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Changes in Cell Wall Polysaccharides of Green Bean Pods during Development

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The changes in cell wall polysaccharides and selected cell wall-modifying enzymes were studied during the development of green bean (*Phaseolus vulgaris* L.) pods. An overall increase of cell wall material on a dry-weight basis was observed during pod development. Major changes were detected in the pectic polymers. Young, exponentially growing cell walls contained large amounts of neutral, sugar-rich pectic polymers (rhamnogalacturonan), which were water insoluble and relatively tightly connected to the cell wall. During elongation, more galactose-rich pectic polymers were deposited into the cell wall. In addition, the level of branched rhamnogalacturonan remained constant, while the level of linear homogalacturonan steadily increased. During maturation of the pods, galactose-rich pectic polymers were degraded, while the accumulation of soluble homogalacturonan continued. During senescence there was an increase in the amount of ionically complexed pectins, mainly at the expense of freely soluble pectins. The most abundant of the enzymes tested for was pectin methylesterase. Peroxidase, β-galactosidase, and α-arabinosidase were also detected in appreciable amounts. Polygalacturonase was detected only in very small amounts throughout development. The relationship between endogenous enzyme levels and the properties of cell wall polymers is discussed with respect to cell wall synthesis and degradation.

The texture of processed vegetables and fruits is in part determined by the properties of the cell wall and the middle lamella (Stolle-Smits et al., 1997). The cell wall is not a static structure, it is dynamic in nature. Its composition and structure change continuously during plant development. Plant cell walls consist of cellulose microfibrils coated by xyloglucans and embedded in a complex matrix of pectic polysaccharides (Talbot and Ray, 1992; Carpita and Gibeaut, 1993). Pectic substances are abundant in fruit and vegetable cell walls and are considered to be important in determining the texture of processed vegetables. Cell wall pectin consists of two regions, a linear homogalacturonan (HGA) and a branched rhamnogalacturonan. Neutral side chains, mainly consisting of arabinosyl and/or galactosyl residues, are attached to the rhamnogalacturonan backbone in variable amounts. In addition, xylosyl units can be linked to the GalUA backbone, as was reported for branched apple pectins (Schols et al., 1995).

RGII is a very typical branched pectin that occurs only in minor amounts and is suggested to function as a signal molecule (Darvill et al., 1978). The carboxyl and hydroxyl groups of the GalUA backbone can be substituted with methyl and acetyl esters, respectively. Pectin is believed be connected to other cell wall components or other pectins by ionic and covalent cross-links (Brett and Waldron, 1990). The exact nature of these cross-links is still unknown, but cross-linking esters are thought to be involved. The aim of this study was to analyze the modifications of cell wall composition in green bean (*Phaseolus vulgaris* L.) during pod elongation and senescence, with emphasis on the pectic substances.

Plant development involves a coordinated series of biochemical processes that, among other things, result in the biosynthesis and degradation of cell wall components. During cell expansion, non-cellulosic polymers are cleaved by enzymes and internal osmotic pressure pushes the fibrillar components apart. New microfibrils and associated polymers are subsequently deposited on the innermost surface of the wall, forming a highly stratified and cross-linked matrix (Carpita and Gibeaut, 1993). The precise role of the pectin matrix in controlling growth is not known. In addition to cell wall polysaccharides, specific structural proteins called expansins may be involved in cell expansion by breaking the H-bonds between hemicellulose and cellulose and allowing shear of the cellulose fibrils. When elongation is complete, the resulting cell wall has to be "locked." This is probably brought about by embedding of other structural proteins or lignin, depending on the type of plant and tissue, in the cell wall matrix (Carpita and Gibeaut, 1993).

Substantial research has been performed on the role of pectins in the softening of fruits during ripening (Seymour et al., 1990; Barrett and Gonzalez, 1994; Lurie et al., 1994; Martin-Cabrejas et al., 1994; Ali et al., 1995; El-Buluk et al., 1995). It has been demonstrated that pectin depolymerization and high levels of *endo*-polygalacturonase (PG) occur simultaneously during ripening in many fruits. However, the role of PG in fruit softening has been questioned, since transgenic tomato fruits with only 1% of the original fruit-specific PG activity levels soften normally (Smith et al.,

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1992). The partial breakdown of other wall components such as hemicellulose may be required in addition to the degradation of pectic materials to bring about the extensive softening observed (Maclachan et al., 1995). Very few data are available concerning cell wall modifications during final senescence of plant tissue.

In this study the changes in cell wall polysaccharides and selected pectin-modifying enzymes were studied during the development of green bean pods from the onset of pod growth, throughout elongation and maturation, until senescence. The relationship between endogenous enzyme levels and structural properties of cell wall polysaccharides will be discussed with respect to cell wall synthesis and degradation.

MATERIALS AND METHODS

All analyses except extraction were performed in duplicate, and the coefficients of variation were in all instances less than 10%.

Plant Material

The green bean (*Phaseolus vulgaris* L.) cvs Masai and Odessa were grown under standard greenhouse conditions. Green beans were harvested at different sequential developmental stages and classified in days after flowering (daf). Immediately after harvest, the pods were frozen in liquid nitrogen and stored at −50°C. Next, the seeds were manually removed from the frozen pods if possible (i.e. from stage IIb [10 daf] onward), and the pods were ground in liquid nitrogen.

Dry Matter Determination

The dry matter content of the samples was determined by drying a known fresh weight of homogenized samples overnight at 70°C, followed by 3 h at 105°C. After cooling to room temperature, the samples were reweighed. The dry matter and water content were calculated from the difference.

