Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 65 (2006) 165–173
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Changes in esterified pectins during development in the flax stems and leaves

L. Bédouet [a,*], E. Denys [b], B. Courtois [b], J. Courtois [b]

[a] *Muséum National d'Histoire Naturelle, USM 0401, 7 rue Cuvier, Paris F-75231, France*
[b] *Laboratoire des Glucides, EPMV CNRS FRE 2779, IUT d'Amiens, Département de Génie Biologique, Avenue des Facultés, Le Bailly F-80025, France*

Received 14 September 2005; received in revised form 7 December 2005; accepted 28 December 2005
Available online 10 March 2006

Abstract

Winter oil flax species (*Linun usitatissimum* L) seedlings were harvested along the development at the dormancy, elongation, maturation and senescence periods, and pectins in stems and leaves were extracted in acid and analysed. The monosaccharides analyses indicated presence of the specific sugar of pectins in the extracts. Co-extraction of arabinogalactan polysaccharides with pectins was possible whereas extraction of hemicellulose material seemed unlikely in regard to the absence of xylose and fucose. The yields of pectins extraction from the stems changed over time and decreased along the development, 7% at the dormancy stage and 2% at senescence, whereas the leaves led to constant yields of pectins over ageing (~4%). In the stems, pectins incorporated galacturonic acid as maturation process, from 53% at dormancy to 66% during senescence. In the leaves, the pectins appeared more acidic than in the stems, and changes along development in composition and size were not significant. During development, methylation of acid-soluble pectins was high (80–100%) and varied moderately, whereas the degree of acetylation was important (23–40%) and increased slightly. Globally, modifications of pectins from leaves were more limited compare to stems, and mild acid extraction indicated that high molecular weight esterified pectins are present or still synthesised in flax until senescence.

Keywords: Acetylation; Flax; Homogalacturonan; *Linun usitatissimum* L; Methylation; NMR; Pectins; Rhamnogalacturonan

1. Introduction

The cell wall is a dynamic structure where a coordinated series of biochemical processes including biosynthesis, changes and degradation of components occurs (Ridley, O'Neill, & Mohnen, 2001). It has been shown that the main changes in the cell wall composition during the development of the green bean pod concerns pectins rather than hemicellulose material. The size of pectins increased together with the modifications of the degrees of methylation and acetylation. As elongation proceeds a shift from ramified RG-I to the synthesis of non-branched homogalacturonan was noted (Stolle-Smits et al., 1999).

Different pectic components were characterised along flax development. In the hypocotyls of 3-day plantelets, ramified and highly acetylated RG-I, short low methylated homogalacturonans, free arabinans and galactans were isolated upon CDTA extraction (Rihouey et al., 1995). Acidic pectins were mainly found in the intercellular junctions and in the middle lamella whereas neutral pectins were found in the primary cell wall. During the development, the parenchymal cells of the phloem differentiate leading to the formation of long fibres of cellulose. At maturity, fibres become organised as bundles and hold together by an interstitial pectic cement which represent 26% of the fibres total mass (Morvan, Abdul Hafez, Morvan, Jauneau, & Demarty, 1989). Additional structural information on flax pectins was obtained with partialy degraded pectins after the dew-retting process in the field. The telluric micro-organisms led to the sequential removal of the methyl esters, the GalA residues of the smooth homogalaturonan, and finally the arabinosyl and galactosyl residues (Morvan, Jauneau, Morvan, Voreux, & Demarty, 1989). In the retted fibres, β-galactans and RG-I like polysaccharides substituted with short galactosyl side chains were characterised (Davis, Derouet, & Hervé Du Penhoat, 1990). Soluble β-galactans were found to be localised specifically in flax stem at a region called the 'snap point' (transition point from cell elongation to cell wall thickening). They progressively declined during the development, they were either degradated or fixed within the cell wall (Gorshkova et al., 2004).

Abbreviations Ara, arabinose; BSTFA/TMCS, bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane; CDTA, *trans*-1-2-diaminocyclohexane-*N*,*N*,*N*′*N*′-tetraacetic acid; DA, degree of acetylation; DM, degree of methylation; Gal, galactose; GalA, galacturonic acid; Glc, glucose; *m*-HBP, *meta*-hydroxydiphenylphenol; NS, neutral sugar; Rha, rhamnose; RG-I, rhamnogalacturonan I; UA, uronic acid.

* Corresponding author. Tel.: +33 1 40 79 37 02; fax: +33 1 40 79 56 20.
E-mail address: bedouet@mnhn.fr (L. Bédouet).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2005.12.041

For the present study, flax plants of an oil flax variety sowed in open field were harvested at four periods of the life cycle: winter dormancy, growth, maturation and senescence. A particular attention was paid to the esterification degrees of pectins. Alkaline conditions of extraction using NaOH or carbonate buffer were avoided. The esterified pectins from the stems and the leaves were extracted in mild condition using hot diluted acid. Such treatment led to an efficient release of esterified pectins from apple (Renard, Voragen, Thibault, & Pilnik, 1990). The treatment of the cell wall material with HCl is a mild acid hydrolysis, to which glycosidic linkages involving arabinofuranosidic residues are particularly sensitive. Then, the sugar composition, size and degrees of esterification of flax pectins were measured and a comparison between the pectins from the stems and the leaves was undertaken.

