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Pectins, Pectinases and Plant-Microbe Interactions

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Introduction

Pectins contain two types of covalently linked backbones. Homopolygalacturonic acid (HG) is a linear helix, α(1→4)–linked, that contains a few xylose substitutions in the proximity where the rhamnogalacturonan (RG) is connected. Rhamnogalacturonans consist of linear α(1→4)-linked repeats of the dimer of galacturonic acid linked α(1→2) to rhamnose (Rha) residues forming α(1→2)-linked dimers. Methyl esterification is found on HG segments. RGs are acetylated and frequently substituted with galactans, arabinans and arabinogalactans linked to Rha residues. Plant cell wall degrading enzymes, including pectinases, are ubiquitous among pathogenic or saprophytic bacteria and fungi. Pectate lyases cleave non-esterified HG-pectate chains by elimination, resulting in unsaturated reaction products. Pectin lyases are able to cleave highly methoxylated HG-pectin and pectin methylesterases remove methyl groups rendering HG-pectin polymers accessible to other depolymerizing enzymes. RG specific enzymes such as rhamnogalacturonase, galactanase, arabinase, arabinosidase and galactosidase have frequently been reported. There are only a few links for which we remain uncertain about the existence of a specific cleaving enzyme. These links are; Ara-Rha, Gal-Rha, Xyl-GalA and unsaturated rhamnose containing products. Microorganisms recognize pectins as complex bonded carbon sources, respond by activating the synthesis and secretion of proteins involved in degradation and metabolize released sugar residues. Pathogenic bacteria and fungi induce pectinases early during infection (penetration). Even though genetically it remains unclear whether these activities are absolutely required for infection, it seems clear that plants respond to invasive pectin degradation by producing polygalacturonase inhibiting proteins (PGIP), indicating a specific biochemical interplay at the pectin degradation level.

Higher plants have developed complex defense systems against potential pathogens. There are numerous structural and chemical features present in plants that discourage

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discourage visitors from establishing a parasitic interaction. With few exceptions, plant cells are enclosed by multi-layered walls with specialized structures that confer protection against invaders, mechanical strength, shape and size to tissues, organs and entire plants. The primary, secondary and middle lamella are sub-structural levels found in typical plant cell walls. Primary and secondary layers contain variable amounts of cellulose, hemicellulose and pectins. The secondary wall is not always present and is usually involved in providing structural support. Therefore, the cellulose content is increased and pectin content diminished in secondary walls. The middle lamella, also known as the intercellular substance, fills the spaces between primary walls of adjacent cells.

Carbohydrates are the main components of plant cell walls that form the bulk of the supporting structure. As a result, plant cell walls represent the most abundant natural and renewable organic material available on earth. This resource will be used increasingly as a source of energy, as well as raw materials for biotechnological processes.

When microorganisms initiate a colonization cycle on plants, regardless of whether a pathogenic or saprophytic (*i.e.* biomass decay) association, the first complex carbon source they encounter is the pectin present in the middle lamella. Since we know that bacteria and fungi are effective in depolymerizing pectin and other polysaccharides into metabolizable energy sources, the natural interactions that take place at this level are of importance to several aspects of biology.

Complete hydrolysis of pectins involves numerous enzymes – several of them are at least partially dependent on the outcome of one or more prior enzyme/substrate interactions. Thus, natural interactions between pectin degrading enzymes and structural pectins in plants are complex, but specific, and in many instances exhibit 'quasi' redundant functions (e.g. lyases and hydrolases). Moreover, pectin/pectinase interactions between entire plants (tissue) and microorganisms (bacteria or fungi) are highly specific and localized, contingent upon the expression of an infection regulated genetic circuit.

In this discussion we will initially focus on key aspects of pectin structure, then discuss in detail microbial pectin degrading systems, and finally analyze several examples of natural pectin/pectinase interactions. Even though pectins are important industrial food additives and pectinases are at the core of developments in fruit and vegetable preservation technologies, we will not focus on applied aspects of pectins. The discussion presented here is intended to be an updated description of what we know about interactions between plants and microbes that occur in nature. This information should be complementary to several excellent reviews that have recently been published, describing the economics and the utility of pectins (Sakai *et al.*, 1993; Hamilton *et al.*, 1995; Sutherland, 1995; Hugouvieux-Cotte-Pattat *et al.*, 1996; De Lorenzo and Cervone, 1997; Thakur *et al.*, 1997; Hadfield and Bennett, 1998)].

Pectin structure

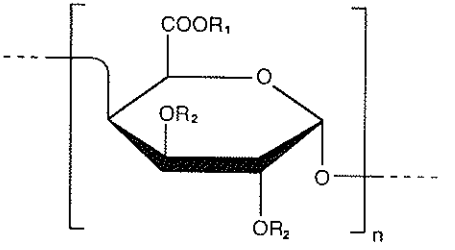
Pectin means different things to different people. In this discussion, we will describe pectins as those cell wall polysaccharides containing a relatively high proportion of $\alpha(1\rightarrow4)$-linked galacturonic acid (GalA). Others have often used operational definitions of pectin as those polysaccharides extracted from cell walls by chelators or hot water.

The definition is complicated further by having to decide how far, out from the GalA-containing region, in the molecule is still pectin. Within the pectin molecule, extensive side chains can be attached to the GalA-containing backbones, and these might be cross-linked to other polymeric cell wall components, such as proteins (Qi *et al.*, 1995), and hemicellulose (Keegstra *et al.*, 1973; Fu and Mort, 1996). In an attempt to be consistent and perfectly general, we will consider first the two major types of pectin backbone structures, and then describe the many substituents found on them.

PECTIN BACKBONES

There is strong evidence for two types of backbones in pectins. The best known, homogalacturonan (HG), depicted in *Figure 1*, contains long stretches of α(1→4)-linked D-galactopyranosyl uronic acid residues (Thibault *et al.*, 1993). The other, rhamnogalacturonan (RG), depicted in *Figure 2*, contains stretches of the disaccharide α-D-GalA*p* (1→2) α-L-Rha*p* linked (1→4) to each other (Lau *et al.*, 1985). Both backbones show a wide variety of decorations on them such as acylations on the 2-, or 3-hydroxyl positions of galacturonic acid residues (GalA), esterifications of GalA acidic groups, and glycosyl side chains on 2- or 3-hydroxyl positions of GalA or 4-hydroxyl position of rhamnose (Rha). Three major questions remain about pectin backbone structures: a) the length and variability of polyGalA stretches, b) the length and variability of polyGalA-Rha stretches, and c) whether or not HG and RG regions are covalently linked together.

The question of how long and uniform HG regions are is difficult to address. Determining the molecular weight of pectin fragments is tricky due to their polyanionic character (Fishman *et al.*, 1984; Mort *et al.*, 1991). In addition, RG regions (as suggested by the presence of rhamnose) are often found in association with HG regions. Thus, it has been difficult to determine whether or not HG regions are interrupted by RG regions or vice versa. Thibault *et al.* (1993) found that prolonged mild acid hydrolysis of de-esterified pectins causes solubilization of most of the

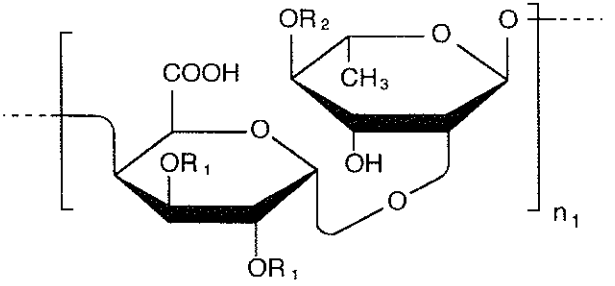


(galacturonic acid)

R_1 = H, Me or non-methyl esters

R_2 = H or acetyl

Figure 1. The Homogalacturonan (HG) Region. Homogalacturonan (HG) regions contain most of the galacturonic acid content found in native pectin molecules. They form long linear helical, often heavily methylesterified (R_1) and rarely acetylated (R_2) structures with essentially no side chains. n, indicates the degree of HG polymerization which can be greater than 100 residues (Chambat and Joseleau, 1980).