Starch Content

To solubilize starch, 5 mL of HCl (8 M) and 20 mL of DMSO were added to 250 mg of sample, and the mixture was placed in a 60°C water bath. After an incubation period of 60 min under continuous shaking, 5 mL of NaOH (8 M) and citrate buffer (Titrisol, pH 4.0, catalog no. 9884, Merck, Rahway, NJ) was added to a final volume of 100 mL. After filtration, 0.1 mL of filtrate was used to quantify the starch content in the sample using test combination no. 207748 from Boehringer Mannheim (Basel).

Protein Content

The nitrogen content of the alcohol-insoluble residue (AIR) fractions was measured using an elemental analyzer (model CHNS-OEA 1108, Carlo Erba, Milan). The protein content was estimated by multiplying the nitrogen value by 6.25.

Purification and Fractionation of Cell Walls

Frozen tissue was immersed in 180 mL of cold (−30°C) ethanol (96%, v/v), homogenized (Ultra-Turrax T25, Jancke und Kunkel, IKA Labortechnik, Staufen, Germany) by four bursts of 45 s, and collected on a GF/C filter (Whatman, Clifton, NJ). The material was suspended in 50 mL of cold (−30°C) aqueous ethanol (80%, v/v) and stirred for 1 h at 4°C. The material was filtered again, washed twice with 50 mL of 100% (v/v) acetone until the filtrate was colorless, and dried overnight to yield the AIR. The AIR was subsequently ground in a ball mill (model MM2, Retsch, Ochten, The Netherlands). To remove starch, the AIR (2 g) was suspended in 150 mL of 90% (v/v) DMSO and stirred for 16 h at 20°C. The suspension was centrifuged (7,000*g* for 15 min) and the pellet washed twice with 90% (v/v) DMSO and three times with 80% (v/v) ethanol. The supernatant, which contained predominantly starch, was discarded. Pectic polymers were extracted from the resulting CWM using a method from Selvendran et al. (1985) with minor modifications. To the pellet, 100 mL of 0.05 M ammonium acetate buffer (pH 4.7) was added and the suspension was stirred for 16 h at 4°C. The suspension was centrifuged and the pellet was washed twice with acetate buffer.

The supernatants were then combined and this "buffer"-soluble fraction was dialyzed exhaustively against deionized water at 4°C. Then, 100 mL of 0.05 M CDTA (pH 6.5) was added to the pellet, and the suspension was stirred for 16 h at 4°C. The suspension was centrifuged and the pellet was washed once with the CDTA solution and once with deionized water. The supernatants were combined and dialyzed at 4°C for 14 d against deionized water (CDTA-soluble fraction). The pellet was subsequently extracted with 100 mL (O_2-free) of 0.05 M Na_2CO_3 containing 0.01 M $NaBH_4$ at 4°C and 20°C. Next, hemicelluloses and residual pectins were extracted with, respectively, 0.5, 1.0, and 4.0 M KOH containing 0.01 M $NaBH_4$ and 4.0 M KOH containing 0.65 M H_3BO_3 and 0.01 M $NaBH_4$. All extractions were performed with constant stirring under N_2 for 16 h at 20°C to leave a residue consisting mainly of cellulose. All Na_2CO_3 and KOH supernatants were filtered, adjusted to pH 5.0 with acetic acid, dialyzed exhaustively against deionized water, and lyophilized. During neutralization of the 0.5 and 1.0 M KOH supernatants a precipitate formed, which was isolated and analyzed separately.

Methyl and Acetyl Substituents

The amount of methyl and acetyl groups was determined after saponification by HPLC as described by Voragen et al. (1986).

Monosaccharide Composition

All polysaccharides, including cellulose, from the AIR and residue after extraction of pectin and hemicellulose

were solubilized by dispersing the dried samples in cold 11.5 M H_2SO_4 for 2 h at 20°C, followed by hydrolysis in 1 M H_2SO_4 for 2 h at 100°C under continuous stirring (Seaman hydrolysis). The hydrolysates were filtered through a glass fiber filter, and neutralized with $BaCO_3$. Pectic and hemicellulosic fractions were hydrolyzed by stirring in 2 M TFA for 2 h at 121°C. Samples were dried under N_2 gas at 45°C, washed with 1 M NH_4OH, dried under N_2 gas, and dissolved in deionized water. Samples (10 μL) of the neutralized hydrolysates were analyzed for neutral sugars by using HPLC as described by Stolle-Smits et al. (1997). Uronic acids in AIR and cell wall fractions were determined as described by Ahmed and Labavitch (1977).

Size Exclusion Chromatography

High-performance size exclusion chromatography (HPSEC) was performed using a HPLC system (UK6 injector and 510 HPLC pump, Waters, Milford, MA) equipped with a guard column (7.8 × 300 mm; Ultrahydrogel and Ultrahydrogel 500, Waters) and elution with 0.4 M acetic acid/sodium acetate (pH 3.0) at 0.8 mL min^{-1}. For the measurement of enzyme activity, the same system except with two columns in series (Ultrahydrogel 2000 and Ultrahydrogel 500, each 7.8 × 300 mm, Waters) were used. The eluate was monitored using a refractive index detector (Pharmacia, Uppsala). The system was calibrated using linear pullulans (Shodex P-82, Waters) with molecular masses ranging from 6 to 1,660 kD. Data analysis was performed using Millennium 2010 software (Waters).

Enzyme Activity Assays

All procedures were performed at 4°C. Ground, frozen pods were immersed in 2 M NaCl and homogenized using an ultra Turrax (IKA Labortechnik, Staufen, Germany) by three bursts of 30 s each. After centrifugation, low-molecular-mass compounds were removed from the salt-soluble extracts by elution over a prepacked Sephadex G-25 column (Pharmacia PM10). Fractions containing proteins were pooled and assayed for enzyme activities and protein. Activities are expressed in katals (1 kat = 1 mol product formed s^{-1}).

