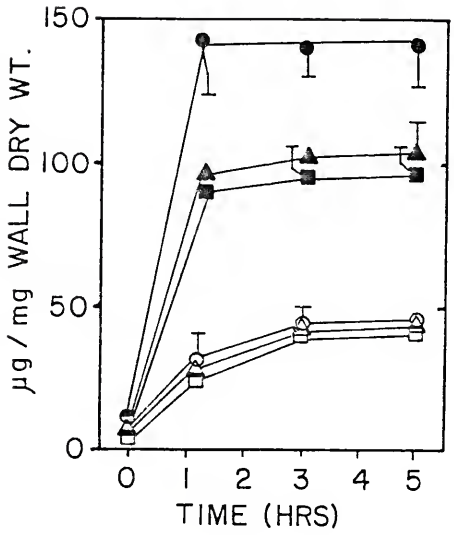


Figure 4-2. Effect of acetone-washing and drying on release of polyuronide from cell-walls isolated from 'Rutgers' pink or red pericarp. Data points represent the mean of duplicate samples and standard error bars, when absent, are smaller than the symbol (●,▲,■ = red; ○,△,□ = pink; ●,○ = freshly prepared, hydrated, cell-walls; ■,□ = acetone-washed and dried cell-walls 24 hrs after preparation; ▲,△ = dried cell-walls 2 weeks after preparation).

Whatman GF/C filters, and Spectramesh (Spectrum Medical Industries, Los Angeles, CA; 10 micron pores) became plugged by eluted products soon after the reaction was initiated. Results presented here were obtained by using a double layer of Miracloth that was carefully mounted so that it was not stretched and, presumably, its filtration properties were not altered.

Profile for elution of acid sugars from predried, column-packed walls from pink pericarp shows that most acid sugars were solubilized within 2 hours (Fig. 4-3B). Soluble acid sugars and protein account for 24% and 11%, respectively, of the preautolysis dry weight (100 mg). After 5 hours elution, 49.2 mg insoluble wall material was recovered from the column (Table 4-3).

Cell walls pretreated with boiling 80% EtOH exhibited a profile of acid sugar solubilization (Fig. 4-3C) similar to the profile obtained for enzymically active walls (Fig. 4-3B). The preautolysis dry weight (100 mg) of pretreated walls decreased to 80.3 mg after 5 hrs elution (Table 4-3). Soluble acid sugars account for 12.4 mg of the decrease during autolysis.

For comparison, wall samples were allowed to autolyze in a static system (centrifuge tubes) with soluble product sampling by aliquot (0.5 ml) removal. Approximately 10% of the preautolysis dry weight (100 mg) was released from enzymically active walls as soluble acid sugar during 7 hrs incubation (Fig. 4-3A) compared to 24% with the column sampling system. Inactivated walls in the static system released only 3% (dry wt) as acid sugar (Fig. 4-3A) compared to 12.4% on the column. Rate of acid sugar solubilization declined to near

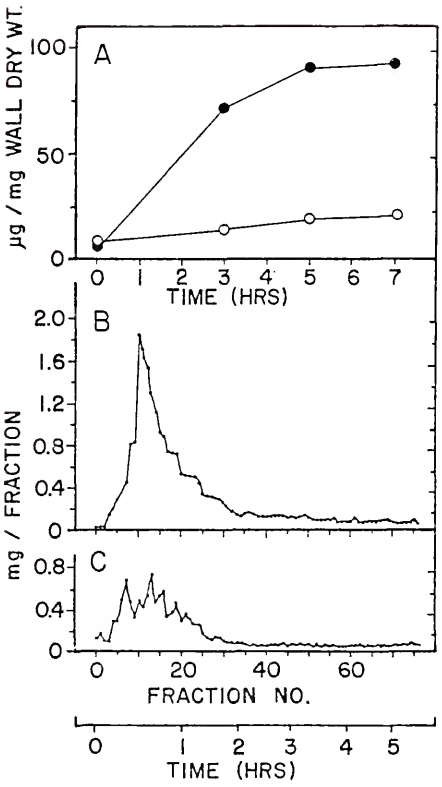


Figure 4-3. Comparison of methods of sampling for soluble autolysis products from cell walls isolated from 'Rutgers' pink pericarp. Each sample consisted of 100 mg dry cell walls and experiments were performed at 34°C. These data are from a single experiment that was repeated with similar results.

A. Cell-walls incubated in static system (centrifuge tubes) with sampling by aliquot (0.5 ml) removal. Data points are means of duplicate samples and standard error is within each symbol (● = enzymically active walls; ○ = pretreated with boiling 80% EtOH).

B. Profile of acid sugar released from enzymically active walls placed on a column.

C. Profile of acid sugars released from cell walls that were pretreated with boiling 80% EtOH.

Table 4-3. Dry weights and acid sugar content of cell-wall samples described in Fig. 4-3.

Sample	Dry weight (mg)	Acid Sugar (% dry wt)
preautolysis	100	32.2
postautolysis		
A (●)	80.0	22.5
(○)	93.0	29.0
B	49.2	8.2
C	80.3	19.8

zero after 5 hrs incubation in the static system compared to 2 hrs elution time on the column (Fig. 4-3). Postautolysis dry weight of samples incubated in static system were 80.0 and 93.0 mg, respectively, for enzymically active and boiling 80% EtOH inactivated samples (Table 4-3).

Experiment 4

An additional method of soluble product sampling involved centrifugation of the reaction mixture and removal of supernatant at each sampling throughout the experiment. This was compared to the previously described technique of aliquot removal. Enzymically active walls from pink tissue that were repeatedly subjected to centrifugation, supernatant removal, and pellet resuspension in fresh buffer exhibited a 50% increase in both rate of release and total amount of acid sugars and acid reducing sugars released during autolysis compared to those that were sampled by aliquot removal (Fig. 4-4A,B). Samples that were pretreated with boiling 80% EtOH were more drastically affected, releasing almost 3 times more product

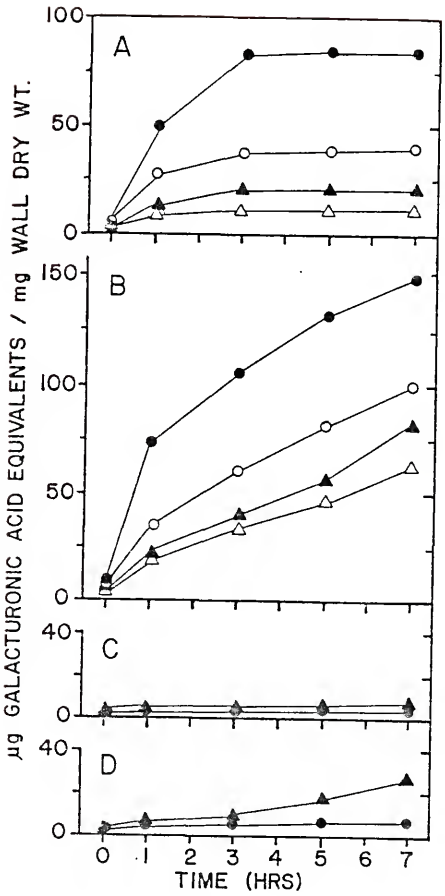


Figure 4-4. Comparison of aliquot removal to centrifugation and supernatant removal for sampling of solubilized acid sugars in cell walls from 'Rutgers' pink and mature-green pericarp incubated at 34°C. Each sample consisted of 100 mg dry cell walls. Results are from a single experiment that was repeated twice with similar results each time.
A. Pink, aliquot removal; B. Pink-centrifugation; C. Mature-green, aliquot removal; D. Mature-green, centrifugation (●,▲= total acid sugars; ○,△= acid sugar reducing groups; ●,○ = no inactivation pre-treatment; ▲,△= pretreated with boiling 80% EtOH).

if sampled by centrifugation (Fig. 4-4A,B). Walls from mature-green tissue released acid sugars only if pretreated with boiling 80% EtOH and centrifugation used for soluble product sampling (Fig. 4-4C,D). Enzymically active samples that were centrifuged and resuspended in the same buffer did not release more acid sugars than uncentrifuged samples (data not shown).