2. Materials and methods

2.1. Material

A winter oil flax *(Linun usitatissimum* L*),* the variety Oliver was sowed at a density of 400 plants/m^2 at the INRA station of Estrées-Mons (Somme, France). Flax samples, approximatively 200 plants, were harvested at four steps of the development: in January during the 'Dormancy stage', in March at the beginning of the 'Growth stage', in June during the flowering time, the 'Maturation stage' and in July at 'Senescence stage'. Samples were washed under water, roots were removed and the leaves were detached from the stems. Leaves and stems were frozen and lyophilised during 1 week before being stored at 40 °C.

2.2. Extraction of the pectins

Dried flax stems and leaves (5 g) were powdered and grinded to powder in 50 mM sodium acetate buffer, pH 4.5 (200 ml) for 1 min at 4 °C using a domestic liquidiser. After filtration, through a Whatman filter (no. 3), the insoluble fraction was washed twice (10 min) with 200 ml of 1:1 (v/v) chloroform/methanol solution. Then, 100 ml of 50 mM HCl was added to the fraction and the mixture was incubated during 10 min at 70 °C under magnetic stirring. The solution was then cooled and the pH adjusted to 5 with 1 M NH_4OH. Upon centrifugation (10 min, 12,000*g*, 15 °C), the supernatant was recovered and 3 vol of ethanol were added. After overnight incubation at 4 °C, the pectins were collected upon centrifugation (10 min, 12,000*g*, 4 °C). The pellets were solubilised in water and the pectin solutions were stored at −20 °C. These extracts were named HCl 1. A second extraction of pectins (20 min at 70 °C) was performed using the insoluble fraction obtained after the first extraction. This second pectin extract was named HCl 2. The HCl 1 extracts were used for biochemical characterisation (neutral sugars composition, size-exclusion chromatography and esterification analyses) because they contained major part of the total acid-soluble pectins.

2.3. Sugar assays

Uronic acids and neutral sugars contents were determined by using *meta*-hydroxydiphenylphenol (Van den Hoogen et al., 1998) and resorcinol (Monsigny, Petit, & Roche, 1988) micromethods performed on micro-titration plates (Nunc, Maxisorp). L-Ara (Sigma) and D-GalA (Sigma) were used as standards. The uronic concentration was determined with the *m*-HBP test. Under the present conditions, there was no interference between neutral sugar in the *m*-HBP test. The neutral sugar content was calculated after correction due to the interference of uronics (30–50%) in the resorcinol assay. The assay performance was tested using known mixtures of larch wood arabinogalactan (Sigma) and sodium polygalacturonate (Sigma), and errors of 5 and 8% were found for the determination of neutral sugars and uronic acids, respectively. These errors concerned the correction for the uronics interference and the different responses of arabinose and galactose in the resorcinol test.

2.4. Low pressure size-exclusion chromatography

Size fractionation of pectins was performed at room temperature using a glass column (100×1 cm) containing Sephacryl S-400 gel (Pharmacia) at a flow rate of 6 ml/h in 50 mM sodium acetate buffer, pH 5, 5% ethanol. The exclusion and inclusion volumes were estimated using amylopectin and GalA, respectively. Three milliliters of non-lyophilised pectin containing extract were injected onto the column. Fractions of 2 ml were collected and were five times concentrated in a speed-vac. The elution patterns of uronic acids and neutral sugars were drawn after the assays had been performed in duplicate using 40 μl of the concentrated fractions. The yields of recovery of pectins following the chromatography separations were around 75%.

2.5. Sugar composition determination of pectin extracts

The protocol was adapted from Quemener and Thibault (1990). Pectins were digested with pectinase containing an endopolygalacturonase activity (Sigma) (0.1 U/mg of pectin) for 24 h in 50 mM pyridine acetate, pH 5 at 37 °C. The product was lyophilised, and methanolysis in 0.5 M HCl (Methanolic instant kit, Alltech) was performed onto the hydrolysates for 10 h at 80 °C with mesoinositol (Sigma) as internal standard. The methanol/HCl solution was evaporated under argon and *O*-methyl-glycosides were derivatised at 4 °C in pyridine with BSTFA/1% TMCS solution (Alltech). Silylated derivatives were injected in splitless mode on a HP 1 capillary column (25 m×200 μm) of a HP 6890 chromatograph (Hewlet–Packard).