R_1 = H or acetyl

R_2 =

1) α-L-Araf-1[→5-α-L-Araf-1→]n_2 5-α-L-Araf-1→
2 or 3
1
α-Araf or H

2) β-D-Galp-1[→4-β-D-Galp-1→]n_2 4 -β-D-Galp-1 →
2 or 3
1
Galp , Araf or H

3) α-L-Araf-1[→5-α-L-Araf-1→]n_2 5 -β-D-Galp-1 →

4) α-L-Araf-

5) β-D-Galp-

6) H

Figure 2. The Rhamnogalacturonan (RG) Region. Rhamnogalacturonan (RG) regions contain galacturonic acid, rhamnose (GalA-Rha) dimer repeats with frequent and variable side chains containing arabinose (Ara) and galactose (Gal). n_1, indicates the number of GalA-Rha disaccharide repeat units, reported to be up to at least 30 (Zhan *et al.*, 1998) and as high as 200 (McNeil *et al.*, 1980). n_2, indicates the number of sugar residues in the side chains (from 0 to 40).

neutral sugars, leaving insoluble the majority of GalA. Pectic acids containing 23 or more residues are acid insoluble (Hotchkiss and Hicks, 1990). In addition, the rhamnose content also decreases with time in these acid precipitates, indicating gradual destruction of RG containing regions. Thus, it is likely that most of the insoluble material is composed of HG. Furthermore, this insoluble precipitate is soluble after neutralization, and the approximate molecular weight of 20,000 suggests HG fragments containing, on average, 115 GalA residues.

It has been generally accepted that HGs contain periodic interruptions caused by single rhamnose residue insertions which would produce kinks in an otherwise extended helical structure (Rees and Wright, 1971; Powell *et al.*, 1982; Jarvis, 1984). However, we were unable to find the expected rhamnose containing GalA oligomers

in enzyme-digested citrus pectin (Zhan *et al.*, 1998). The existence of Rha inserts has not yet been investigated in other pectins.

The helical nature of pectic acid (HG with no esterifications or sugar side chains) has been determined by three-dimensional structural modeling (Rees and Wright, 1971), investigated by x-ray diffraction, and analyzed by various forms of spectroscopy (Rees, 1982). Even though there seems to be agreement on the helical nature of pectic acid chains, there remains the question as to whether there are two or three GalA residues per helix turn. Ca^{2+} ions interact with pectic acid molecules to form strong complexes, if the participating GalA-oligomers are 14 residues or longer. Grant and coworkers (Grant *et al.*, 1973) proposed an 'egg box' type of model in which two helical polyGalA fragments interact with each other via ionic interactions and coordination of Ca^{2+} ions between chains.

The length of RG backbones has also been elusive. In sycamore the length of RG backbones was calculated to be around 200 GalA-Rha repeats, based on gel permeation chromatography elution profiles (McNeil *et al.*, 1980). The above number of GalA-Rha repeats is an approximate prediction, because it was based on dextran standards and side chains had to be artificially discounted. In addition, in most plant cell wall extracts the RG region cannot be separated from HG regions. In apple (Schols *et al.*, 1990), various vegetables (Schols *et al.*, 1994; Schols *et al.*, 1995), watermelon, and cotton (Yu and Mort, 1996), RG appears to be strongly associated with a portion of HG heavily substituted with $\beta(1\rightarrow3)$-linked xylose. The xylose substitution on the HG fragment prevents endopolygalacturonase (EPG) digestion, making it difficult to determine whether a stretch of RG contains HGs at one or both ends, or if the backbone consists of several interspersed RG sections flanked by xylosylated HG segments. In beet pectin, RGs contain at least ten GalA-Rha repeat units (Renard *et al.*, 1995) while citrus pectin has up to at least 30 units (Zhan *et al.*, 1998). RG backbone oligomers of up to these lengths have been detected as the result of controlled (partial) acid hydrolysis that causes selective release of neutral sugar side chains from Rha before affecting the majority of Rha-GalA linkages of the backbone, producing a homologous series of RG oligomers. High performance anion exchange chromatography (HPAEC) or capillary zone electrophoresis (CZE) can further analyze these oligomers. Each oligomer can also be compared to known standards, and in the case of HPAEC, eluted for further compositional and structural analysis.

So far, all of the evidence indicates the presence of covalent linkage between HG and RG regions. All methods of extracting pectins yield preparations containing GalA and Rha along with varying amounts of arabinose (Ara), galactose (Gal), xylose (Xyl), mannose (Man), or glucose (Glc). Usually, there is a great preponderance of GalA, indicating the dominance of HG, but the presence of Rha shows RG is also present. Gel permeation or ion exchange chromatography does not yield fractions containing only GalA if the extraction did not involve HG cleaving enzymes, or if the tissue from which the pectin was extracted was not rich in pectinases.

DECORATIONS ON HG BACKBONES

Methyl esters

Methanol, as the methyl ester of carboxylic acid groups, is a well studied adornment

on HGs. The degree of methyl esterification (DM) varies widely from plant to plant, cell to cell, and from one location within a cell wall to another (Liners and Van Cutsen, 1992; McCann *et al.*, 1994; Femenia *et al.*, 1998). The pattern of esterification has been suggested to be random in some cases (de Vries *et al.*, 1983; Mort *et al.*, 1993) and well defined in others (de Vries *et al.*, 1986; Mort *et al.*, 1993). The activity of enzymes that degrade pectin is directly affected by the presence of methyl esters (Chen and Mort, 1996). It is widely believed (convincing evidence is available for only a few cases) that newly synthesized pectin has a high degree of esterification and that pectin methylesterases (PME) produce the lower DM pectins (Goldberg *et al.*, 1986).

Plant PMEs are reported to cause blockwise de-esterification (Taylor, 1982), and PMEs of pathogenic or saprophytic microbes are thought to be random de-esterifiers. Grasdalen and collaborators (Grasdalen *et al.*, 1988) found that the chemical shift of the H-5 and H-1 signals in the proton NMR spectrum of GalA reflects its esterification and esterification of neighboring residues. DeVries and collaborators (de Vries *et al.*, 1986) used the digestion products from pectin lyase to infer patterns of esterification. The lyase was postulated to require three or four adjacent esterified GalA residues for activity. Mort and coworkers (Mort *et al.*, 1993) devised a quantitation method for the various lengths of contiguous non-esterified GalA residues in a pectin fragment. The experiment was based on a combination of reduction of esterified residues to galactose, specific hydrogen fluoride (HF) solvolytic cleavage of galactosyl glycosidic linkages and separation of the resulting GalA containing oligomers. We found that commercial pectins with around 50% esterification have a random pattern, but some HGs extracted from cotton suspension cultures show an almost strictly alternating pattern of esterified and non-esterified residues.