Experiment 5

The procedures for cell-wall preparation and handling described for data presented in Fig. 4-4B were used to further characterize autolysis products. Solubilized products from enzymically active walls were sampled at intervals throughout the time course. Enzymically-inactive (pretreated with boiling 80% EtOH) walls were sampled at time zero and at the end of the experiment.

Acid-sugar reducing groups were solubilized from enzymically active walls (Fig. 4-5) in quantities equal to the earlier trial (Fig. 4-4B). However, the total soluble acid sugar was about 25% lower the first time. Protein was released from the wall preparation at a fairly constant rate (approx. 1 mg/hr) throughout the incubation period (Fig. 4-5) and the total released accounted for almost 7% of the preautolysis dry weight (Table 4-4).

Samples inactivated with boiling 80% EtOH lost 9.0% of their dry weight during incubation, with the predominant products being acid sugar and protein (Table 4-4). Total acid sugar content of the cell-wall preparation was 34.5% before autolysis and decreased to

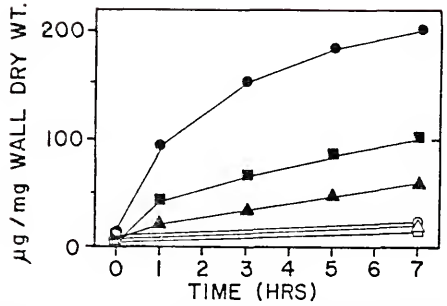


Figure 4-5. Solubilization of uronic acids and proteins during autolysis of cell walls extracted from 'Rutgers' pink pericarp. Duplicate samples each contained 100 mg (preautolysis dry weight) cell walls and standard errors are smaller than the symbols (● = total acid sugar; ■ = acid reducing sugars; ▲ = protein; ○,□,△ = pretreated with boiling 80% EtOH).

Table 4-4. Dry weights of individual components of the autolytic reaction described in Fig. 4-5 above.

	Dry Weight (mg)	
	Enzymically active	Pre-treated (Boiling 80% EtOH)
Cell-walls		
preautolysis	100.0	100.0
postautolysis	74.6	91.0
Soluble products		
acid sugar	21.0	3.2
protein	6.8	2.9
neutral sugar	2.4	0.8

13.5% and 31.3%, respectively, for enzymically active and inactivated samples recovered postautolysis.

Neutral sugars solubilized during autolysis made up slightly over 2% of the preautolysis wall dry wt (Table 4-4) and were released at a rate of 5 µg/hr the first hour. This rate decreased to 2.5 µg/hr by the end of the incubation period (Fig. 4-6). The acid: neutral sugar ratio was 10.8 after the first hour but had decreased to 2.9 by the seventh hr of incubation (Fig. 4-6). Analysis of individual neutral sugars revealed that galactose was the predominant product, constituting approximately 50% of the total neutral sugar released (Fig. 4-7). Arabinose accounted for 30% and minor quantities of rhamnose, xylose, glucose, and mannose were also detected (Fig. 4-7).

Gel filtration chromatography was used to analyze samples from the reaction described in Fig. 4-5. Bio-Gel P-10 was chosen to facilitate this analysis since Gross and Wallner (1979) found it appropriate for fractionating enzyme (PG)-solubilized-pectin (ESP) from cell walls from mature-green tomatoes. Pectic polymers solubilized during autolysis had a size distribution (Fig. 4-8) quite similar to the profile obtained for ESP by Gross and Wallner (1979). However, no apparent shift in polymer size during the time course was observed with either Bio-Gel P-10 (Fig. 4-8) or Fractogel HW-50 (Fig. 4-9). A portion of the supernatant was removed from sample t_t and incubated an additional six hours at 34°C, during which no increase in acid reducing sugars was observed.

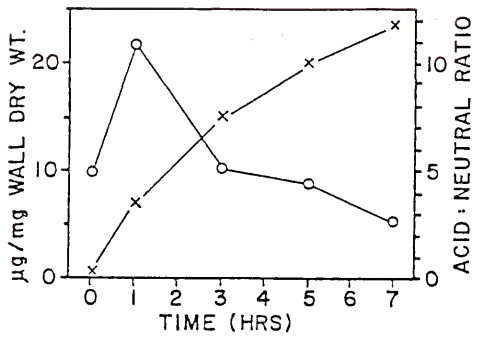


Figure 4-6. Solubilization of neutral sugars and acid:neutral sugar ratio during autolytic reaction described in Fig. 4-5 (× = total neutral sugar; O = acid neutral ratio).

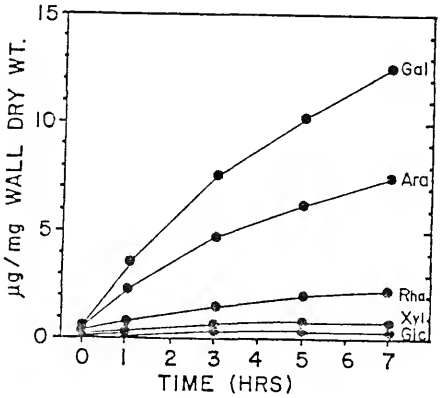


Figure 4-7. Neutral sugars released in the autolytic reaction described in Fig. 4-5 (Gal = Galactose; Ara = Arabinose; Rha = Rhamnose; Xyl = Xylose; Glc = Glucose; a detectable trace of mannose was present).

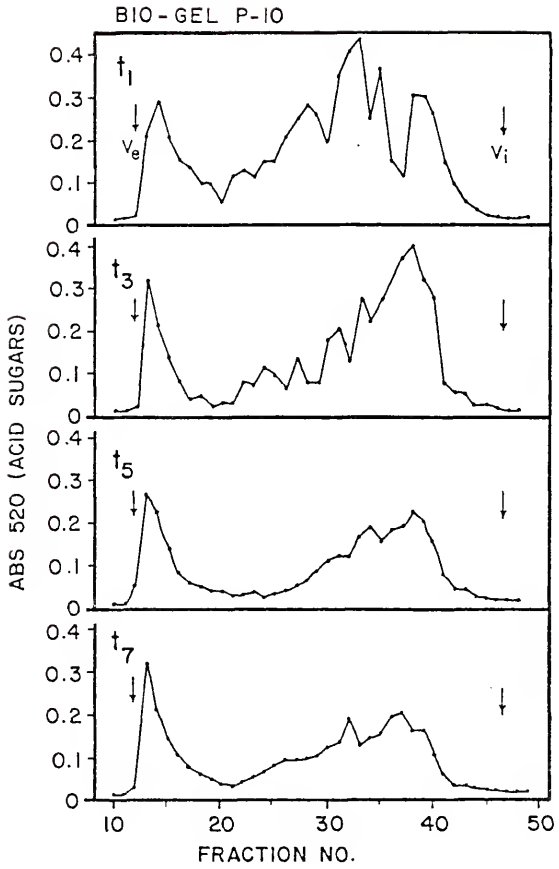


Figure 4-8. Bio-Gel P-10 chromatography of polyuronides released in the autolytic reaction described in Fig. 4-5. (V_e = exclusion limit; V_i = inclusion limit; subscripts following t = sampling time)