2.6. NMR analyses

^1H experiments were analysed on a Bruker Avance 300 spectrometer operating at a frequency of 300 MHz. Dry pectins samples (5 mg) were dissolved in D_2O (Sigma) at 10 mg/ml

concentration. The proton chemical shifts were determined from the ^{1}H NMR experiments using the conventional pulse sequences provided by Bruker. The spectral window was 3000 Hz for 8 k data point with a pulse of 7 μs, an acquisition time of 1.36 s and a relaxation delay of 1 s. A number of 256 scans were recorded.

2.7. Pectin esterification analyses

After a first recording at 80 °C, in order to check the absence of free methanol and acetate, 55 μl of 1 M NaOD (Sigma) dissolved in D_2O were added in the NMR tube and 256 scans were recorded. Quantification of methanol and acetate was done relative to the H-4 of GalA after three manual integrations (Bédouet, Courtois, & Courtois, 2003). During the analyses (~20 min), a 3% loss of methanol was noted. The accuracy of the method was validated using a standard pectin from citrus (Sigma P-9561) with a degree of methylation of 72% (Mannes, Ryan, & Mort, 1990). The NMR method gave a value of 71 ± 0.6% for the methyl-esterification degree of the control pectin sample.

3. Results

3.1. Evolution of stems and leaves weight during growth

The flax plants consist of a central stem with two lateral ramifications bearing leaves. At the senescence period, stems were broken during the pulling up and they were dehydrated (45% of water content) and looked like to straw without leaves (Table 1). The ratio between the leaves and the stems progressively decreased during the growth, leaves represented 66% of the weight of the plants at the dormancy stage, and they disappeared after the maturation period. The stems weight increased 20 times from the dormancy period to the maturation one.

3.2. Pectin extraction yields

It was verified that the acidic condition of the extraction slightly degraded the neutral oligosaccharides. Thus, from the larch arabinogalactans incubated during 30 min at 70 °C in 50 mM HCl, less than 0.5% (w/w) of Ara and Gal were liberated. This limited removal of neutral monosaccharides did not impaired the further analyses of the acid-soluble extracts. Furthermore, incubation of sodium polygalacturonate in 50 mM HCl at 70 °C with methanol and acetic acid during 10 min did not esterify the GalA residues (data not shown). The acidic medium did not lead to the release of methanol and acetate as shown after incubation of a flax pectins sample with 50 mM of DCl at 70 °C in an NMR tube (data not shown).

Table 1
Evolution of the stems and the leaves for one flax plant during the growth

Stages of flax development	Dry weigh of the stem (mg)	Dry weigh of the leaves (mg)	Ratio leaves/stems
Dormancy (198)[a]	34[b] (80%)[c]	63[b] (83%)	1.85
Growth (628)	80 (80%)	100 (80%)	1.25
Maturation (118)	702 (70%)	167 (80%)	0.23
Senescence (100)	676 (45%)	No leaf	0

After the pulling up, leaves and stems were separated, washed and lyophilised. The roots were removed. The dried organs were weighted and stored at 40 °C.
[a] Number of the plants recovered.
[b] Dry weight values obtained for 1 plant.
[c] Content in water.

3.2.1. Pectins from the stems

Pectin extraction ratio after the first 10 min of incubation was twice higher than after the second treatment of 20 min (Table 2). At the dormancy stage, it was observed that a third acid treatment of 20 min gave only half of the second pectin extract amount (data not shown). Consequently, only two successive extractions were done in routine.

The yield of extraction declined after the growing period of flax. By adding the pectins of the first and the second extract, a yield of 7.3% was obtained for the dormancy stage, 4.6% for the growth stage, 2.5% for the maturation stage and only 2.1% for the senescence stage. The decrease in the amount of pectins extracted from the stems along the development, on a dry weight basis, can result from synthesis of other components such cellulose and lignins with ageing, or to the pectins cross-linking with cell wall materials.

3.2.2. Pectins from leaves

A yield of 4.1% was obtained at the dormancy period, 4.5% at the growth stage and 3.5% at maturation. For every stages, acidic sugars were approximatively twice more abundant than neutral sugars. About 60% of the extracted pectins were obtained after the first 10 min of extraction.