Protein Content

Protein in the enzyme extracts was analyzed with the Coomassie Plus protein assay reagent (catalog no. 23236, Pierce Chemical, Rockford, IL) using BSA as a reference protein.

Pectin Methylesterase (PME) Activity

PME activity in the supernatant was determined using a continuous spectrophotometric assay with bromothymol blue as a pH indicator (Hagerman and Austin, 1986).

PG Activity

PG activity was determined spectrophotometrically following derivatization of the reaction product with UV-absorbing 2-cyanoacetamide as described by Gross (1982). In addition, the decrease in molecular mass of polyGalUA caused by PG action was analyzed qualitatively using HPSEC.

Peroxidase (POD) Activity

POD activity was determined using a continuous spectrophotometric assay. The reaction mixture (3.0 mL) consisted of 0.1 M citric acid buffer (pH 4.5) containing 0.05 mM 2,2′-azinobis-3-ethylbenzthiazolinesulfonic acid and 0.25 mM H_2O_2. The reaction was started by adding 50 μL of sample solution, and the decrease in A_{414} was monitored using a spectrophotometer (Perkin-Elmer UV/VIS spectrofotometer lambdalb, Nieuwerkerk a/d Ijsel, The Netherlands). POD activities were determined using the molar extinction coefficient of 2,2′-azinobis-3-ethylbenzthiazolinesulfonic acid (3.6×10^{-4} M^{-1} cm^{-1}).

Glycosidases

The activities of β-galactosidase and α-arabinosidase were analyzed using the corresponding *p*-nitrophenyl derivatives of α-L-arabinofuranoside, β-D-galactopyranoside (Sigma, Zwyndrecht, The Netherlands) as substrates. The reaction mixture consisted of 1.5 mL of 33 mM acetate buffer of optimum pH for each enzyme (pH 3.5 for galactosidase, pH 4.0 for arabinosidase), 50 mM NaCl, and 3 mM of the corresponding PNP derivative. The reaction mixture was incubated at 30°C before the addition of sample solution. After 20 min of incubation at 30°C, the reaction was terminated by the addition of 1.5 mL of 0.2 M Na_2CO_3. The activity was calculated from the amount of para-nitro phenol formed using the molar extinction coefficient of para-nitro phenol at 420 nm (4.8×10^3 M^{-1} cm^{-1}).

Pectinase

Enzyme activity of enzyme extracts from stage Ib and IV using native bean pectin as a substrate was tested using a purified green bean pectin (extracted with Na_2CO_3 at 4°C) containing 48% (mol %) GalUA, 41% (mol %) Gal, 10% (mol %) Ara, and 1% (mol %) Rha. Enzyme extract (1.0 mL) was incubated with 2.0 mL of 200 mM acetate buffer and 150 mM NaCl (pH 4.0) containing 30 mg of pectin for 16 h at 30°C. All samples were then analyzed qualitatively by HPSEC to determine if their molecular mass distributions had changed upon incubation.

RESULTS

Development of the Pods

Pod length was determined to monitor the overall development of green beans during the sampling period (Fig. 1). A previous study demonstrated that pods of green beans

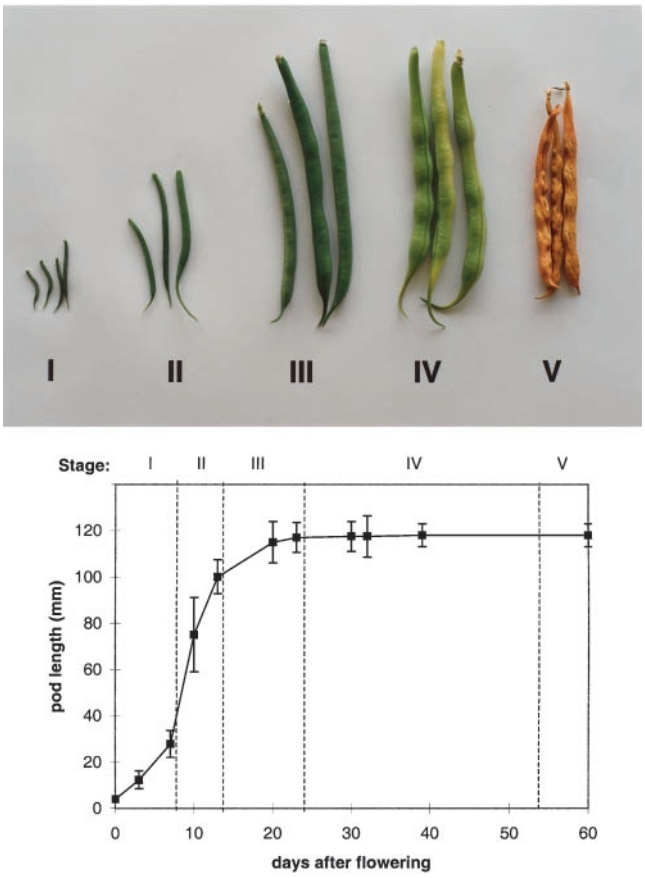

Figure 1. Appearance and average length of green bean pods during sequential developmental stages.

developed similarly on separate plants and during different seasons (Ebbelaar et al., 1996). The pattern of growth followed a single sigmoid curve. Development of bean pods was preceded by white flowers turning yellow. This yellow flower stage was assigned as 0 daf. Based on growth characteristics, the developing pods were classified intofive stages (Fig. 1). Stage I (0–7 daf): initial exponential growth phase, the seeds could not be separated from the pods during this stage. This stage was subdivided into two samples: 0 to 5 daf and 6 to 7 daf. Stage II (8–13 daf): linear growth phase, pods extended approximately 10 mm in length per day. This stage was subdivided into three samples: 8 to 9 daf, 10 to 11 daf, and 12 to 13 daf. From this stage on, the seeds could be separated from the pods and were discarded. Stage III (14–23 daf): cessation of pod elongation, resulting in pods averaging 117 mm in length. Stage IV (24–55 daf): "maturation," further development of seeds, degradation of the inner parenchyma tissue or seed cushion, pods start to turn yellow. Stage V (>55 daf): "senescence," dehydration and browning of the pods and, in some cases, spontaneous release of the mature seeds.