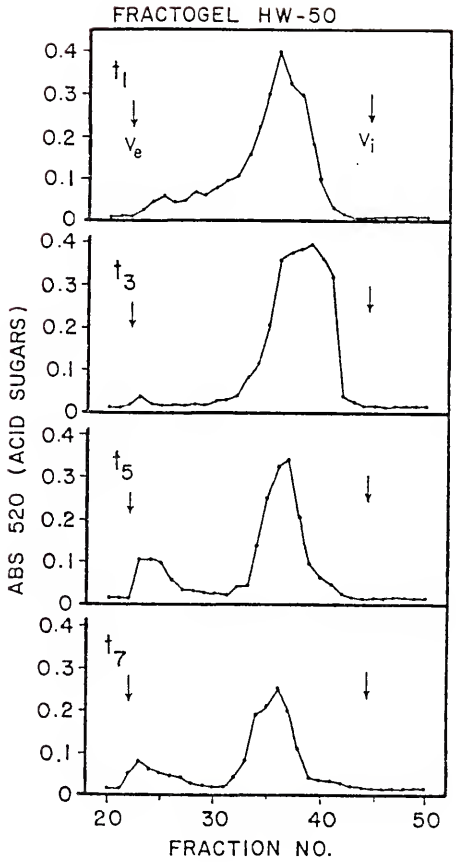


Figure 4-9. Fractogel HW-50 chromatography of polyuronides released in the autolytic reaction described in Fig. 4-5. (V_e = exclusion limit; V_i = inclusion limit; subscripts following t = sampling time)

Discussion

The use of Miracloth for postautolysis recovery of cell walls (Chapter 3) was based on the assumption that cell-wall material other than that enzymically solubilized in autolytic reactions would be quantitatively retained. Data presented in Table 4-1 do not support that assumption. The autolysis reaction apparently generated fragments that were not soluble in the supernatant of a centrifuged sample yet were able to pass through Miracloth. Fragments lost through Miracloth were unaccounted for and the resulting estimates of dry weights were low (Table 4-1), leading to an overestimation of the relative amount of soluble product (Fig. 4-1). There was an apparent increase in the quantity of these fragments during the incubation period between 5 hrs and 22 hrs since considerably lower amounts of cell walls were recovered on Miracloth after extended incubation (Table 4-1). The use of Miracloth for postautolysis cell-wall recovery was discontinued because it does not appear to adequately retain cell walls once a degree of degradation has occurred.

A significant source of error in estimating soluble products was identified and, to some extent, resolved. However, the precise preautolysis dry weight of the freshly prepared, hydrated cell walls used in the previous experiment could not be ascertained. That problem was circumvented by using acetone-dried walls prepared as described in Materials and Methods.

Yields (dry wt) of acetone-washed cell walls from frozen pericarp decreased approximately 50% as fruit ripened and senesced (Table 4-1). Wallner and Bloom (1977) reported similar values and

suggested that the yield decrease is a consequence of *in situ* cell wall hydrolysis associated with fruit softening. Acid sugar content (% dry wt) of isolated walls decreased with fruit development in the present study, from approximately 34% in mature-green to 32% in overripe. Gross and Wallner (1979) reported a similar decrease (34% to 30%).

The acetone washing and drying procedure had no apparent effect on subsequent autolysis of walls from pink pericarp (Fig. 4-2); however a decrease (25%) in activity was noted in dried walls compared to freshly prepared, hydrated walls from ripe tissue (Fig. 4-2). A possible explanation for that difference is that softening in the later stage of development is more closely associated with an acetone soluble factor such as membrane lipid. Data presented here do not address the involvement of factors other than those which are wall-bound. All remaining experiments were performed with acetone-washed and dried cell walls.

Several techniques for sampling soluble autolysis products were evaluated in order to ascertain the most appropriate technique to utilize for soluble neutral sugar analysis. Packing cell walls into a column and eluting autolysis products with reaction buffer seemed to offer the advantage of continuous removal of end-products that might accumulate and inhibit autolysis. Almost double the amount of acid sugar was released from column-packed walls compared to walls in a static system (centrifuge tubes) with both enzymically active and inactivated (boiling 80% EtOH) samples (Fig. 4-3). However, these results must be interpreted with caution because Miracloth was used

to retain cell-walls in the column and an earlier study suggested it may be inadequate for that purpose (Table 4-1). It was employed here because conditions were such that the material was not subjected to undue strain that might alter its filtration properties. Results of the present study seem to confirm the earlier observation that Miracloth does not adequately retain some fragments in the cell-wall preparation. A clear disadvantage of the column sampling system is that it is limited to analysis of only one cell-wall sample each trial. An additional criticism is that the cell walls may function as a gel filtration matrix, delaying the elution of small molecules.

A more practical method of sampling for soluble autolysis products is centrifugation of the reaction mixture and complete removal of the supernatant at each sampling. Cell walls handled and sampled in that manner released 50% more uronic acid during autolysis than cell walls incubated in a static system and sampled by aliquot (0.5 ml) removal (Fig. 4-4). This difference cannot be attributed to the additional manipulation involved in resuspension of the walls since enzymically active walls that were centrifuged and resuspended in the same buffer released approximately equal amounts of acid sugar compared to uncentrifuged samples (data not shown). Perhaps a more reasonable hypothesis for the increased polyuronide solubilization in centrifuged cell-wall samples is that a soluble inhibitor is removed with the supernatant each time the reaction mixture is centrifuged. Once resuspended in fresh buffer, autolytic reactions resume. The inhibitor hypothesis is supported by the fact that centrifuged samples of enzymically active walls had a higher rate of polyuronide

solubilization than uncentrifuged samples throughout the time course (Fig. 4-4A,B). Further, uncentrifuged samples ceased to release acid sugars within 3 hrs after start of incubation (Fig. 4-4A) compared to centrifuged samples that were autolyzing at a rate of 25 µg galacturonic acid/mg wall dry wt/hr after 7 hrs incubation (Fig. 4-4B). Products solubilized from cell wall during softening include polyuronide (Hobson, 1964; Pressey and Avants, 1971), neutral sugar (Huber, 1983a; Gross and Sams, 1984), protein (Strand et al., 1976; Hobson, 1983), calcium (Rigney and Wills, 1981), and possibly other compounds. Of these, only calcium has been reported to inhibit PG in vitro (Wills and Rigney, 1979; Ali and Brady, 1982), although the precise nature of that inhibition is incompletely understood (Huber, 1983b). Calcium was demonstrated to inhibit cell-wall autolysis in an earlier study (Fig. 3-2) and further identification of autolysis inhibitors was not performed in these studies.

Pretreatment of enzymically active walls with boiling 80% EtOH inhibited polyuronide solubilization approximately 50% when soluble products were recovered by centrifugation (Fig. 4-4B) compared to 80% inhibition if sampled by aliquot removal (Fig. 4-4A, Fig. 3-2). The appearance of reducing groups suggests that some PG activity remained after the hot EtOH treatment (Fig. 4-4B). However, residual PG activity probably does not account for all of the release since enzymically-inactive walls from mature-green tissue released a significant amount of acid sugar if exposed to the same handling regime, e.g., pretreated with boiling 80% EtOH and soluble product sampling by centrifugation and supernatant removal (Fig. 4-4C,D).