3.3. GalA content of pectins

No glucuronic acid was detected in the pectin extracts after pectinase digestion followed by methanolysis. GLC analyses

Table 2
Yields of pectin extraction and quantification of the uronic acid and neutral sugars in the pectin extracts

	HCl extract	Neutral sugar (mg)	Uronic acid (mg)	Yield of pectin (%)
S-dormancy[a]	1	23.8	25.9	4.9
	2	8.4	16.3	2.4
S-growth	1	14.7 ± 1	20.6 ± 1.4	3.4
	2	2.9 ± 0.4	10.3 ± 2	1.2
S-maturation	1	5.4 ± 0.8	10.4 ± 1.4	1.6
	2	2.4 ± 0.1	6.5 ± 2.2	0.9
S-senescence	1	5.8 ± 1	11.2 ± 0.3	1.5
	2	1.3 ± 0.1	5 ± 1.3	0.6
L-dormancy[a]	1	12.2	20.4	3.2
	2	3.4	5.5	0.9
L-growth	1	11.2 ± 0.4	23 ± 0.1	3.4
	2	3.7 ± 0.2	7.5 ± 0.05	1.1
L-maturation	1	6.1 ± 0.1	16.9 ± 1.5	2.2
	2	2.7 ± 0.1	10.5 ± 3.2	1.3

The sugar content in the extracts (S, in stems; L, in leaves) were determined according to resorcinol and *m*-HBP micro-methods. The yield of extraction was calculated as the total extracted sugar mass (UA + NS) per gram of material (dry weight). Arabinose and galacturonic acid were used as standards. Values are the mean of two independent extractions.
[a] One extraction was performed due to the sacrcity of material.

Table 3
Monosaccharides composition in stem and leaf pectin extracts at different stages of plant development

	Pectins of stems				Pectins of leaves		
	Dormancy	Growth	Maturation	Senescence	Dormancy	Growth	Maturation
Uronic acid	53	59±4	66±8	66±1.7	62.6	67.3±0.3	74±6
Neutral sugars (%)	47	41±2.7	34±5	34±5.8	37.4	32.7±1.1	26±2.6
ara (%)	19.2±1.1	15.2±0.6	11.5±1.5	10.5±0.4	10.5±1.3	10.4±0.1	ND
rha (%)	3.9±0.6	3.4±0.1	2.4±0.2	4.4±0.4	4.5±0.7	3.9±0.2	
Gal (%)	19.7±1.3	18.8±0.1	17.2±0.6	16.2±0.5	18.3±0.1	15.1±0.9	
Glc (%)	3.7±0.7	2.2±1	2.9±0.1	2.7±0.1	3.9±0.2	3.7±0.1	

The uronic acid content was determined using the *m*-HBP micro-colorimetric test and neutral sugars using a resorcinol micro-assay, after correction of UA interference. The neutral sugars composition was determined by GLC after pectinase pre-digestion followed by methanolysis (10 h at 80 °C in 0.5 M methanolic HCl) and trimethylsilylation. Values are the means of two experiments. S, stems; L, leaves; ND, not determined.

after trimethylsilylation led to the identification of the four isomers of the GalA indicating that the acidic sugars detected in the *m*-HBP colorimetric assay corresponded to GalA. According to the colorimetric assay, pectins in the stems became more acidic during ageing with 53% of GalA at the dormancy period to 66% at the maturation and senescence stage (Table 3). For the leaves, the GalA concentration in the HCl 1 extracts was stable (62 and 67%) at the dormancy and the growth periods and increased moderately up to the maturation stage (74%).

3.4. Neutral sugars composition

The centesimal compositions indicated that sugars specific of pectins were present in the stem and leaf extracts, i.e. Ara, Rha and Gal (Table 3). Traces of Glc could have come from a contamination from some residues of starch. No xylose or fucose was identified, indicating that hemicellulose was not solubilised during the acidic extraction, even during the second acid extraction of 20 min (data not shown).

3.4.1. Pectins from the stems

The relative proportion in neutral sugars changed over time, Ara decreased, from 19.2% at dormancy down to 10.5% at the senescence stage. The diminishing of the Gal proportion was less important, from 19.7 to 16.2%. The level of Glc was stable, with an average of 2.8% of the neutral sugars. The Rha content in the pectin extract was low, averaging of 3.7% of the neutral sugar at the dormancy stage. However, the proportion of Rha changed with time, it decreased at the maturation stage (2.4% of sugars) and increased again at the senescence stage (4.4%). A synthesis of rhamnogalacturonans before the senescence and after the maturation stages seemed to occur.

3.4.2. Pectins from leaves

Glc content was higher (~4% of the neutral sugars) in the leaves than in stems. This could be due to a contamination with starch which could be more concentrated in the leaves. Contamination with xyloglucans seemed unlikely due to the absence of xylose residues determined after monosaccharide analyses. Over the two first periods, the relative proportion in sugars did not change: Ara was stable (10% of the neutral sugars) and Gal slightly decreased (from 18.3 to 15.1%). For Rha, a faint decrease was observed, the same as for the stem pectins.

3.5. Size-exclusion chromatography analyses

Analyses were performed with non-lyophilised extracts. Indeed, the lyophilisation induced the formation of high molecular weight complexes whatever the sample investigated (data not shown).

3.5.1. Pectins from the stems

The low pressure size-exclusion chromatography performed on a S-400 column indicated that the size of pectins increased parallel to the development periods (Fig. 1). In the young stems, small and heterogeneous pectins composition were evidenced. Colorimetric assay indicated that uronic acids were associated in high molecular weight polysaccharides, whereas neutral sugars were rather eluted in the low molecular weight region. However, neutral sugars in lower amounts were also found in the high molecular weight fractions, they could correspond to free neutral polysaccharides or to the lateral chains of pectins.