Non-methyl esters on GalA carboxyl groups

There are several reports of esters in pectins involving alcohols other than methanol. In no case however, has the type of alcohol been determined. The presence of these esters was implied indirectly, and in some cases up to 30% of the esters has been presumed to be something other than methyl ester (Kim and Carpita, 1992; McCann *et al.*, 1994; Needs *et al.*, 1998). When Needs and collaborators (Needs *et al.*, 1998) attempted to isolate oligosaccharides containing putative non-methyl esters from carrot roots after driselase (a cocktail of digestive enzymes) digestion, they failed, suggesting that driselase may contain activities which hydrolyze non-methyl esters or that they do not exist. Additional evidence for the presence of a small amount of alcohols other than methanol esterified to pectin was presented by Brown and coworkers (Brown and Fry, 1993) who detected anomalous behavior on thin-layer-chromatography (TLC) of driselase digested pectin fragments. Saponification of the oligomers converted them to $GalA_3$. In our opinion, the presence of non-methyl esters in pectins will not be certain until they have been identified by direct methods.

Finally, it has been suggested multiple times that GalA carboxyls could be esterified to other sugar hydroxyls to form cross-links (Fry, 1986). However, no convincing evidence has been published, although there is an intriguing suggestion that pectin from peas can be cross-linked by a trans-esterification induced by pectin methylesterases (Hou and Chang, 1996).

Acetate esters

Acetate esters on the 2- and 3- hydroxyl position of GalA residues in HGs have been reported frequently on sugar beet pectin (Rombouts and Thibault, 1986; Thibault *et al.*, 1993), but they are only a minor substituent on most HGs. Ishii has isolated small amounts of acetylated GalA trimers from bamboo (Ishii, 1995) and potatoes (Ishii, 1997) and characterized them by extensive NMR and mass spectral analysis.

Xylogalacturonan

Xylose has been recognized as a minor component in most pectin preparations and is found concentrated at particular sites, often referred to as xylogalacturonan, XGA (*Figure 3*). Bouveng (1965) described a xylogalacturonan extracted from pine pollen as being an HG with xylose linked β(1→3) to approximately one in every two GalA residues. In their generalized pectin structure, Cook and Stoddart (1973) included side chains of up to three sugar residues linked to HG regions via the β(1→3)-xylose residues. These included: β(1→2)-galactosyl-xylose, α(1→2)-fucosyl-xylose, and both of these oligomers elongated by addition of a β(1→3)-glucuronosyl residue on the fucose or β(1→6)-glucuronosyl residue on the galactose. In recent review articles, the presence of xylogalacturonan regions is mostly ignored (McNeil *et al.*, 1984; Bacic *et al.*, 1988; Carpita and Gibeaut, 1993; Albersheim *et al.*, 1996). However, recent work from Voragen's (Schols and Voragen, 1994; Schols *et al.*, 1995) and Mort's (Yu and Mort, 1996) groups has shown the presence of xylogalacturonans in

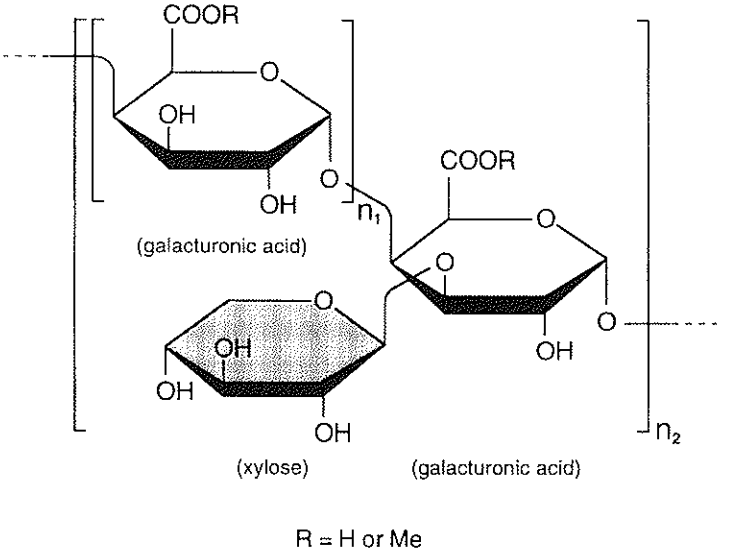


Figure 3. The Xylogalacturonan (XGA) Region. Xylogalacturonan (XGA) regions are HG segments containing xylose substituents located close to the region where the rhamnogalacturonan (RG) region is believed to interact in native pectin molecules. n_1, indicates the number of non-substituted, interspersed GalA residues in the XGA backbone (average of 1) and n_2, the number of Xyl-GalA-$GalA_{n1}$ repeated units (Yu and Mort, 1996).

many fruit pectins. Kikuchi and coworkers (Kikuchi *et al.*, 1996) have also found substantial amounts of xylogalacturonan in cultured carrot cells.

Rhamnogalacturonan II

In 1978 Darvill and coworkers (Darvill *et al.*, 1978) reported that endopolygalacturonase digestion of sycamore cells releases a polymeric region of pectin rich in GalA, Rha, and a variety of other sugars. They designated this region rhamnogalacturonan II (RGII) because of its high Rha and GalA content and to distinguish it from the rhamnogalacturonan region which had already been designated rhamnogalacturonan I (RG). *Figure 4* shows a structural description of RGII. Over the last 20 years, members of what now is named the CCRC (Complex Carbohydrate Research Center, Athens GA, USA) have increasingly refined the structure of this region (O'Neill *et al.*, 1996 and references therein). RGIIs have been found in all plants in which its presence was investigated, and it appears that all are almost structurally identical. Moreover, it appears that RGII is the site at which most of the boron found in plants is bound (Matoh *et al.*, 1993). Thus, it is tempting to conclude that RGIIs form cross-links between pectin molecules (Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996) via borate interactions between apiose residues from independent RGII sections.

Apiogalacturonan

In a few plant species such as *Lemna minor* and *Zostera nana* there is a high degree of apiose substituents on the HG backbone, and the apiose occurs as apiobiose linked directly to an as yet unidentified position on GalA residues (Hart and Kindel, 1970a; Hart and Kindel, 1970b).

DECORATIONS ON RG BACKBONES

Acetate and methyl esters

Komalavilas and Mort (1989) first reported acetylation specifically on rhamnogalacturonan after they isolated the GalA-Rha disaccharide repeat unit acetylated at position O-3 of the GalA after HF solvolysis of cotton suspension culture walls. Their conclusion that position O-2 was not acetylated in native RGs was discounted by Lerouge and collaborators (Lerouge *et al.*, 1993) who showed, using ^{1}H NMR spectroscopy, acetylation on both O2- and O3- positions of GalA residues recovered from EPG solubilized sycamore RG fragments. Ishii also found acetate esters on both, 2- and 3-hydroxyl positions in bamboo RGs (Ishii, 1995) and potato (Ishii, 1997).