These data suggest that exposure of cell walls to boiling 80% EtOH in some way enhances polyuronide solubilization from samples that are repeatedly centrifuged and resuspended regardless of the presence of residual PG activity (Fig. 4-4).

Previous experiments were directed toward finding the most suitable handling technique for the enzymically active cell-wall preparation prior to undertaking the more extensive neutral sugar analyses. The scheme described for Fig. 4-4B was chosen, e.g., acetone-washed and dried cell walls with all soluble product sampling by centrifugation and supernatant removal and no exposure to Miracloth once autolysis is initiated. That system was selected because it maximizes the amount of soluble product and eliminates product overlap in the intervals of a time-course experiment.

Accountability for cell-wall constituents in the autolysis system (Table 4-4) compares quite favorably with other studies. Numerous workers have based their calculations of soluble cell-wall constituents on the amount of cell-wall material recovered after enzymic hydrolysis (Chapter 3; Wallner and Bloom, 1977; Gross and Wallner, 1979) which confounds any effort to account for all of the dry wt of a given sample. In a more thorough study, Gross (1984) reported 92.7 ± 7.3% average total recovery of fractionated tomato cell walls. In the autolysis system, close to 100% of the initial dry weight is accounted for (Table 4-4). Efforts to refine the methods involved in the study of autolysis apparently met with a degree of success.

Dry cell walls (preautolysis) were composed of approximately 8% (dry wt) protein, most of which was solubilized during autolysis (Table 4-4, Fig. 4-5). Hobson et al. (1983) addressed the significance of protein released from cell walls as a result of PG action. They suggested that the process is consistent with Tigchelaar's (1978) hypothesis that specific proteins released from the wall might initiate ripening. The main concern in including the measurement here was to account for as much of the initial wall dry wt as possible.

The acid:neutral sugar ratio of soluble autolysis products (Fig. 4-6) is higher than generally reported by other workers. Gross and Wallner (1979) determined the acid:neutral hexose ratio to be 6.4 in water-soluble pectin released from cell walls extracted from light red tomatoes. Wallner and Bloom (1977) used a crude enzyme extract from ripe tomatoes to hydrolyze cell walls from mature-green fruit and reported on acid:neutral sugar ratio for soluble products of from 6 to 9. In each of these examples the cell wall was exposed to a more rigorous extraction scheme than was employed in the present study.

Galactose was the predominant neutral sugar found in soluble autolysis products (Fig. 4-7). This correlates well with Gross and Wallner's (1979) observation that galactose is solubilized most rapidly <u>in situ</u> during the transition from pink to ripeness. Galactan and arabinan serve as cross-links for the rhamnogalacturonan backbone of pectin (Albersheim, 1976). PG is believed to act upon a region of rhamnogalacturonan with a relatively low degree of

cross-linking (Gross and Wallner, 1979). Although all of these neutral sugars were present in the autolysis reaction mixture, no conclusions regarding PG specificity can be drawn.

The low amount of soluble glucose (Fig. 4-7) is indicative of little or no readily soluble starch in the cell-wall preparation. Gross and Wallner (1979) reported no detectable starch in a similar extract but Pressey and Avants (1982b) found high levels of non-cellulosic glucose in tomato cell walls and employed an amylase treatment to remove it.

Gel filtration chromatography of polyuronides released throughout autolysis was performed to determine if a shift in polymer size occurs as wall degradation becomes more extensive. Although polymer size did appear to decrease slightly over the time-course, a significant amount of acid sugar always voided Bio-Gel P-10 (Fig. 4-8), indicating that the molecular weight of some pectin products exceeded 20,000. Gross and Wallner (1979) used a PG extract to hydrolyze walls from mature-green tissue and were able to fractionate enzyme-soluble pectin on P-10. However, they found that water-soluble pectin (autolysis products) released from cell walls of pink tissue all voided P-10, in contrast to results of the present study. This inconsistency may be explained by two important differences in methods: first, Gross and Wallner (1979) subjected their cell-wall preparation to more extensive mechanical disruption which may have allowed removal of smaller polyuronide during extraction and, secondly, they did not include 150 mM NaCl, which enhances PG activity, in the reaction mixture. It has been suggested

that little, if any, degradation of polyuronide occurs once solubilized from the larger wall matrix in vitro (Wallner and Bloom, 1977). That hypothesis is supported, but not confirmed, by gel filtration chromatography data presented here (Figs. 4-8 and 4-9).

The primary objectives of this study were to examine methods of handling enzymically active cell walls and to choose an appropriate system for determination of neutral sugar content and relative polymer size of pectins released during autolysis. These objective were satisfied and new questions were raised. It appears that almost any handling technique introduced into a cell-wall study is worthy of considerable investigation before its implementation in large scale experiments.

Analyses of soluble autolysis products provided data that are in general agreement with other tomato cell-wall studies. Experiments in progress are directed toward more detailed characterization of the effects of calcium, pH, and NaCl on cell wall autolysis.

CHAPTER 5
EFFECTS OF NaCl, pH, AND CALCIUM ON AUTOLYSIS OF ISOLATED TOMATO CELL WALLS

Variables that are often tested in in vitro cell-wall studies of fruit softening are pH (Patel and Phaff, 1960a; Hobson, 1964; Pressey, 1984), NaCl (Pressey and Avants, 1973; Ali and Brady, 1982), and calcium (Rigney and Wills, 1981; Jarvis, 1982; Demarty et al., 1984). All have been implicated in regulation of softening but the role of each is poorly understood because interactions are complex. In systems that employ model substrates to study the activity of cell-wall hydrolases, the effects of a single variable on enzyme activity are relatively easy to isolate. For example, the discovery of differential pH optima in a crude polygalacturonase (PG) extract from tomato fruit was an important clue leading to the discernment of PG isozymes (Patel and Phaff, 1960a; Hobson, 1964). Once purified, different isozymes responded maximally to different concentrations of NaCl, the requirement for Na being quite specific (Pressey and Avants, 1973). The effects of calcium on PG activity present more of a challenge, even in assays that employ relatively simple substrate, because calcium has the capacity to interact with both protein and polyuronide (Ferguson, 1983). Specific regulatory roles for calcium in fruit softening are speculative (Demarty et al., 1984; Ferguson, 1984).

Cell-wall autolysis may offer additional insight into the regulation of polyuronide hydrolysis by pH, NaCl, and calcium. Earlier autolysis experiments addressed those variables to a limited extent and confirmed that enzymically active cell wall is a useful tool for investigation of fruit softening (Chapters 3 and 4). The objective of the present study is to extend the scope of earlier experiments with additional emphasis on interactions of the variables discussed.

Materials and Methods

Plant Material

Tomato (Lycopersicon esculentum Mill. cv 'Rutgers') plants were grown at the University of Florida Horticultural Unit near Gainesville. Fruit were harvested at pink or red (ripe) developmental stages based on a United States Department of Agriculture visual aid for color classification of tomatoes (Anon., 1975). Fruit were surface sterilized with 100 ppm NaOCl and sectioned; seed and placental tissue were removed and pericarp stored at -20°C.