At the start of the growth, an increase in the molecular weight was observed with a closer co-elution of uronic acids and neutral sugars. However, an important polydispersity still remained. The change in molecular mass of the pectins may correspond to the elongation of the polygalacturonic core rather than to the addition of neutral sugars.

After the growth period, acid and neutral sugars were gathered in a few fractions associated together as high molecular weight polysaccharides leading to the formation of a unique peak (68% of the sugars). The remaining sugars (32%) tailed up to the total volume of the column. Thus, at the maturation period, the stems contained an homogeneous population of pectins in regard to the size. Degradation of pectins seemed to be blocked at this stage.

At the senescence period, a more heterogeneous pattern was observed which could be correlated with a limited degradation process. Neutral sugars appeared to be removed from the high molecular weight pectins, and were recovered in low molecular weight fractions. The presence of uronic acids in intermediate and in low molecular weight fractions can indicate the beginning of fragmentation of large acidic pectins.

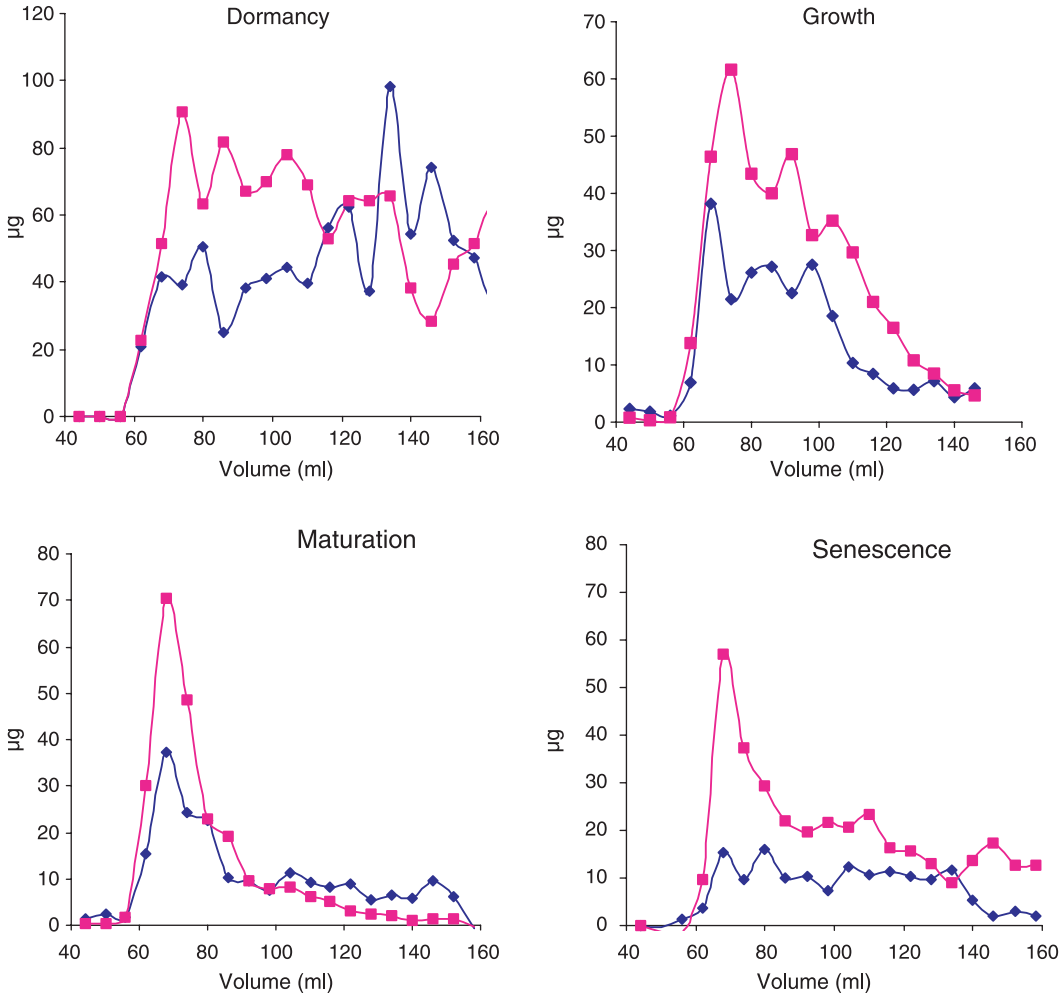

Fig. 1. Size-exclusion chromatography of the pectins extracted from the stems. Non-lyophilised pectins (3 ml) of the HCl 1 extracts were injected on a Sephacryl S-400 column (100×1 cm). Elutions were performed in 50 mM sodium acetate buffer, pH 5 (5% of ethanol) at a flow rate of 6 ml/h. Sugar assays were performed in duplicate on 40 µl in micro-titration plates. Elution of neutral sugars were drawn after correction due to the interference of the uronic acids (—◆—, NS; —■—, UA).