Composition of Bean Pods

During the first stages of pod development the water content increased and the AIR content declined (Table I). The AIR contains all of the high-molecular-mass components of the beans, including CWM, proteins, and starch. The initial decrease in AIR was mainly due to a strong reduction of protein during these stages. The proportion of the wall material on pod dry weight basis increased after stage IIb. In addition, there was an increase in starch content during stage IIb, IIc, III, and IV, followed by a strong reduction during senescence. All of this together resulted in a higher yield of AIR. During the last stage of development, i.e. senescence, the pods were dehydrated and contained less than 40% (w/w) water. The remaining dry matter contained much AIR, mainly consisting of CWM.

Cell Wall Composition and Changes during Pod Development

The sugar composition of the AIR was analyzed to obtain information about the overall features of the cell wall during development (Table I). The uronic acid was shown to be mainly (>98%) GalUA by HPLC analysis (data not shown). Major changes were detected in the pectic sugars Ara, Gal, and GalUA. The percentage of Ara in the AIR declined during pod development. The percentage of Gal also decreased, particularly after stage IIc. The GalUA content increased during exponential growth and during the last two stages, but remained constant during linear elongation of the pods (stages II and III). In contrast, Glc

Table I. *Composition of green bean pods during sequential developmental stages and overall changes in cell wall sugars*

Stage	daf	Water	AIR	Starch	Protein	CWM[a]	Fuc	Rha[b]	Ara	Gal	Glc[c]	Xyl	Man	GalUA	DM[d]	DA[e]
		mg g^{-1} pod fresh wt		*mg g^{-1} pod dry wt*			*mol % in AIR*									
Ia	0–5	867	539	10	257	243	1	2	17	27	27	8	4	16	62	6
Ib	6–7	891	529	9	219	251	1	2	13	27	25	7	3	24	51	9
IIa	8–9	911	518	12	175	272	1	2	10	25	24	9	5	25	40	8
IIb	10–11	912	463	33	111	260	1	2	9	24	28	7	2	30	45	8
IIc	12–13	913	557	87	101	325	1	2	7	20	37	6	3	26	49	8
III	14–23	913	620	100	101	396	1	1	6	18	40	5	3	27	58	8
IV	24–55	869	665	143	88	347	1	2	5	14	40	5	6	29	50	8
V	>55	379	857	20	68	474	1	1	5	10	37	6	7	34	74	9

[a] The amount of CWM was calculated from the total amount of cell wall sugars after hydrolysis of the AIR. [b] Rha was determined after TFA hydrolysis, the other neutral sugars with Seaman hydrolysis. [c] All Glc values have been corrected for contributions made by starch. [d] Mole percentage methyl to GalUA. [e] Mole percentage acetate to cell wall sugar residues.

Table II. *Yields of the fractions after extraction of the CWM from green bean pods during sequential developmental stages*

Developmental Stage	daf	Buffer	CDTA	Na_2CO_3 (4°C)	Na_2CO_3 (20°C)	0.5 M KOH		1.0 M KOH		4.0 M KOH	KOH/ Borate	Residue	Total
						Soluble	Precipitate	Soluble	Precipitate				
						mg g^{-1} pod dry wt							
Ia	0–5	2	23	32	33	25	93	3	23	19	3	20	276
Ib	6–7	13	26	41	35	20	79	5	33	13	2	37	304
IIa	8–9	10	28	40	39	28	54	6	18	16	3	34	276
IIb	10–11	13	23	37	28	14	25	6	10	14	2	32	204
IIc	12–13	18	26	45	33	27	25	4	11	21	3	62	275
III	14–23	27	31	52	33	40	30	4	2	20	4	63	306
IV	24–55	68	31	45	4	36	17	12	0	18	1	84	316
V	>55	22	113	70	7	39	0	3	1	13	2	94	364

percentages increased during the linear elongation phases (stages II and III). As the beans aged (stages IV and V), the levels of Man increased. The Fuc, Rha, and Xyl contents remained relatively constant throughout development.

A decrease in the degree of methylation (DM) of pectins was observed at the beginning of development (stage I to IIa), and was followed by an increase during elongation and senescence of the bean pods (Table I). The degree of acetylation (DA) was shown to increase rapidly to a level of 8% to 9%, calculated as the ratio of acetate to cell wall sugar residues. The DA was not calculated on a GalUA basis, because in addition to pectins, other cell wall components such as xylans and xyloglucans are also known to be substituted with acetyl groups (Carpita and Gibeaut, 1993).