Cell Wall Preparation

Approximately 100 g of frozen pericarp were partially thawed, peeled, and homogenized in 400 ml distilled H_2O (4°C) for 1 min in a Sears blender set at maximum speed. The homogenate was transferred onto Miracloth and washed with 3 liters of distilled H_2O (4°C) and 1 liter of acetone (1°C) with continuous stirring. Cell walls were suspended in 100 ml of acetone for 1 hr then filtered through Whatman

GF/C filters and washed with acetone (approximately 500 ml) until pigment was no longer visible. Walls were transferred to a beaker and dried at 30°C with occasional stirring to reduce formation of aggregates. Dry cell walls were stored in sealed containers at 22°C and were used for autolysis experiments within 2 weeks following preparation. All wall extracts were analyzed for total acid sugar content (Ahmed and Labavitch, 1977).

Autolysis Experiments

Cell walls (100 mg dry wt) were placed in a 50 ml centrifuge tube in an ice bath and rehydrated by dropwise addition of 15.0 ml cold (1°C) reaction buffer (50 mM Na acetate, pH 4.5, 150 mM NaCl, 0.02% Thimerosal) unless otherwise indicated. Samples were held at 1°C for 30 min with occasional stirring, then centrifuged (10 min at 10,000 RPM) with a Beckman Model J2-21 centrifuge equipped with a JA-20 rotor. The supernatant was filtered through Whatman GF/C filters to obtain a time-zero sample. The pellet (cell wall) was resuspended in 15.0 ml of fresh reaction buffer and incubated at 34°C. At selected intervals during incubation, centrifugation, supernatant removal, and pellet resuspension were repeated. All supernatants were analyzed for total acid sugars (Blumenkrantz and Asboe-Hansen, 1973) and acid reducing sugars (Milner and Avigad, 1967). Cell walls were recovered postautolysis by suspension in 30.0 ml acetone and filtration through tared Whatman GF/C filters; walls were analyzed for total acid sugar content (Ahmed and Labavitch, 1977).

In some experiments, cell walls were suspended in hot 80% EtOH and boiled 15 min under reflux. The mixtures were cooled, centrifuged, the supernatant removed, and the cell walls resuspended in reaction buffer as described above.

Experiment 1. Effect of NaCl concentration on cell-wall autolysis was observed by rehydration of cell walls from either pink or red pericarp in either distilled H_2O, reaction buffer, or buffer containing 0.15, 0.30, 0.45, or 0.60 M NaCl. At intervals during autolysis, reaction mixtures were centrifuged, the supernatant removed, and the pellet resuspended in either distilled H_2O or the appropriate ionic strength buffer.

Experiment 2. The interaction of pH and 1 M NaCl treatment prior to autolysis was investigated using cell walls from pink pericarp. Walls were rehydrated in one of the following buffers with or without 1 M NaCl: pH 2.5, 5 mM Na_2-acetate titrated with glacial acetic acid; pH 4.5, 50 mM Na_2-acetate; pH 6.5, 50 mM MOPS; pH 8.5, 50 mM HEPES. After 30 min in pretreatment solution (1°C), mixtures were centrifuged (10 min at 10,000 RPM) and supernatants removed. Walls were resuspended in reaction buffer (50 mM Na_2-acetate, pH 4.5, 150 mM NaCl, 0.02% Thim.) and incubated at 34°C. The pH of all supernatants was checked to ensure that buffer strength was adequate.

Experiment 3. In some studies, treatment of a cell-wall extract with 1 M NaCl was employed to enhance dissociation of bound protein, including active wall hydrolases (Hobson, 1964; Ali and Brady, 1982). In the present experiment, exposure to 1 M NaCl was introduced at selected steps during extraction of cell walls from red pericarp (Table 5-1). Autolysis of the resulting wall extracts was

Table 5-1. Protocol for cell-wall extraction in Experiment 3.

Sample	Homogenization Medium	Miracloth Filtration	Cell-Wall Yield (mg/100 g fresh wt)	Acid Sugar Content (% dry wt)
A	H_2O	H_2O (4 L) → subsample I; acetone (1 L)	690.6	30.3
B	H_2O	H_2O (3 L); NaCl; H_2O (1 L); acetone (1 L)	575.2	32.8
C	H_2O	H_2O (3 L); acetone (1 L); NaCl; H_2O (1 L); acetone (1 L)	591.7	34.7
D	1 M NaCl	1 M NaCl (3 L); H_2O (1 L) → subsample J; acetone (1 L)	593.0	23.1
E	1 M NaCl	H_2O (4 L); acetone (1 L)	573.9	29.2
F	1 M NaCl	acetone (1 L); H_2O (3 L); acetone (1 L)	623.7	30.1
G	acetone	H_2O (4 L); acetone (1 L)	773.2	32.7
H	acetone	NaCl; H_2O (4 L); actone (1 L)	720.0	34.1
I	refer to A			
J	refer to D			

observed after pretreatment of some samples with 1 M NaCl or boiling 80% EtOH.

Extracts A, B, and C were homogenized in distilled H_2O (Table 5-1) using a blender as described. Sufficient NaCl was added to D, E, and F prior to tissue disruption to adjust homogenate to 1 M NaCl

(Table 5-1). Extracts G and H were homogenized in acetone in a Sorvall blender that would not accommodate the full 100 g of frozen tissue. Those lots were subdivided for homogenization; however, in all extractions the ratio of tissue:homogenization medium was 1:4.

The cell-wall preparation procedure for extracts B, C, D, and H included exposure to 1 M NaCl during Miracloth filtration (Table 5-1). This was performed by rinsing walls with 1 liter of 1 M NaCl (1°C), then lowering the Miracloth and its contents into a beaker containing 500 ml of 1 M NaCl (1°C) for 20 min followed by distilled H_2O rinse (Table 5-1).

All extracts were rinsed with 1 liter acetone on Miracloth prior to dry wt analysis (Table 5-1). In order to observe autolysis of walls that were not exposed to acetone, aliquots of approximately 50 mg (dry weight) hydrated cell walls were removed from extracts A (labeled subsample I) and D (labeled subsample J) immediately after the first aqueous rinse on Miracloth (Table 5-1). These aliquots (subsamples) were placed in 10.0 ml of either distilled H_2O or 1 M NaCl (1°C). Additional aliquots (subsamples) were adjusted to 80% EtOH and boiled 15 min. After pretreatment, all subsamples were centrifuged (10 min at 10,000 RPM), the supernatant removed, and the pellet resuspended in 10.0 ml distilled H_2O for 5 min. That mixture was centrifuged (5 min at 5,000 RPM), the supernatant removed, and the pellet resuspended in 10.0 ml reaction buffer (50 mM Na_2 acetate, pH 4.5, 150 mM NaCl, 0.02% Thimerosal) before incubation at 34°C.

Experiment 4. A factorial experiment was designed to test the effects of pH (2.5, 4.5, 6.5, 8.5; same buffers as experiment 2),

NaCl concentration (0, 150, 300 mM) and calcium (0, 5 mM $CaCl_2$) on autolysis of cell walls extracted from ripe pericarp. All buffer solutions were prepared individually and contained 150 mM NaCl and 0.02% Thimerosal. Some cell-wall samples were boiled in 80% EtOH and allowed to cool slowly to room temperature prior to centrifugation, supernatant removal, and resuspension in appropriate buffer solution for autolysis.

Results

Experiment 1

Autolysis of cell walls extracted from red pericarp was optimally stimulated by 150 mM NaCl, compared to 300 mM for walls from pink fruit (Fig. 5-1). Acid reducing sugar analysis correlated with total acid sugar assays (data not shown). Walls from both stages of development released 3 times more acid sugars in the presence of NaCl than was released in distilled H_2O (Fig. 5-1). At 600 mM NaCl, over 50% inhibition of autolysis was observed compared to the optimum NaCl level (Fig. 5-1). Dry wt of walls incubated at 600 mM NaCl increased 30-35% above the preautolysis dry weight during the 7 hrs incubation period (Table 5-2).