GalA linked sugar side chains

Most, or all, of the side chains found in RGs are connected to the backbone through Rha residues. There have been reports about a small proportion of the GalA residues in the rhamnogalacturonan region having xylose linked to them (Cheethan *et al.*, 1993; An *et al.*, 1994). However, we strongly suspect that, since in most pectins

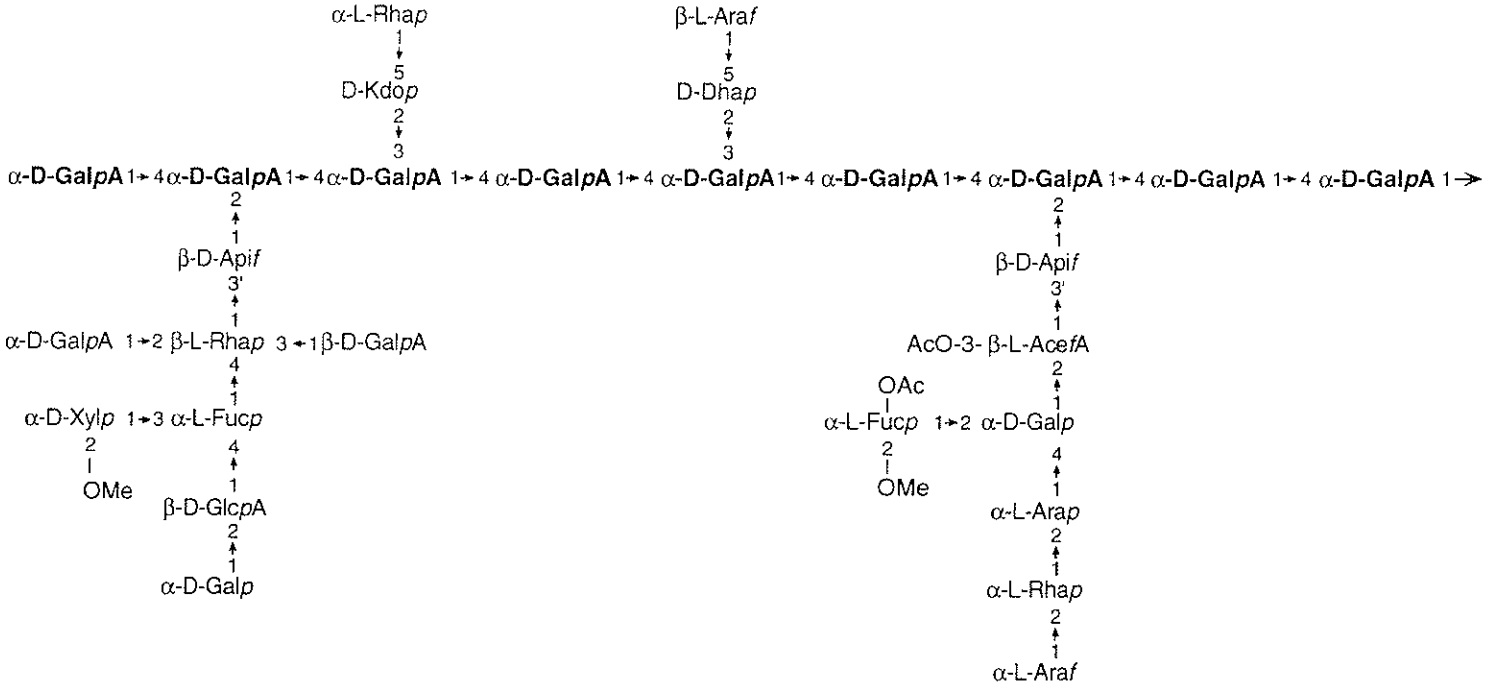


Figure 4. The Rhamnogalacturonan-II (RGII) Region (partial structure). Rhamnogalacturonan–II (RGII) is found as oligomeric regions present in all native pectins, with an almost identical structural configuration. This region contains a GalA backbone with extensive side chains containing rhamnose (Rha), galactose (Gal), xylose (Xyl), glucose (Glc), fucose (Fuc), apiose (Api), arabinose (Ara), 2-keto-3-deoxy-D-manno-octulopyranosylonic acid (Kdo) and 3-deoxy-D-lyxo-2-heptulopyranosylaric acid (Dha) and aceric acid (AceA). RGIIs are resistant to endopolygalacturonase (EPG) digestion. The order and position of side chain attachments on the GalA backbone has not been determined and were arbitrarily assigned. Structural representation redrawn from O'Neill *et al.* (1996).

xylogalacturonans are localized in close proximity to the RG-HG junction, the xylosylated GalA is probably not internal to the RG region itself.

Rha linked sugar side chains

Since the discovery that RGs make up a substantial fraction of most pectins (McNeil *et al.*, 1980), it has been shown that many of the Rha residues have sugar side chains attached to them at position O-4. These side chains are quite variable in length and sugar composition. The predominant sugars are arabinose and galactose, but fucose, glucuronic acid, 4-O-methyl glucuronic acid, xylose, and rhamnose have also been reported. Lau and coworkers (Lau *et al.*, 1987) isolated and characterized a large variety of oligomers linked to rhamnitol after they destroyed the GalA residues in the RG backbone using a dissolving metal reduction reaction (Mort and Bauer, 1982). The arabinose appears most often as $\alpha(1\rightarrow5)$-linked arabinofuranans with $\alpha(1\rightarrow3)$ arabinose and some $\alpha(1\rightarrow2)$ side branches. Galactose is found predominantly as a single galactose residue, or $\beta(1\rightarrow4)$-linked galactopyranose chains with galactose or arabinose side branches through the O-3 positions. An arabinogalactan based on a backbone of $\beta(1\rightarrow3)$-linked Gal with $\beta(1\rightarrow6)$-linked side branches of Gal and/or Ara is also quite common.

Pectin nomenclature

There are quite a few names used to describe pectins and sections of pectins. Protopectin is an old term used to refer to pectin that is in its insoluble native state in cell walls. Protopectinases are enzymes which solubilized pectins from plant material (Sakai *et al.*, 1993). Pectate, or polypectate, is the salt of de-esterified pectin. Pectic acid is the acid form of de-esterified pectin. Within pectins there are usually various regions, some of which have been given very specific names such as rhamnogalacturonan II, and I (McNeil *et al.*, 1984), and some more general, such as hairy and smooth regions (Schols and Voragen, 1996). The smooth regions consist of homogalacturonan (HG) which may be methylesterified or acetylated but have few, if any, side chains. The hairy regions are very complex, consisting of both rhamnogalacturonan and xylogalacturonan regions. Within the rhamnogalacturonan (RG) regions there may also be subdivisions into sections with long side chains and others with short side chains. Rhamnogalacturonan I (RG) is defined as a rhamnogalacturonan with a perfectly repeated disaccharide backbone with side chains attached to O-4 positions of Rha residues. Thus, hairy regions contain RGs, but RG is only a section of the hairy region. On the other hand, RGIIs were named based on their GalA and Rha content. Structural studies, however, revealed that RGIIs have an HG backbone with side chains containing several sugars including rhamnose. Localization of RGIIs in native pectin molecules and its possible relationship to hairy or smooth regions remains unknown.

Microbial pectin degrading systems

Pectins make up a substantial fraction of the total sugar content present in plant materials collectively termed biomass. As described earlier in detail, pectins basically

utilize the majority of their sugar residues in the assembly of a linear molecule with a homopolygalacturonic (HG), non-branched methyl-esterified segment, and a rhamnogalacturonan (RG) branched and acetylated fragment. A simplified and schematic representation of a generic pectin molecule is shown in *Figure 5*.