Changes in Yield and Composition of the Pectic Fractions

During pod development there were differences in the amounts of material solubilized with the different chemical extraction methods, which indicated changes in bonding of the various cell wall polymers to each other (Table II). There was an increase in the amount of buffer-soluble material, from 0.2% to 7.0% of AIR, with a maximum during maturation (24–55 daf). The amount of CDTA-soluble material was constant during almost all stages except for a large increase during senescence. The amount of the 4°C Na_2CO_3-soluble fraction increased slowly during pod development, from 3.2% to 7.0% of pod dry weight. The amount of the 20°C Na_2CO_3-soluble fraction was approximately constant during growth, but was almost absent during maturation and senescence. All frac-

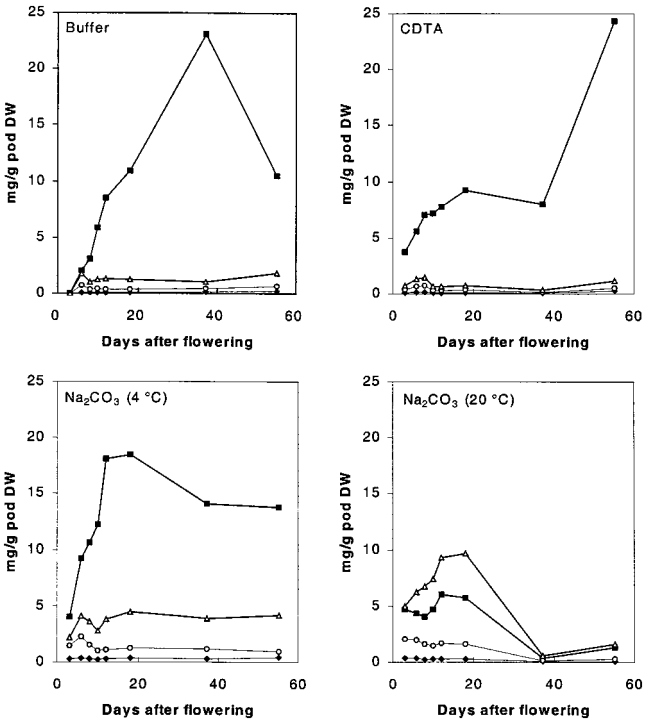

Figure 2. Yield and composition of pectic fractions from green bean pods during sequential developmental stages. ■, GalUA; ◆, Rha; ○, Ara; △, Gal. DW, Dry weight.

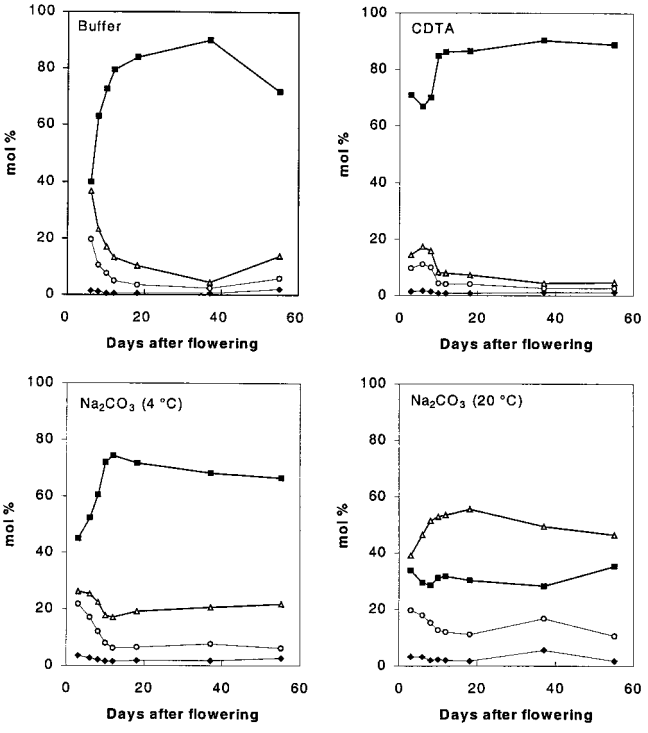

Figure 3. Molar composition of pectic fractions from green bean pods during sequential developmental stages. ■, GalUA; ◆, Rha; ○, Ara; △, Gal.

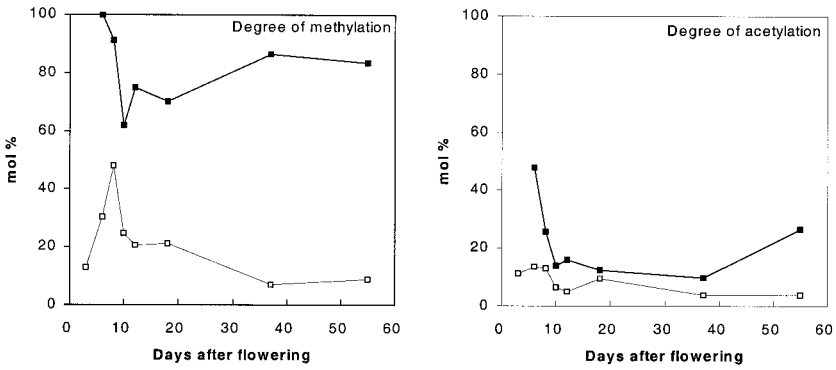

Figure 4. Degree of methylation (left) and degree of acetylation (right) of buffer-soluble (■) and CDTA-soluble (□) pectic fractions from green bean pods during sequential developmental stages.

tions also appeared to contain various amounts of protein and minor amounts of starch (data not shown).

Figure 2 shows the amounts of pectic sugars in each fraction during development. Figure 3 displays the mol % of the sugars, focusing more on the pectic composition, for each fraction during development. Both are presented because the yield of pectins in a fraction can change, while the composition of the pectins (mole percentage) in that particular fraction stays the same (e.g. CDTA-soluble fraction, 55 daf; 20°C Na_2CO_3-soluble fraction, 34 daf). The major changes in cell wall composition occurred during the first stages of development, i.e. pod elongation. GalUA content increased during these stages in all pectic fractions except the 20°C Na_2CO_3-soluble fraction. The increase in yield of the buffer-soluble fraction up to this stage was mainly caused by an absolute increase of GalUA (Fig. 2).