Rate of uronic acid solubilization was not drastically affected by slight changes in NaCl concentration. A gradual increase in rate of release was observed as NaCl was increased to the optimum level, followed by a gradual decline as salt became inhibitory (Fig. 5-1). The pH of supernatants recovered from walls incubated in distilled H_2O varied from 4.43 to 4.48.

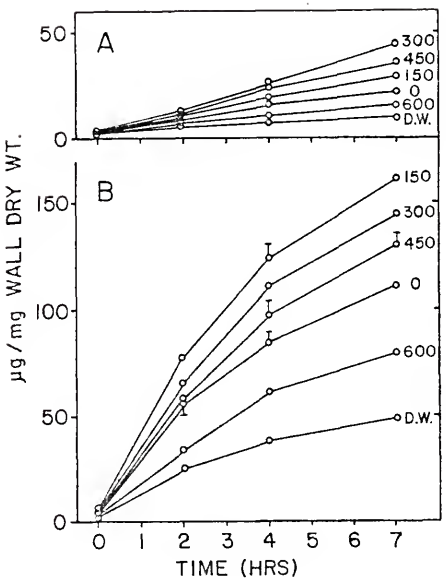


Figure 5-1. Effect of NaCl on release of polyuronide from cell walls of pink (A) or red (B) tomato pericarp. Results are from a single experiment with duplicate samples that was repeated with similar results each time. Bars represent standard error of the mean and, when absent, are smaller than the symbols. (D.W. = distilled H_2O; numbers represent M^{-3} NaCl in reaction buffer)

Table 5-2. Postautolysis dry weight of cell walls recovered at termination of experiment 1.

Reaction Medium	Postautolysis Dry Wt (mg)	
	Pink	Red
distilled H_2O	87.3 ± 0.7	81.6 ± 0.6
buffer	86.9 ± 0.5	75.1 ± 2.2
buffer + 0.15 M NaCl	87.8 ± 2.1	75.7 ± 1.3
buffer + 0.30 M NaCl	94.7 ± 1.2	91.1 ± 2.2
buffer + 0.45 M NaCl	97.6 ± 2.3	105.9 ± 0.6
buffer + 0.60 M NaCl	129.1 ± 0.7	136.7

Experiment 2

Cell walls exposed to pH 6.5 or 8.5 prior to incubation at pH 4.5 (Fig. 5-2 C,D) released acid sugars in a manner similar to walls exposed to pH 4.5 throughout the experiment (Fig. 5-2 B). If pretreatment buffers (pH 4.5, 6.5, or 8.5) contained 1 M NaCl, total acid sugar released at pH 4.5 was nearly doubled compared to pH pretreatment without NaCl (Fig. 5-2 B,C,D). However, no increase in acid sugar reducing groups resulted from the 1 M NaCl pretreatment at either pH (Fig. 5-2 B,C,D).

Walls exposed to pH 2.5 without 1 M NaCl prior to autolysis at pH 4.5 (Fig. 5-2 A) exhibited a 3-fold increase in total acid sugar solubilization compared to all other pH pretreatments without 1 M NaCl (Fig. 5-2 B,C,D). Pretreatment with 1 M NaCl at pH 2.5 caused approximately 25% increase in acid sugar solubilization compared to

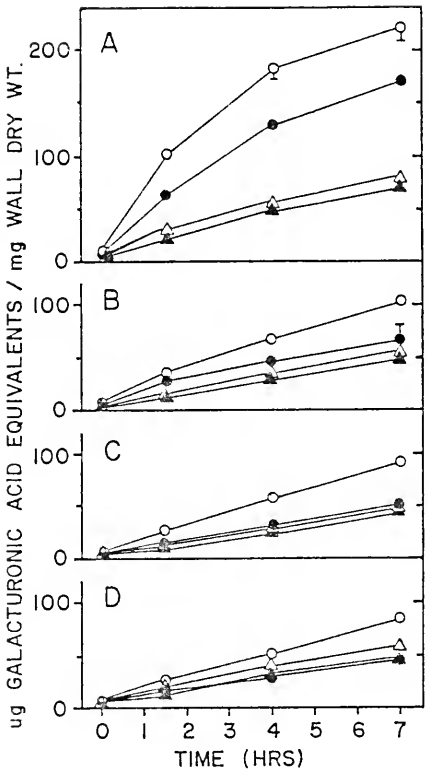


Figure 5-2. Autolytic activity in cell walls from pink pericarp incubated at pH 4.5 after pretreatment with 1 M NaCl at different pH values. (A = pH 2.5; B = pH 4.5; C = pH 6.5; D = pH 8.5; ●,○ = total acid sugars; ▲,△ = acid reducing sugars; ○,△ = 1 M NaCl; ●,▲ = no NaCl)

pH 2.5 pretreatment without 1 M NaCl (Fig. 5-2 A). Release of acid sugar reducing groups was not significantly affected by either pH or 1 M NaCl pretreatments (Fig. 5-2).

Experiment 3

The least rigorous extraction procedure for preparation of dry cell walls is represented by scheme A (Table 5-1 A), i.e., cold aqueous extraction with no exposure to buffers or other salts. Walls prepared in that manner released approximately 10% of their dry weight as acid sugars during autolysis (Fig. 5-3 A). Pretreatment with 1 M NaCl caused approximately 25% increase in acid sugar solubilization (Fig. 5-3 A) and pretreatment with boiling 80% EtOH caused 85-95% inhibition of autolysis in all wall extracts (Fig. 5-3). Autolytic activity in subsamples removed from extract A prior to the acetone wash and drying procedure (Table 5-1 A) is diagrammed in Fig. 5-3 I. Exposure of freshly prepared, fully hydrated walls to 1 M NaCl caused approximately double the release of acid sugars compared to walls that were maintained at the ionic strength of reaction buffer (Fig. 5-3 I).

Exposure of cell wall homogenates to 1 M NaCl at selected points in the extraction scheme did not eliminate autolytic activity in the resulting wall preparations (Fig. 5-3 B-J) when compared to the low ionic strength extract (Fig. 5-3 A). The most rigorous extraction procedure in terms of ionic treatment is represented by scheme D (Table 5-1), i.e., homogenization and Miracloth filtration with 1 M NaCl. Autolysis of walls prepared in this manner (Fig. 5-3 D) was