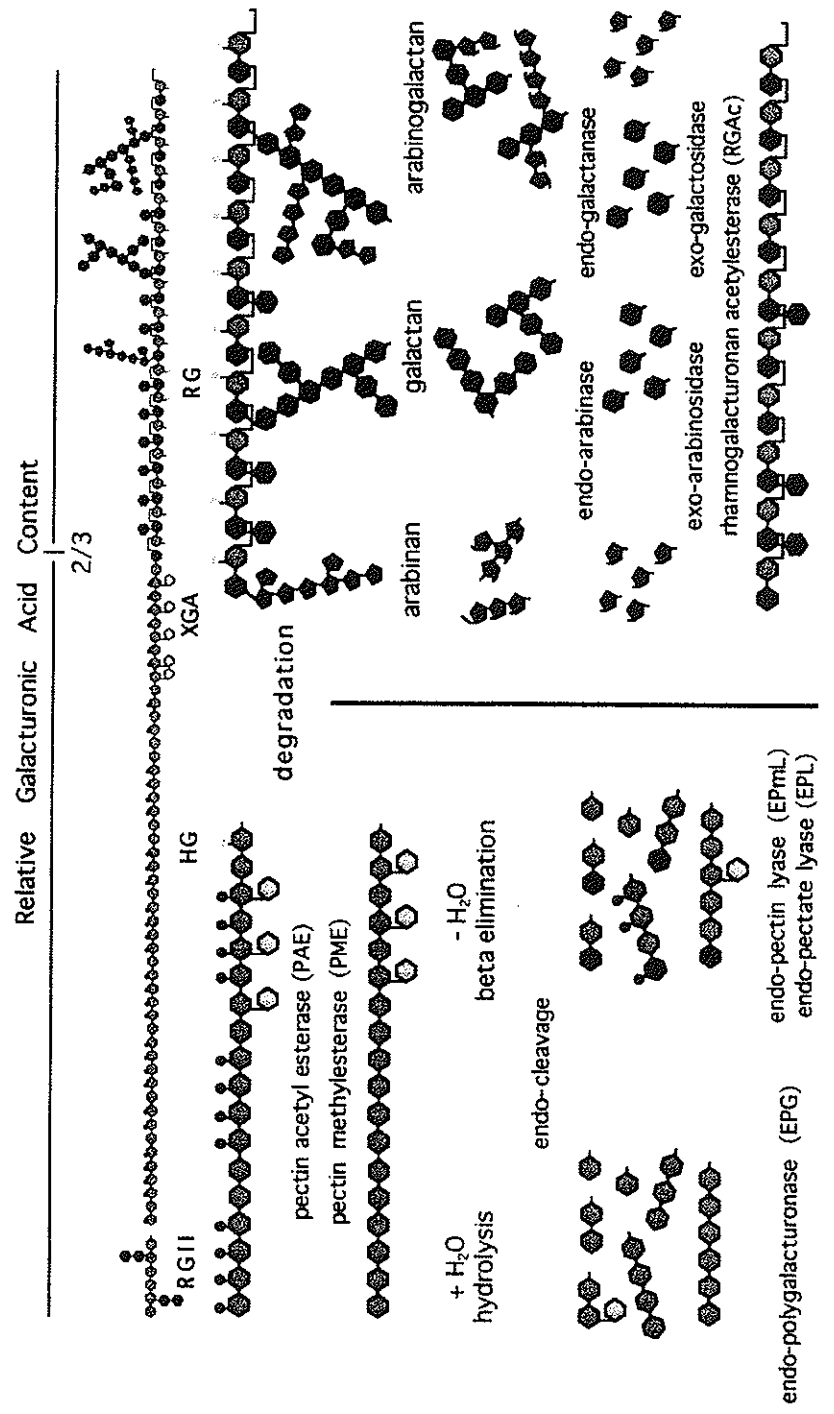
Microorganisms in general recognize pectins, under a variety of physiological circumstances, as a potential, but complex bonded carbon source. They respond positively to the extracellular presence of pectins by activating the synthesis and secretion of a wide range of proteins (all enzymes that degrade a portion of a polysaccharide defined as pectin) involved in modification, degradation, transport, assimilation and metabolism of the released sugar residues. Complete enzymatic degradation by pectinases results primarily in galacturonic acid and rhamnose with production of lesser amounts of galactose, arabinose, methanol, acetate and traces of numerous other sugars (e.g. xylose, fucose, apiose), all of which are assimilated and metabolized by microorganisms, to promote vegetative growth.

The foremost biochemical activity of a microbial pectinase is to cleave glycosidic bonds in native pectin molecules, initially resulting in the production of intermediate sized fragments containing multiple residues followed by the release of individual sugars. In general, enzymes involved in depolymerization are extracellular and the final products are assimilated and metabolized. Typically, monomers and dimers are transported into the cytoplasm, which are then degraded by cytoplasmic enzymes. However, in certain bacteria (e.g. *Erwinia* and *Bacterioides*) specific enzymes are found in association with the outer membrane, periplasm or cytoplasmic membrane. In *Figure 5*, the mode of action and predicted products for most types of microbial pectinases is shown. In addition, an extensive survey of bacterial pectinases, including extracellular and intracellular steps can be found in *Table 1*.

Pectinolytic microorganisms produce an array of pectin degrading enzymes reflecting the complexity of the natural substrate. Pectinases are classified according to their site of cleavage, endo or exo (if they cleave within or at the end of the substrate chain, respectively), preferred substrate (pectin or pectate) and the mode by which they cleave the glycosidic bond (hydrolase or lyase). Pectin hydrolases transfer a single proton from a donor acidic amino acid to the glycosidic oxygen and cleavage involves the net consumption of a water molecule. Eliminases, commonly known as lyases, cleave glycosidic bonds via an elimination mechanism that generates unsaturated oligomeric or monomeric reaction products. The proton, α to the carbonyl group, is abstracted by a basic amino acid and followed by elimination of the sugar, resulting in a 4,5 GalA unsaturation (Linhardt *et al.*, 1986).

Pectin lyases act on highly methyl-esterified pectins and do not require calcium for enzymatic activity. Pectate-lyases are catalytically similar to pectin-lyases, except that they cleave non-esterified GalA residues and require calcium for optimal activity (Pickersgill *et al.*, 1994; Mayans *et al.*, 1997). Until recently, it was not clear whether calcium is part of the enzyme (Rao *et al.*, 1996) or is involved in substrate binding (Crawford and Kolattukudy, 1987). Recent x-ray studies on a pectate lyase from *Bacillus subtilis* complexed with calcium, shows that calcium interacts with an arginine residue that is conserved across all pectin and pectate lyases (Pickersgill *et al.*, 1994).