The Na_2CO_3-soluble fractions, especially the fraction extracted at 20°C, contained significantly more neutral sugars compared with the buffer and the CDTA-soluble fractions. The pectic sugars in both Na_2CO_3-soluble fractions decreased during maturation and senescence (Fig. 2). In addition to changes in sugar composition, there was also variation in the DM and DA of the pectins (Fig. 4). These values were determined only in the buffer- and CDTA-soluble fractions, since the esters are saponified during the alkaline extraction procedures. The DM and DA of the buffer-soluble pectins were initially high: 100% and 50%, respectively. During the linear growth stage they declined to 70% and 12%, respectively, but increased again slightly during maturation and senescence. For the CDTA-soluble pectins a different trend was noted, with the DM and DA being very low throughout development, about 10% each. During initial growth the DM of the CDTA-soluble fraction increased only temporarily to a value of 50%. On average, only 18% and 7% of the total cell wall methyl- and acetylesters, respectively, were recovered in these fractions.

Molecular Mass Distribution of the Pectic Fractions

The change in the molecular mass distribution of the GalUA-rich fractions during development was determined by HPSEC (Fig. 5). The peak appearing after 13 min in the CDTA-soluble fraction was caused mainly by residual CDTA present in the sample. The 20°C Na_2CO_3-soluble fractions were very difficult to dissolve, so the resulting elution patterns showed no clear peaks and are therefore not shown. The changes in molecular mass during development were comparable for the different pectic fractions. During exponential elongation (stage I), there were large amounts of low- and intermediate-molecular-mass mate-

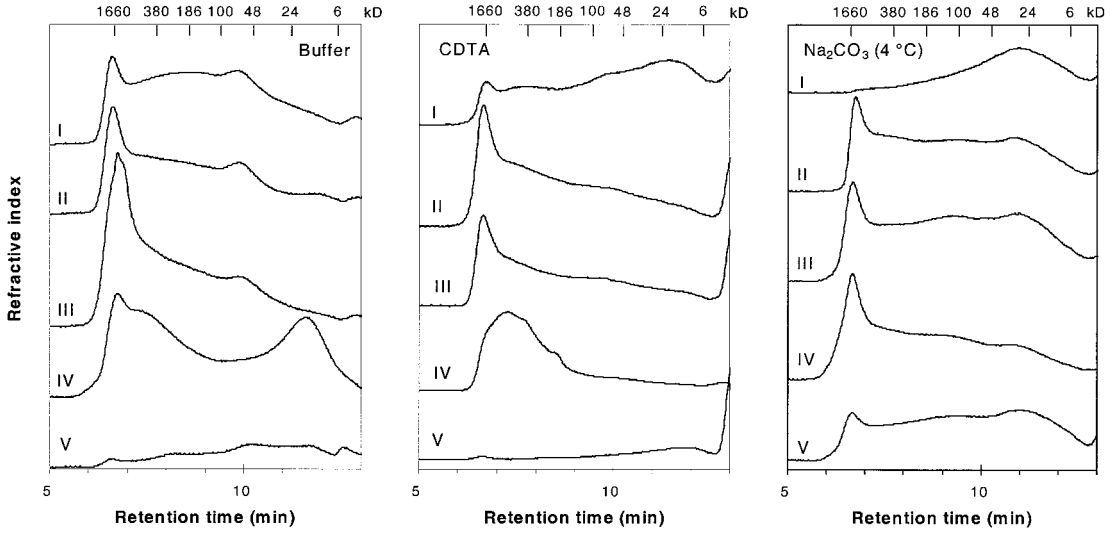

Figure 5. HPSEC elution patterns of various pectic fractions from sequential developmental stages (I–V) from green bean pods.

Table III. *Carbohydrate composition of the KOH-soluble fractions and residue after extraction of the CWM of green bean pods at stage III (cessation of elongation)*

Fraction	daf	Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalUA
					mol %				
0.5 M KOH soluble	14–23	0.2	0.8	8.6	26.4	49.0	6.0	1.0	8.0
0.5 M KOH precipitate	14–23	0.7	3.1	21.9	45.1	9.1	3.9	3.8	12.5
1.0 M KOH soluble	14–23	2.2	0.9	9.7	24.9	28.0	24.5	2.2	7.6
1.0 M KOH precipitate	14–23	0.8	3.1	18.7	42.3	12.5	5.3	1.6	15.8
4.0 M KOH	14–23	4.6	1.3	6.5	22.9	28.2	22.2	3.5	10.9
4.0 M KOH/borate	14–23	0.8	2.7	13.6	50.6	8.1	4.7	4.0	15.6
Residue	14–23	0.0	0.0	3.7	6.3	71.3	2.7	10.3	5.7

rial. During linear elongation and cessation of growth (stages II and III), considerable amounts of high-molecular-mass material were present, while during maturation (stage IV) the molecular mass decreased again. This was especially evident in the buffer-soluble fraction. At the senescent stage (stage V), all fractions were heterogeneous in molecular mass, resulting in a very low, broad peak.

Yield and Composition of the Hemicellulosic Fractions and Cellulose Residue

Overall yields of the various KOH-soluble fractions showed no clear trend (Table II). However, protein content decreased during development from 75% to 30% and 50% to 25% in the 0.5 M and 1.0 M KOH-soluble fractions (not shown). The protein content of the 4.0 M KOH-soluble fractions and the residue were invariably much lower, 8.9%, 7.0%, and 2.9%, respectively. The yields of the KOH-precipitate fractions, being the major fractions in stage I, declined during growth. The protein content of both the 0.5 and 1.0 M KOH-precipitate fraction was constant (average 65%). The amount of cellulose residue increased during growth and became one of the major cell wall fractions after pod elongation stopped (stages IV and V).