3.5.2. *Pectins from leaves*

At every stage when leaves are present, low-, intermediate- and high molecular weight acidic and neutral polymers were eluted (Fig. 2). At the growth stage, more high molecular weight material appeared but an important heterogeneity remained. At the maturation stage, less size heterogeneity was observed with the presence of high molecular weight pectins. At each period, a close co-elution between neutral and acidic sugars was noted.

3.6. ^{1}H NMR analyses

Extracts recovered from the stems during the first extraction step (HCl 1 extract) were easily analysed by ^{1}H NMR at 80 °C (Fig. 3). Unresolved spectra were obtained with the second extracts (HCl 2). On the other hand, no well resolved spectra for the pectins of leaves were recorded probably due to an important viscosity (data not shown).

At each stage a complex zone was observed in the low field region (5.2 and 4.7 ppm), which probably contained the H-1 and H-5 of methyl esterified GalA (Grasdalen, BakØy, & Larsen, 1988) and the H-1 of the α-Ara residues (Fig. 3A). An acetylation area composed of signals from 2.04 to 2.11 ppm, a methyl ester at 3.76 ppm, and the doublet of β-Gal ($J^{3}_{1,2} = 7.2$ Hz) which resonated at 4.51 ppm were identified. Methyl groups of rhamnosyl residues were not detected at 1.2 ppm confirming the low content in rhamnose determined during monosaccharide analyses (Table 3).

Identification of acetyl and methyl esters was confirmed upon saponification with 100 mM NaOD directly in the NMR tube (Fig. 3B). The action of NaOD led to the immediate release in the medium of methanol (3.28 ppm) and sodium acetate (1.82 ppm). Furthermore, the analysis of the leaf pectins from the leaves after NaOD addition was made possible since the saponification suppressed immediately the samples viscosity allowing the recording of spectra.

The degree of substitution of the stem and leaf pectins were measured, taking the GalA residues as reference, by NMR after saponification had been performed at 80 °C. The accuracy of the method was verified with a standard citrus pectin (Bédouet

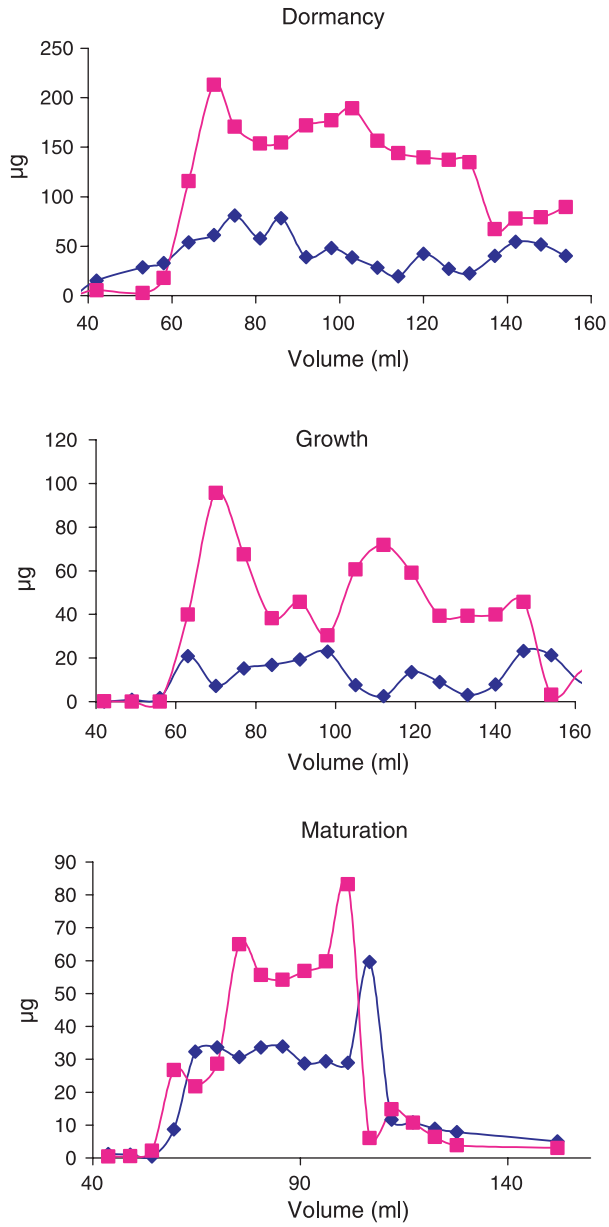

Fig. 2. Size-exclusion chromatography of pectins extracted from the leaves. For conditions see Fig. 1.

et al., 2003). The direct quantification of methanol and sodium acetate relative to the H-4 (4.34 ppm) of GalA was possible since no signals from other components resonated in this area. Values of DM and DA indicated that esterification of flax pectins was important at the four periods of the development, even after plants had stopped their elongation (Fig. 4).