Pectin- and pectate-lyases are common among fungi and have been found in most species where they have been looked at (Dean and Timberlake, 1989; Gysler *et al.*,


Relative Galacturonic Acid Content
2/3
RGII
HG
XGA
RG
degradation
pectin acetyl esterase (PAE)
pectin methylesterase (PME)
+ H_2O
hydrolysis
- H_2O
beta elimination
endo-cleavage
endo-polygalacturonase (EPG)
endo-pectin lyase (EPmL)
endo-pectate lyase (EPL)
arabinan
galactan
arabinogalactan
endo-arabinase
endo-galactanase
exo-arabinosidase
exo-galactosidase
rhamnogalacturonan acetylesterase (RGAc)

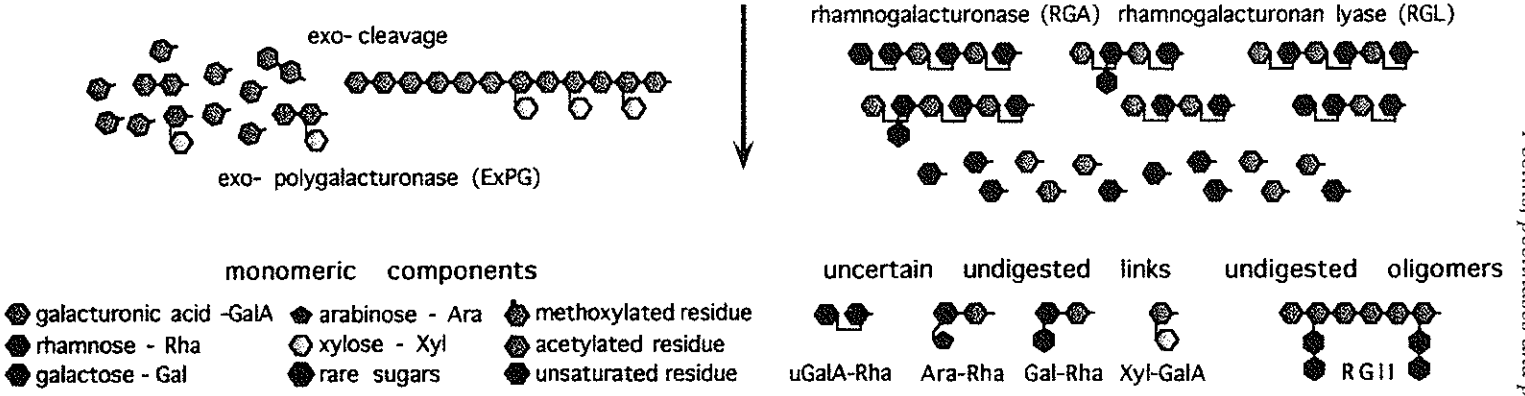


Figure 5. Mode of Action and Predicted Products for Known Pectinases. A schematic of a generic pectin molecule and its complete degradation by extracellular enzymes is shown. Length and distribution of linear and branched portions were based on the relative content of galacturonic acid recovered from backbones containing exclusively polygalacturonic acid (HG, XGA and RGII) and rhamnogalacturonan (RG). Only enzymes directly involved in degradation are listed. For detailed information of related metabolic activities refer to *Table 1*. At the bottom of the figure sugar residues found as constituents in native pectins and fragments that cannot be degraded with known enzymes are shown. HG, homogalacturonan (polygalacturonic acid), RG, rhamnogalacturonan (region 1 or hairy region), RGII, rhamnogalacturonan region 2 as defined by Darvill and collaborators (Darvill *et al.*, 1978) and XGA, xylogalacturonan. Positions of covalent links are drawn as reported for the backbone regions only. Covalent link representations in side chains are approximations.

1990; Polizeli *et al.*, 1991; Gonzalez-Candelas and Kolattukudy, 1992; Bowen *et al.*, 1995; Ho *et al.*, 1995; Kopecny and Hodrova, 1995). In bacteria, pectate lyases represent the largest group of bacterial pectinolytic enzymes and are directly involved in the destruction of plant tissues. Endo-pectate lyases cleave non-esterified pectate chains at internal sites within the chain resulting in a mixture of unsaturated oligomers. Typically, bacterial exo-pectate lyases cleave the non-reducing end of non-esterified pectate chains resulting in the release of unsaturated di- or trigalacturonide (*Table 1*). Interestingly, many phytopathogenic bacteria produce more than one pectate lyase. For example, *Erwinia chrysanthemi* secretes eight endo-pectate lyase isozymes and only a single exo-pectate lyase which is retained in the periplasm (Shevchik and Hugouvieux-Cotte-Pattat, 1997). Based on amino acid sequence relatedness, bacterial pectate lyases are currently separated into five different classes (Shevchik and Hugouvieux-Cotte-Pattat, 1997). This functionally redundant group of enzymes apparently reflects, in part, differential enzymatic sets recruited under varying physiological conditions – *e.g.* saprophytic versus pathogenic growth (Salmond, 1994). Phytopathogenic *Erwinia* species and *Pseudomonas marginalis* also produce extracellular endo-pectin lyases which, unlike the pectate lyases, are induced by DNA damaging agents and possibly by plant host phytoalexins (Tsuyumu and Chatterjee, 1984; Zink *et al.*, 1985; Sone *et al.*, 1988).

Pectin methylesterases remove methyl groups rendering pectin polymers accessible to the depolymerizing enzymes such as pectate lyases and polygalacturonases. Two different pectin methylesterases have been identified from *E. chrysanthemi*. The first was identified as an extracellular pectin methylesterase (*PmeA*) (Laurent *et al.*, 1993) while the second, *PmeB*, was identified as an outer membrane lipoprotein (Shevchik *et al.*, 1996). *PmeB* has a greater activity on small pectic oligomers which can easily diffuse into the periplasm (Shevchik *et al.*, 1996). Pectin methylesterases have also been found in other phytopathogenic bacteria (Schell *et al.*, 1994), several intestinal bacteria (Cornick *et al.*, 1994; Tierny *et al.*, 1994) and in fungi (Khanh *et al.*, 1991; Wojciechowski and Fall, 1996).

Polygalacturonases (PG), poly (1,4-α-D-galacturonide) glycan hydrolases, catalyze the cleavage of glycosidic α(1→4) linkages on HG backbones. Polygalacturonases are of the endo (EPG) type if they cleave internal glycosidic bonds of the HG chain, the products are oligomeric and monomeric, and are of the exo (ExPG) type when they cleave at the non-reducing end of the substrate chain. Polygalacturonases are only active on non-esterified pectin regions, thus demanding the removal of methylester groups for complete depolymerization (Khanh *et al.*, 1991; Wojciechowski and Fall, 1996).

In the literature there is a disproportional number of reports on endo- versus exo-acting fungal pectinases. For example, endo-polygalacturonases have been cloned and genetically studied in a large number of species (Keon and Waksman, 1990; Bussink *et al.*, 1991; Reymond *et al.*, 1994; Whitehead *et al.*, 1995; Centis *et al.*, 1996; Gao *et al.*, 1996; Guo *et al.*, 1996; Centis *et al.*, 1997), and also have received considerable attention from physiologists (Kester and Visser, 1990; Scott-Craig *et al.*, 1990; Polizeli *et al.*, 1991; Clay *et al.*, 1997; Patino *et al.*, 1997; Takasawa *et al.*, 1997). In contrast, less attention has been given to the genes for exo-polygalacturonases (Scott-Craig *et al.*, 1998), although this activity is consistently detected in physiological

studies (Kester and Visser, 1990; Polizeli *et al.*, 1991; Aguilar and Huitron, 1993; Di Pietro and Roncero, 1996; Patino *et al.*, 1997).