With the exception of the 0.5 M KOH extraction, which solubilized appreciable amounts of pectin, the sequential KOH extractions solubilized a range of hemicellulosic polymers and small amounts of acidic polymers (Table III). The composition of the fractions was quite stable throughout development, so only the data from stage III are presented. The precipitates contained significant amounts of Ara, Gal, and GalUA. From the molar proportion of the sugars in the 1.0 and 4.0 M KOH fractions, the major hemicellulosic component can be inferred to be a xyloglucan (O'Neill and Selvendran, 1983, 1985). In the 1.0 M KOH-soluble fraction the proportion of Xyl was relatively high, especially in the final stage, indicating that this fraction most likely also contained some xylans. (1→4)-Linked Xyl residues, typical for xylans, were detected in the cell walls of green bean in an earlier study (Stolle-Smits et al., 1995). The 4.0 M KOH/borate-soluble fraction contained mainly Ara- and Gal-rich polymers. As expected, the residue consisted mainly of Glc, but, interestingly, also contained appreciable amounts of Man in the final stage.

Enzyme Activities during Development

The specific activities (nanokatals per milligram of protein) of the pectin-modifying enzymes that we tested for (except PG) were in all cases highest in the extracts from senescent bean pods (Table IV). To estimate the effect of the enzymes during development, the enzyme activities were also calculated on basis of CWM (nanokatals per milligram of CWM), with the cell wall being the potential substrate in vivo. In contrast to the specific activities, the activities on basis of CWM were not only relatively high during senescence but also during the initial stages of pod development (<10 daf).

The highest activity during all developmental stages was found for PME. The specific activity of PME increased from 20 to 80 nkat mg^{-1} of protein during development. β-Galactosidase, α-arabinosidase, and POD, which plays a

Table IV. *β-D-Galactosidase (β-Gal), α-L-arabinosidase (α-Ara), PME, PG, and POD activities in green bean pods during sequential developmental stages*

The activities are expressed on basis of protein and on basis of CWM, its potential substrate in vivo.

Developmental Stage	daf	α-Ara		β-Gal		PME		PG		POD	
		Protein	CWM	Protein	CWM	Protein	CWM	Protein	CWM	Protein	CWM
						nkat mg^{-1}					
Ia	0–5	0.7	0.22	1.3	0.41	16.3	5.1	0.016	0.0059	8.6	2.7
Ib	6–7	0.8	0.23	1.6	0.46	19.2	5.5	0.015	0.0044	6.9	2.0
IIa	8–9	0.9	0.18	2.1	0.44	25.8	5.4	0.017	0.0056	7.4	1.5
IIb	10–11	1.0	0.11	3.1	0.36	32.0	3.7	0.010	0.0011	9.1	1.1
IIc	12–13	0.8	0.08	3.3	0.30	30.4	2.8	0.019	0.0017	8.5	0.8
III	14–23	0.8	0.05	2.7	0.21	22.3	1.8	0.015	0.0012	6.7	0.5
IV	24–55	1.4	0.03	6.5	0.14	33.4	0.7	0.014	0.0003	10.1	0.2
V	>55	6.5	0.13	29.0	0.68	80.5	1.9	0.020	0.0005	13.6	0.3

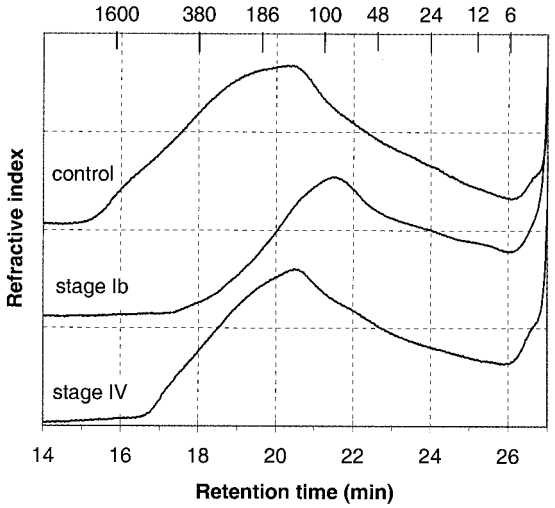

Figure 6. HPSEC elution patterns of green bean pectin after digestion for 16 h at 30°C with enzymes extracted from exponentially elongating (stage Ib) and maturing (stage IV) green bean pods. The control represents the elution pattern of the green bean pectin after incubation for 16 h at 30°C without the addition of enzymes.

role in the cross-linking of pectins and cell wall proteins by catalyzing the formation of phenolic-coupling activity, were also present throughout pod development. β-Galactosidase specific activity increased the most (>20-fold) during development. PG activity was detectable only in extremely low amounts (10–20 pkat mg^{-1} of protein) during all developmental stages. Nevertheless, small levels of *endo*-PG activity can have a large effect on the chain length of pectins. HPSEC analysis of HGA before and after incubation with the enzyme solution showed no shift in molecular mass distribution (not shown), which implied that the observed low activity did not represent *endo*-PG activity, but may have been caused by *exo*-PG activity.