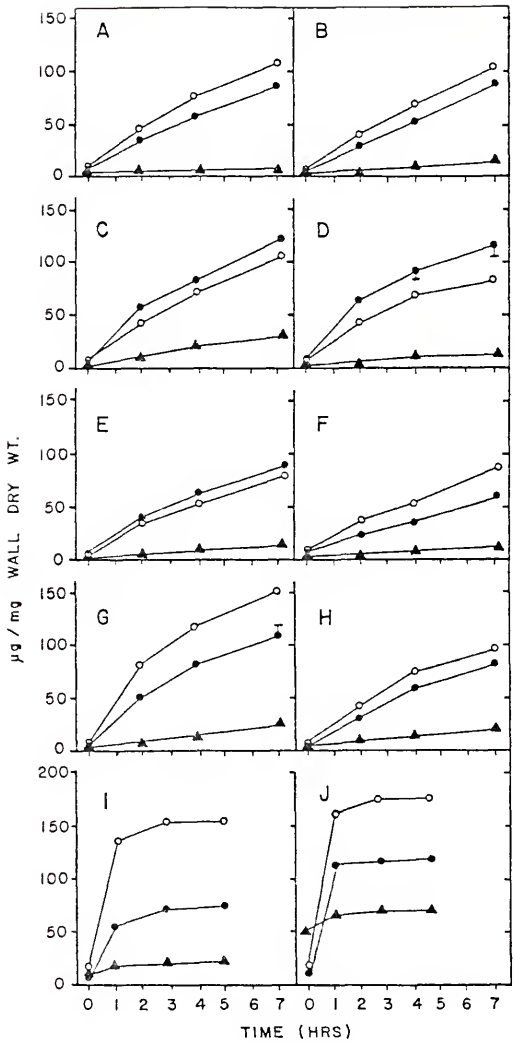


Figure 5-3. Effect of exposure to 1 M NaCl on solubilization of acid sugars from cell walls of red tomato pericarp. Letters (A-J) correspond to those that designate extraction protocol described in Table 5-1. (O = 1 M NaCl pretreatment; ● = no pretreatment; ▲ = boiling 80% EtOH treatment)

not significantly different from autolysis of the aqueous wall extract (Fig. 5-3 A) except that an additional 1 M NaCl pretreatment of walls from extract D did reduce (25%) autolytic activity (Fig. 5-3 D). Autolysis of cell walls that were removed from extract D after 1 M NaCl treatment on Miracloth but prior to acetone wash (Table 5-1 D) is diagrammed in Fig. 5-3 J. These walls released approximately 30% more acid sugars than similarly sampled walls from the aqueous extract A (Fig. 5-3 I, J). An additional 1 M NaCl treatment of wall sample J further enhanced autolysis by about 50% (Fig. 5-3 J) and boiling in 80% EtOH inhibited autolysis, although time zero values for boiled walls was unusually high (Fig. 5-3 J).

The highest cell-wall yield (773 mg/100 g fresh wt) was obtained by homogenization of pericarp in acetone with no exposure to NaCl (Table 5-1 G). Yield was lower (720 mg) if acetone-homogenized walls were treated with 1 M NaCl on Miracloth (Table 5-1 H). The aqueous extract yielded 690 mg walls (Table 5-1 A) and all other extracts that received 1 M NaCl treatments yielded from 575 to 625 mg walls/100 g pericarp (Table 5-1 B-F).

Acid sugar content (% dry wt) of cell walls was lowest (23.1%) in walls exposed to the most rigorous extraction scheme (Table 5-1 D). All other extracts varied from 29-34% acid sugars (Table 5-1).

Experiment 4

Cell walls incubated at pH 8.5 released negligible quantities of acid sugars regardless of presence of NaCl or $CaCl_2$ (Fig. 5-4 D). At

pH 6.5 detectable amounts (1-2% wall dry wt) of acid sugars were solubilized and calcium inhibition of autolysis was apparent (Fig. 5-4 C). At pH 4.5, autolysis was approximately doubled by the presence of either 150 or 300 mM NaCl compared to walls incubated without NaCl (Fig. 5-4 B). Calcium inhibited autolysis at pH 4.5 by 60-70% at all three levels of NaCl (Fig. 5-4 B).

Stimulation of autolysis by NaCl was most apparent at pH 2.5, at which either concentration of NaCl caused a 3-fold increase in uronic acid solubilization compared to walls incubated without NaCl (Fig. 5-4 A). Calcium did not significantly inhibit autolysis at pH 2.5 in buffer without NaCl or with 150 mM NaCl; approximately 15% inhibition was observed at 300 mM NaCl (Fig. 5-4 A).

Cell walls that were boiled 20 min in 80% EtOH and cooled slowly (approximately 1 hr to reach 34°C) were over 95% inactivated in terms of acid sugar release at all pH, NaCl, or calcium levels (Fig. 5-4). Boiling EtOH treatments were not as effective (85-95%) in inhibition of autolysis in earlier studies (Figs. 3-2, 4-4 and 5-3) probably because high temperature exposure was of shorter duration.

Discussion

Autolysis data presented here (Fig. 5-1) are generally consistent with Pressey and Avants (1973) work with purified PG isozymes from tomato. The authors assayed the activity of PGs I and II over a range of NaCl concentrations at pH 4.5 and reported optimal NaCl concentrations of approximately 200 and 300 mM, respectively, for PG I and PG II. More realistically, they observed a range of

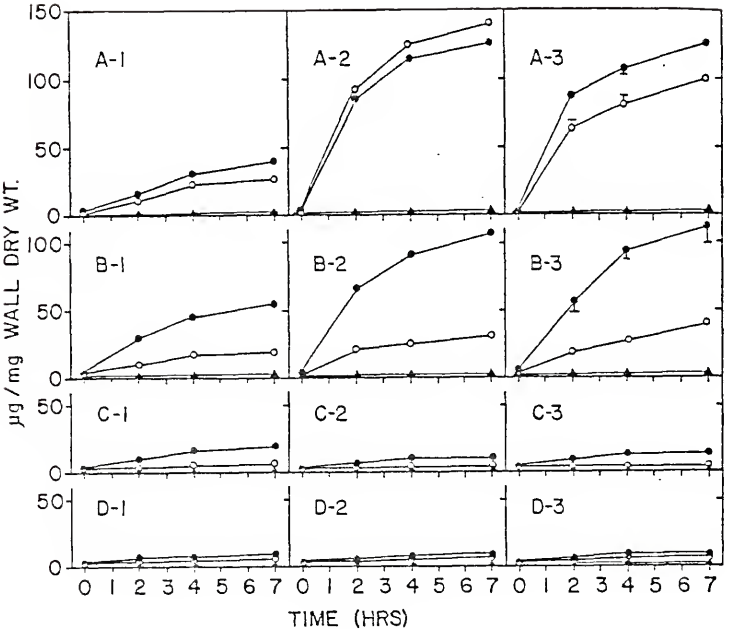


Figure 5-4. Effects of pH, NaCl, and calcium on solubilization of acid sugars from cell walls of red tomato pericarp. Results are from a single experiment with duplicate cell wall samples. Experiment was repeated with similar results each time. Bars represent standard error of the mean and, when absent, are smaller than the symbol. (A = pH 2.5; B = pH 4.5; C = pH 6.5; D = pH 8.5; 1 = no NaCl; 2 = 150 mM NaCl; 3 = 300 mM NaCl; ● = no calcium; ○ = 5 mM calcium; ▲ = pretreated with boiling 80% EtOH).

activity from approximately 150 to 300 mM for PG I and 200 to 300 mM for PG II. A similar range of activity was observed using autolysis of enzymically active cell walls as a measure of PG activity (Fig. 5-1). Concentrations of 600 mM NaCl or higher inhibited purified PG activity (Pressey and Avants, 1982b) as well as cell-wall autolysis (Fig. 5-1).

PG I is reported to appear during the earliest color development associated with ripening, but by the time fruit are fully ripe PG II is the predominant species (Tucker et al., 1980). Enzymically active walls from the two stages of development used in the present study probably contained both PG isozymes. Although differential NaCl optima may exist (Pressey and Avants, 1973), they do not seem distinct enough to serve as a means of conclusively ascertaining the presence, or absence, of a specific PG isozyme in enzymically active walls.

The increase in dry weight observed with cell walls incubated at higher NaCl concentrations (Table 5-2) was possibly due to Na^+ binding to demethylated galacturonic acid residues. This illustrates an inherent complexity of many cell-wall studies, e.g., the capacity for pectins to bind extensively, and perhaps indiscriminately, to cationic molecular species.