3.6.1. Pectins from the stems

The pectins DM greatly increased when the plants grow up (103% at the growth stage) and moderately decreased after the plants had stopped their growth (83% at the maturation stage). The methylation level was stable in pectins extracted from dead plants (80.5% at the senescence stage) but higher than the value determined for the pectins extracted at the dormancy period (72%), when the growth was blocked. Pectins acetylation also increased from the dormancy period (23.5%) to the growth stage (32.3%), when the development of the plant was active. After the growing period, the DA was stable at maturation (34%) and even at the senescence period (30%).

3.6.2. Pectins from leaves

The DM also varied as observed for the pectins from the stems, 83% at the dormancy period up to 100% at the growth and maturation periods. The DA evolved from 31% at dormancy up to 40% at the maturation stage.

4. Discussion

This study report that the pectins present in the stems and in the leaves of the same plant of flax can be different in several features. Flax acid-soluble pectins underwent changes along the development of the plants: in the stems, the size of pectins synthesised increased with the ageing of the plant, whereas evolution of pectins extracted from leaves was more limited. The acid-soluble pectins from stems and leaves displayed important degrees of esterification, which changed moderately over time.

The switch between low molecular weight pectins in young stems to high molecular weight pectins in mature stems can be correlated with the increase in size of the stems which occurs between the dormancy and the maturation stages. During the ageing, it seemed that high molecular weight pectins are synthesised. The synthesis of large pectins in the stems during the growth is accompanied by the incorporation of GalA residues. At dormancy, stem pectin extracts contained an equal amount of neutral and acidic sugars. Small neutral polysaccharides and large acidic compounds were evidenced after size-exclusion chromatography. In 3-days-old flax stems, only small RG-I molecules and small homogalacturonans were detected after extraction with *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (Rihouey et al., 1995). It appears that from the early beginning of the development up to the dormancy period, homogalacturonans preferentially undergoes elongation.

After the dormancy period, pectic substances increased in size. A close co-elution between neutral sugars and uronic acid can be the result of the linkage of homogalacturonans and RG-I.

At the maturation period, after the growth had stopped, large pectins were observed, this pattern remained at senescence. Pectins were not degraded and large acidic molecules are present in the stems. It is not the same in ripening fruits (Rose, Hadfield, Labavitch, & Bennet, 1998) or in the buffer- and CDTA-soluble pectins of green pods recovered at senescence where fragmentation of pectic substances was high (Stolle-Smits et al., 1999). Large linear pectins in the senescent stems of the flax can give resistance to the stems and help the bearing of the seed-containing capsules.

The changes in the composition of the sugars in the stem pectins can be correlated to the development of the flax. The overall sugar composition indicated that the Gal content was stable whereas Ara declined. Such an evolution during ageing was already reported in the carrot with the decrease