To test for pectin-degrading activities, the enzyme extracts from stage Ib (exponential growth) and stage IV (maturation) were tested against a native 4°C Na_2CO_3-soluble pectic fraction from green bean. HPSEC elution patterns of these enzyme digests are shown in Figure 6. The peaks of the digested samples had shifted to a lower molecular mass. In particular, the enzyme preparation from exponentially growing bean pods (stage Ib) contained significant pectin-degrading activity. The observed results suggest the presence of another pectic-degrading enzyme such as rhamnogalacturonase or pectate lyase, since the shift in retention times could hardly have been the result of only side chain degradation and endo-PG activity was not detected by HPSEC.

DISCUSSION

The main change in cell wall composition during the development of green bean pods was a change in pectic constituents. Very young, exponentially growing cell walls contained mainly neutral, sugar-rich pectic polymers (rhamnogalacturonan) that were water insoluble and relatively tightly connected to other cell wall components. During linear elongation, additional Gal-rich pectic polymers were deposited. Since the amount of Gal increased mainly in the 20°C Na_2CO_3-soluble fraction (Fig. 2), they were probably cross-linked to other cell wall polymers by ester linkages. Apart from this, the level of neutral, sugar-rich pectins (rhamnogalacturonan) remained relatively constant, while the level of HGA increased steadily. Concurrently, the molecular mass of all of the pectins increased. During the early developmental stages, the pectinase activity was relatively high, suggesting that in addition to synthesis there was also degradation of regions of pectin (Table IV; Fig. 6).

Gal turnover is suggested to be important in prolonging auxin-induced expansion (Brett and Waldron, 1990). During maturation almost 50% of cell wall pectins could be solubilized with buffer and were thus most likely just held in the wall by physical entanglement (Fig. 2). The 20°C Na_2CO_3-soluble, Gal-rich pectic polymers at were degraded, while the accumulation of HGA continued. Interestingly, the mole percentage of the various pectic fractions did not change very much. This was surprising, since the 20°C Na_2CO_3-soluble pectins consisted of more than 50 mol % of Gal, and if they were solubilized, one would expect that some of this Gal would end up in the more soluble fractions. However, this Gal was not recovered in the buffer or in the CDTA-soluble fraction. This could have been the result of *exo*-galactanase action, with the resulting Gal monomers being lost during preparation of the AIR and dialysis of the cell wall fractions. Solubilization of galactan has been demonstrated to be a general feature of ripening fruit such as tomato, mango, apple, and kiwi (Seymour et al., 1990; Redgwell et al., 1992; MacLachan and Brady, 1994; Yoshioka et al., 1994; Muda et al., 1995). In nectarines, a decreased degradation of galactan side chains was was associated with the development of mealy fruit (Dawson et al., 1992).

During senescence there was an increase in the amount of CDTA-soluble pectins, mainly at the expense of buffer-soluble pectins. This was accompanied by a decline in molecular mass of the buffer- and CDTA-soluble pectins. This is comparable to results found for many ripening fruits, such as kiwi, nectarine, and melon (Dawson et al., 1992; Redgwell et al., 1992; Rose et al., 1998). The increase of CDTA-soluble pectins during senescence can be explained by overall termination of polymer synthesis, with ongoing demethylation of cell wall pectin by PME, which was shown to remain active (Table IV). The demethylated pectins could subsequently become Ca^{2+}-complexed to each other and thus become extractable with CDTA.

There was no clear relationship between PME activity and the average DM of the pectin during development (Table I and IV). This was also observed for mung bean, tomato, and green bean in a previous study (Ebbelaar et al., 1996). However, after comparison of the DM of buffer- and CDTA-soluble pectins, it is obvious that overall DM values provide very little information about the status of the different pectins in the cell wall in vivo (Fig. 4). The average

DM of the total cell wall (Table I) varied only slightly and was about 50% throughout development. The DM of buffer-soluble and CDTA-soluble pectins, however, showed great variation during initial growth and reached extreme values of, respectively, 80% and 10% after elongation had ceased (Fig. 4). From these data it is clear that in order to understand the way in which PME is involved in development, more information is needed about the localization of the different types of pectin and their DM values.

The changes in hemicellulose during pod development mainly concerned an increase in cellulose content at the end of the elongation phase and a small shift from xyloglucans to more xylans and mannans during maturation and senescence. In addition, there was a decreased amount of precipitate formed during neutralization of the KOH-soluble fractions. The KOH precipitates most likely contained highly branched pectins, as could be deduced from the high levels of pectic sugars and from the high Rha/GalUA ratio. In the present study there was no clear change in xyloglucan and xylan in the bean pods during development. Chanda et al. (1995) reported a decline in 4 M KOH-soluble xyloglucans and concluded that depolymerization of xyloglucan was involved in cell wall loosening during elongation of green bean hypocotyls. Our data do not support their observation, since no significant change in the 4.0 M KOH-soluble fraction was detected. The cellulose content of the pods increased during the later stages of pod elongation and during maturation and senescence (Table III). During maturation and senescence this was accompanied by an increase in Man content, probably originating from glucomannans that occur in moderate amounts in certain secondary cell walls and can crystallize to cellulose (Fry, 1988).

The results of the present study suggest that there is a constant synthesis and degradation of the CWM during pod elongation, with a shift from neutral, sugar-rich, branched pectins (rhamnogalacturonan) to the synthesis of non-branched, high-molecular-mass pectins (HGA) as elongation proceeds. From our results it can be hypothesized that during expansion the cell wall network is probably filled with HGA, while the structure is locked by branched, Gal-rich pectic polymers (rhamnogalacturonan). The synthesis and cross-linking of neutral pectic side chains seems to halt as elongation ceases, while the synthesis of methylated linear pectins appears to continue until early senescence. Cell walls of senescent pods consist mainly of cellulose and relatively linear, ionically linked pectins.

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