In bacteria, endo- and exo-polygalacturonases have been identified as extracellular depolymerizing enzymes of phytopathogenic *Erwinia* (Kotoujansky, 1987; He and Collmer, 1990). In contrast to pectate lyases, polygalacturonases of *Erwinia* do not require calcium but are stimulated by sodium, potassium and ammonium ions (Nasuno and Starr, 1966). Polygalacturonase has also been identified in *Agrobacterium tumefaciens* (Rodrigues-Palenzuela *et al.*, 1991) and several intestinal bacteria (see *Table 1*).

Rhamnogalacturonan acetyl-esterase (Kauppinen *et al.*, 1995), rhamnogalacturonan hydrolase (Mutter *et al.*, 1994) and rhamnogalacturonan lyase (Mutter *et al.*, 1996) de-acetylate and cleave the rhamnogalacturonan backbone (RG) of the substituted regions of native pectins, respectively (Schols *et al.*, 1990; Schols and Voragen, 1994). These enzymes have been found frequently in fungi (Schols *et al.*, 1990; Sakamoto *et al.*, 1994; Suykerbuyk *et al.*, 1995; Chen *et al.*, 1997; Nelson *et al.*, 1997). Except for a pectin acetyl esterase (Shevchik and Hugouvieux-Cotte-Pattat, 1997), we are unaware of similar reports in bacteria. Rhamnogalacturonases cleave de-acetylated regions of the RG backbone, producing linear hexamers and tetramers with Gal branches (see *Figure 5*). Additional enzymes that degrade the RG backbone have been found and include: rhamnogalacturonan α-D-galactopyranosyluronohydrolase, which releases GalA residues from the non-reducing end of RGs but not HGs (Mutter *et al.*, 1998a; Mutter *et al.*, 1998b) and rhamnogalacturonan α-L-rhamnopyranohydrolase, which cleaves Rha residues from the non-reducing end of RGs (Mutter *et al.*, 1994).

Enzymes that degrade RG side chains have also been found and include: arabinases, α-L-arabinofuranosidase (v.d. Veen *et al.*, 1991; Ramon *et al.*, 1993; v. d. Veen *et al.*, 1993; Flipphi *et al.*, 1994; Ruijter *et al.*, 1997), endo-(1→5)-α-L-arabinases (Flipphi *et al.*, 1993), and galactanases, exo-β-(1→4)-galactanase (Bonnin *et al.*, 1995; Christgau *et al.*, 1995), endo-β-(1→4)-D-galactanase (Yamaguchi *et al.*, 1995), exo-(1→3)- β-D-galactanase – able to by-pass the branching points of galactan backbones (Pellerin and Brillouet, 1994) and endo-β-(1→5)-galactofuranase (Reyes *et al.*, 1992). Only arabinases have been found in bacteria so far (McKie *et al.*, 1997; Sakamoto *et al.*, 1997).

Enzymes that remove Xyl residues from the XGA region have not yet been reported. However, activities that release GalA residues from the non-reducing end of XGA trimers, have been observed. This enzyme also acts as an exo-galacturonase, releasing GalA monomers from HGs. Thus, it is likely that this enzyme is an exo-polygalacturonase, not inhibited by xylosylated GalA residues. Other links for which a suitable enzyme has not been identified (or tested) include the entire RGII region, the RG portions, Ara-Rha as well as Gal-Rha branching bonds, and Rha containing unsaturated elimination (lyase) products.

In bacteria, the major end products of polygalacturonases and pectate lyases are saturated and unsaturated di-galacturonate, respectively, which enter cells and are further metabolized intracellularly. Extracellular galacturonate enters the cell through active transport in *E. chrysanthemi* or is produced intracellularly by the action of oligo-galacturonate lyase. Oligogalacturonate lyase is an intracellular enzyme in *E. chrysanthemi* which cleaves the di-galacturonates into two different monomers: 5-keto-4-deoxy-uronate (DKI) and galacturonate. The monomers DKI and galacturonate

are then metabolized in *E. chrysanthemi* by two distinctly different enzyme systems to produce 2-keto-3-deoxy-gluconate (KDG) which is further converted to pyruvate and 3-phosphoglyceraldehyde (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1989). Intracellular di- and tri-galacturonate are hydrolyzed to monomers by oligoglacturonate hydrolase in *Clostridium thermosaccharolyticum*. Galacturonate is further metabolized by a modified KDG pathway similar to that described for *Erwinia* species but involves galacturonate isomerase, tagaturonate reductase and altromate dehydratase (Van Rijssel *et al.*, 1993). While considerable data are available on the uptake and metabolism of pectin hydrolysis products in bacterial systems, no such data are available in fungi.

In most filamentous fungi, pectinases, xylanases, as well as cellulases, are under carbon catabolite repression (Dean and Timberlake, 1989; Dean and Timberlake, 1989; Polizeli *et al.*, 1991; Ho *et al.*, 1995; Reymond-Cotton *et al.*, 1996; Ruijter *et al.*, 1997). Very little is known about the induction phase of these complex and important biochemical activities. We know that induction of cell wall degrading enzymes involves constitutive low level expression of at least some of the structural genes (El-Gogary *et al.*, 1989). In the presence of substrate or partially degraded fragments, these enzymes produce trace amounts of degradation products that are supposedly chemically modified (e.g. via positional isomerization) resulting in a potent inducer. It is assumed that the signal is transduced through several unknown steps and culminates with the massive synthesis of cell wall degrading enzymes (Sternberg and Mandels, 1979; Biely *et al.*, 1980; Polizeli *et al.*, 1991; v. d. Veen *et al.*, 1993). The recognition process remains unclear and no advances have recently been made.

Plant-microbe interactions

SUBSTRATE SPECIFICITY AND PRODUCT DISTRIBUTION

Because of the difficulty in obtaining highly defined pectin substrates and analyzing the exact nature of the products of enzyme digestions, only a few pectin degrading enzymes have been well characterized with respect to their exact substrate specificity and product distribution. Most often, the hydrolytic type of enzymes are assayed by their production of reducing equivalents using a colorimetric assay, and lyases by 236 nm absorbance reflecting the generation of unsaturated GalA residues.

In the last few years, it has become much easier to characterize reaction products because of the high resolving power of high performance liquid chromatography (HPLC) anion exchange columns and the high sensitivity of the pulsed electrochemical, or amperometric, detector commercialized by the Dionex company. It is possible that these will be partially superseded by capillary electrophoresis with fluorescence detection. We have found that fluorescently labelled pectic oligosaccharides are good substrates for most pectin degrading enzymes, and that by capillary electrophoretic analysis the exact nature of the products can be determined quickly, if suitable standards are available (Mort and Chen, 1996; Zhan *et al.*, 1998).

Fungal endopolygalacturonases appear to bind four (or five (Benen *et al.*, 1996)) adjacent GalA residues of the chain and hydrolyze between the last two residues toward the reducing end of the bound cluster (Rexova-Benkova and Markovic, 1976; Thibault, 1983; Mort and Chen, 1996). Some enzymes act randomly, whereas it

appears that others exhibit a multiple attack action. Instead of dissociating from the substrate, the enzyme sometimes 'moves' along the chain by one residue towards the non-reducing end and catalyzes repeated glycosidic hydrolysis (Benen *et al.*, 1996).

Exogalacturonases in fungi hydrolyze single GalA residues from the non-reducing end of polymeric, or oligomeric galacturonan (Kester *et al.*, 1996). In contrast, bacteria usually remove disaccharides from the non-reducing end of pectate by an exo-pectate lyase. The substrate specificity of fungal pectin lyases is not well known. Van Houdenhoven (1975) suggests the enzyme binds between 8 and 10 sugar residues. De Vries and collaborators (de Vries *et al.*, 1983) inferred by modelling pectin lyase degradation of apple pectins, that the enzyme needs three, but perhaps four, adjacent esterified residues.