Additional effects of NaCl on cell-wall autolysis were investigated in experiment 2, in which walls were exposed to 1 M NaCl over a range of pH values prior to incubation at pH 4.5. This salt concentration enhanced extraction of PG from homogenates of tomato pericarp or from cell-walls (Hobson, 1964; Ali and Brady, 1982);

however these studies did not examine the cell wall for residual wall-bound PG activity (autolysis) after ionic treatment. Results of the present study suggest that a significant population of PG molecules remain bound to the wall after 1 M NaCl treatment (Fig. 5-2) and the tenacity of their binding is further illustrated by failure of 2 or 3 M NaCl pretreatment to inhibit subsequent autolysis (data not shown).

Since timing of the 1 M NaCl treatment during wall extraction might be a significant factor in its effectiveness in removing wall-bound PG, experiment 3 was designed to examine the effect of 1 M NaCl treatment on autolysis when performed at selected points in the extraction scheme (Table 5-1). Autolysis was not effectively eliminated by any of the 1 M NaCl treatments (Fig. 5-3). These results are particularly pertinent to fruit softening studies that employ cell walls from ripening fruit tissues (Buescher and Hobson, 1982). Appropriate controls must be presented to ensure that residual wall-bound PG activity (autolysis) does not lead to misinterpretation of data. Unfortunately, treatments that remove or inactivate bound enzymes may also degrade cell wall, thus limiting the interpretation of results.

Autolysis was significantly enhanced by exposure of walls to pH 2.5, whether by pretreatment (Fig. 5-2 A) or by incubating at that pH (Fig. 5-4 A). The data allow two possible explanations. Exposure to pH 2.5 may cause demethylation of galacturonic acid residues (Talmadge et al., 1973) which renders the polygalacturonan more susceptible to PG hydrolysis (Jansen and MacDonnell, 1945;

Pressey and Avants, 1982b). However, it does not seem likely that degree of esterification is limiting to autolysis at pH 4.5 because pretreatment of walls nearer the pH optima (6.5-8.5) of PME isozymes (Pressey and Avants, 1972) slightly inhibited, rather than enhanced, subsequent autolytic activity at pH 4.5 (Fig. 5-2). This inhibition may have been due to perturbation of the PG-substrate complex and diffusion of enzyme from its site(s) of hydrolysis, since exposure of walls to neutral or higher pH reportedly facilitates removal of bound hydrolases (Babbit et al., 1973).

A more plausible explanation for the low pH stimulation of autolysis is that protons enhance solubilization of calcium from polygalacturonan, allowing PG greater access to sites of hydrolysis (Demarty, 1984). This view is supported by failure of calcium to inhibit autolysis at pH 2.5 (Fig. 5-4 A). Additionally, brief (30 min) pretreatment at pH 2.5 (experiment 2) was probably adequate for calcium to dissociate from the wall since it has been demonstrated that solubilized calcium diffuses from isolated wall more rapidly than polymeric wall constituents (Jarvis, 1982). Calcium chelators have been shown to enhance PG activity against isolated cell wall (Buescher and Hobson, 1982) and to cause non-enzymic pectin solubilization from isolated wall (Jarvis, 1982). It has been proposed that soluble apoplastic calcium may be a regulator, if not the initiator, of ripening metabolism (Rigney and Wills, 1981).

Autolytic activity was relatively low at pH 2.5 unless NaCl (150 or 300 mM) was present in reaction buffer (Fig. 5-4 A). Pressey and Avants (1971) reported that NaCl had its greatest effect on <u>in vitro</u>

PG activity when substrate was large and pH was low. They speculated that larger galacturonans form aggregates at low pH by extensive hydrogen bonding, limiting the access of PG to sites of hydrolysis. Presence of salts would effectively disrupt hydrogen bonds. Results of the present study (Fig. 5-4) correlate well with Pressey's data.

Autolysis was affected by calcium, NaCl, and pH similarly to activity of purified PG. A major point of interest is that pretreatment of cell walls with 1 M NaCl did not entirely suppress autolysis. Efforts are currently directed toward examination of mobility and binding characteristics of PG in isolated cell wall and to ascertain the involvement of wall-bound hydrolases other than PG.

CHAPTER 6
SUMMARY

Fruit softening is generally attributed to degradation of the cell wall by hydrolytic enzymes. The most common approach to enzymology of softening has been to extract and purify the hydrolases of interest and assay with a commercially available substrate or with a cell wall preparation. In the present study, cell walls isolated under conditions which minimized the disruption of protein-cell wall interactions were incubated under conditions favorable for continued enzymic activity.

Cell walls isolated from pericarp of ripening tomato (Lycopersicon esculentum Mill. cv 'Rutgers') released acid sugars amounting to 20% cell wall dry wt in a reaction apparently mediated by wall-bound polygalacturonase (PG). Release was negligible in wall preparations from normal green and the ripening mutant rin fruit. Pectin solubilization was most extensive at pH 2.5 with a less prominent peak at 5.5. Exposure of cell wall to low (1.5) or high (7.5) pH resulted in a reduction of autolytic activity. Calcium, high temperature, and treatments employed to dissociate protein from cell wall also inhibited autolytic reactions. Autolysis was enhanced by 150 mM NaCl and by increased temperature within the physiological range.

The rate and extent of polyuronide solubilization was significantly affected by the techniques employed for handling the cell walls. Enzymically active cell walls subjected to either intermittent or continuous removal and replenishment of the bathing medium released up to twice as much polyuronide as walls incubated in a static environment.

A handling system that maximized acid sugar solubilization was selected for experiments to determine the neutral sugar content and size distribution of pectic polymers released during autolysis. Approximately 2% of the initial wall dry wt was released as neutral sugars; the predominant product was galactose (50%), followed by arabinose (30%) and minor quantities of rhamnose, xylose, glucose, and mannose. The acid:neutral sugar ratio of soluble autolysis products decreased from 10.8 to 2.9 as wall degradation became more extensive. Gel filtration chromatography of solubilized pectic polymers did not reveal a temporal relationship between polyuronide size distribution and the autolytic reaction. Approximately 8% of the wall dry wt was protein, most of which was released during autolysis. The techniques developed in these studies for preparation and handling of enzymically active cell walls in vitro allow for an accounting of close to 100% of the preautolysis wall dry wt.

The effects of NaCl, pH, and calcium on the autolytic reaction also were examined. Polyuronide solubilization at pH 4.5 was optimally enhanced by NaCl in the range of 200-300 mM. Brief pretreatment of enzymically active walls with 1 M NaCl in the pH range of 2.5-8.5 did not inhibit subsequent autolysis at pH 4.5. The

tenacity of PG-binding was further illustrated by the fact that inclusion of 1 M NaCl treatments at selected points in the wall extraction scheme also did not eliminate release of acid sugars. A factorial experiment to test for interactions between pH (2.5, 4.5, 6.5, 8.5), NaCl (0, 150, 300 mM), and calcium (0, 5 mM $CaCl_2$) demonstrated that polyuronide solubilization was most dramatically enhanced by NaCl at pH 2.5, at which calcium inhibition was not observed. Inhibition by calcium was most apparent at pH 4.5 in the presence of NaCl. Autolytic activity was negligible at pH 6.5 and 8.5 and no effects of NaCl or calcium were noted.

The results of these experiments support the concept that the release of polyuronide from isolated cell walls in vitro is enzymic and provides a convenient and reliable system for the study of softening metabolism.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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