In bacteria, pectate lyases seem to be the most important enzymes in HG degradation. There is good evidence that several enzymes, all capable of degrading pectate, with different specificities are produced at the same time by the same organism. Preston and collaborators (Preston *et al.*, 1992) compared four pectate lyases from *E. chrysanthemi* acting on pectic acid and found that one (*Pla*) produced random cleavage products, two (*Plb*, *Plc*) produced predominantly unsaturated GalA trimers, and another (*Ple*), unsaturated dimers. The three enzymes that cleave in a non-random fashion are likely to function via a multiple attack (scanning) mechanism observed in some fungal EPGs (Benen *et al.*, 1996). Another comparison testing various pectate lyases from *E. carotovora* on differentially methylesterified substrates resulted in synergistic interactions, indicating that individual enzymes are differentially affected by the amount of methyl ester groups present on the substrate molecule (Bartling *et al.*, 1996).

BACTERIAL INFECTIONS

Bacteria have variable impacts on agriculture. For example, bacterial pectinases are beneficial in processes like biomass utilization, but the same activities are devastating when acting as virulence factors in plant diseases (He *et al.*, 1993). The insoluble plant cell wall polymers are significant barriers to microorganisms, yet represent an abundant source of carbohydrates. The disease process in general requires pectin degradation and is often dependent on plant sensitivity and environmental conditions such as temperature, nitrogen starvation, osmolarity and oxygen limitations (Hugouvieux-Cotte-Pattat *et al.*, 1992).

To address whether or not pectin degradation was required to establish bacterial pathogenesis, Kelemu and collaborators (Kelemu and Collmer, 1993) created an *E. chrysanthemi* recombinant strain, deficient in all known pectate lyases and polygalacturonases. However, the pectinase deficient mutant was still able to degrade the host plant tissue. This initially disappointing observation led to the discovery of a whole 'new' set of pectate lyases that are induced and secreted *in planta*. The fact that bacteria have evolved alternate sets of functionally redundant cell wall degrading enzymes expressed *in planta* illustrates the importance of these enzymes in the natural life cycle of these apparently simple microorganisms.

FUNGAL INFECTIONS

Most filamentous fungi are outfitted with a specific genetic program that allows them

to infect plant cells and colonize specific organs, tissues or entire plants. Although pathogen-host interactions are in many cases determined by very specific biochemical relationships, a more or less morphologically defined developmental programme of events is consistently observed. A typical pathological cycle is initiated when fungal spores land and attach on the surface of a compatible host. During germination, a filamentous cell grows along the plant surface and undergoes several mitotic cell divisions until, in response to physical and chemical signals, a morphologically differentiated cell-type, the *appressorium*, is formed. From the apical vegetative cell, most of the cytoplasm migrates into the forming appressorium (Bourett *et al.*, 1987; Kwon and Hoch, 1991; Kwon *et al.*, 1991; Gross *et al.*, 1993). Penetration of the host is accomplished by the formation of a penetration peg whose production is coordinated by the fully differentiated appressorium. The penetration peg punches and dissolves the thick plant cell wall and seeks the protoplast where the apex penetrates the membrane (Mould *et al.*, 1991; Mould *et al.*, 1991). Once the penetration step has been completed, the infection hypha gains access to host nutrients and returns to the vegetative mode of growth (polar extension).

Compelling physiological evidence for the role of pectin degrading enzymes in pathogenesis has been obtained for many diseases. However, although the physiological evidence is compelling, a satisfactory genetic proof indicating a substantial implication of pectin degradation remains to be produced (Mendgen and Deising, 1993; Walton, 1994; Scott-Craig *et al.*, 1998). There is not much doubt that these enzymes are specifically induced by fungal pathogens during infection and progression of disease (Bateman *et al.*, 1976; Keon and Waksman, 1990; Walton and Cervone, 1990; Van Hoof *et al.*, 1991; Cary *et al.*, 1995; Ho *et al.*, 1995; Wu *et al.*, 1995). However, genetic inactivation (disruption) of individual enzyme-coding loci has shown no detectable phenotypes. The inactivation, in *C. carbonum*, of any one cell wall-degrading enzyme (endo-polygalacturonase, β-(1→4)-xylanase, exo-(1→3)-glucanase or a cellulase) does not abolish the infection process as a whole (Scott-Craig *et al.*, 1990; Apel *et al.*, 1993; Schaeffer *et al.*, 1994; Sposato *et al.*, 1995). Moreover, a *C. carbonum* endo-polygalacturonase and exo-polygalacturonase double 'null' mutant is still pathogenic (Scott-Craig *et al.*, 1998). There are many possible interpretations why the disruption of one gene does not inactivate infection: one is that pectin degradation is not needed for infection and another that genetic redundancy and biochemical overlap found in plant cell wall degrading systems compensates the genetic 'null' phenotype (Prade, 1996).

PLANT PROTEINS THAT INHIBIT PGS

Plants produce a class of proteins that inhibit fungal PGs. These polygalacturonase-inhibiting proteins (PGIPs) were reviewed recently (De Lorenzo and Cervone, 1997). PGIPs were discovered in the early '70s, but have only been studied intensively in the '80s. It has been shown that PGIPs are able to inhibit the progress of the invading pathogen in spreading through the infected plant. One possible and likely explanation for the mode of action of PGIPs is that they attenuate the pectin breakdown rate, thus slowing the reduction of oligogalacturonides into trimers, dimers and monomers (De Lorenzo and Cervone, 1997). The reduced efficiency in breaking down HG fragments increases the lifetime of various intermediate sized GalA fragments (12–14mers) and

it has been shown that these kinds of fragments are able to activate plant resistance responses (Darvill *et al.*, 1994; Cook *et al.*, 1998). PGIPs have been observed in a wide variety of dicots, and in monocots they have been reported in the onion family. We do not know of any attempts to survey plants for PGIPs to see how widely they are distributed. Constitutive levels of PGIP vary with age and cell type, and in some cases are induced through wounding or the presence of elicitors. In beans, three families of PGIP genes that are differentially expressed have been reported (De Lorenzo and Cervone, 1997). Interestingly, known PGIPs do not inhibit PGs produced by plants or bacteria (De Lorenzo and Cervone, 1997).

Conclusions

Finally, on one hand we are able to provide a fairly detailed account on pectin structure and enzyme systems found in microorganisms. Pectinases, in conjunction with other large sets of degrading activities, are likely to entirely degrade any significant structural components of the invaded host into metabolizable products. This one sided view suggests that microbes are able to assemble an impressive biochemical arsenal. On the other hand, we are beginning to appreciate that a full blown microbial attack is not always successful because hosts have evolved specific biochemical barriers. Even though we know little about PGIPs, their existence alone surely tempt the suggestion that additional, yet unknown biochemical plant (host) fungus (pathogen) communication mechanisms may play determining roles in the outcome of disease. Thus, if biochemical defense reactions between pathogens and hosts are in fact more common and widespread, as currently suspected, plant infections and other related interactions (e.g. rotting) may include biochemical interplay reactions that directly interfere with microbial functions such as nutrition and penetration.

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