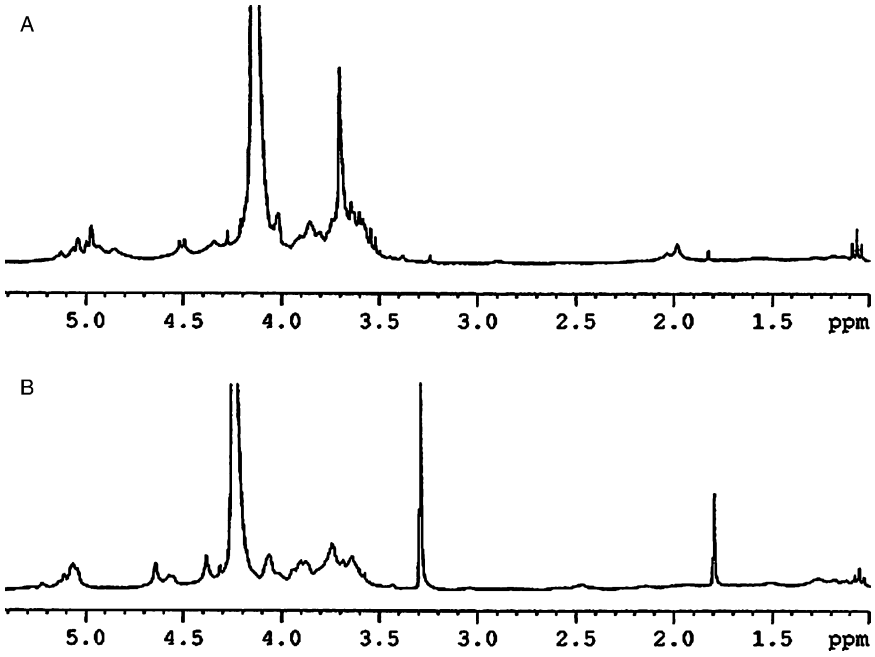

Fig. 3. Demonstration by ^{1}H NMR of the methylation and the acetylation of flax pectins. Pectins extracted from the stems (A) at the dormancy stage (HCl 1 extract) were dissolved in D_2O (10 mg/ml) before to be saponified with 100 mM NaOD in the NMR tube. Methanol ($\delta = 3.28$ ppm) and acetate ($\delta = 1.82$ ppm) were liberated in the medium and further quantified relative to the H-4 signal ($\delta = 4.34$ ppm) of GalA of the pectin core (B). Analyses were performed at 80 °C.

of α-L-arabinan in cell walls, while the transition from cell division to cell elongation occurred (Willats, Steele-King, Marcus, & Knox, 1999). For the stem pectins, the progressive decrease of the Rha concentration relative to the total sugars during the lengthening of the stems (1.8% in the dormancy to 0.81% in the maturation period) may correspond to a need of fluidity for the plant elongation (Ridley et al., 2001). An incorporation of Rha took place before the senescence (1.5% of the total sugars) which supposed an increasing amount of RG-I molecules. The installation of RG-I before the plant death may correspond to a reinforcement of the cohesion between the cells.

The pectins extracted from leaves, displayed different properties than those of the pectins recovered from the stems. Throughout leaf development, the size and the neutral sugars composition did not change. Pectins in the leaves were more acidic as soon as the dormancy period and proportion increase up to the end of the growth period (74% of the total sugars at maturation). The formation of high molecular weight pectins which was evidenced in the stems did not occur in leaves, and a large polydispersity of polysaccharides remained. Mechanisms of the pectin synthesis seems to be different in the two organs for one particular period. Installation of large pectins in leaves seem unnecessary for the strengthening of the wall.

The persistence of heavy substituted pectins at every stage of flax development was unexpected, it did not suited with the general scheme of cell differentiation and elongation where an important decrease of pectin methylation was observed at the end of growth. The non-esterified homogalacturonans are expected to allow the formation of cross-linked structures via calcium bridges and consequently reinforce the cell-to-cell adhesion (Jauneau et al., 1994). The present study report that the degree of methylation of flax pectins varied in small extend along the development, the maximum being at the active growing phase that follows the dormancy period. Then, only a limited decreased in DM from 100 to 80% was observed at the maturation period when the growth had stopped. High DM levels maintained at the senescence period indicated that pectins belonged to the highly methoxy pectins family. These esterified pectins from flax, solubilised in diluted HCl, seemed to be the same as the buffer-soluble pectins which were extracted from green pods after incubation (16 h, 4 °C) of cell

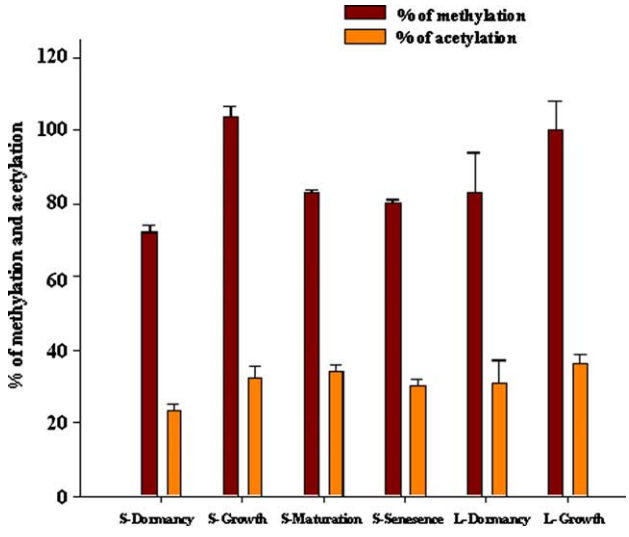

Fig. 4. Evolution of the acetyl and methyl esterification of the flax pectins. Pectins from the HCl 1 extracts were solubilised in D_2O (500 μl) at a concentration of 10 mg/ml, before addition of NaOD and quantification of methanol and acetic acid using ^{1}H NMR with GalA as reference. Values are the means of three manual integrations performed onto two different pectin preparations. S, pectins from stems; L, pectin from leaves.

wall material in ammonium acetate buffer (Stolle-Smits et al., 1999). The localisation of esterified pectins in flax was not identified here, but they can stand near the plasmic membrane where their secretion occurs as highly esterified forms (Micheli, 2001).

The acid-soluble pectic substances from stems and leaves appeared acetylated whatever the stages of development. As for the stems, an increase of the DA was noted during elongation from 23 to 34% and a value of 30% was maintained at senescence. The DA of leaf pectins was higher and rised again during ageing. The increase in DA during the active period of growth was in accordance with an high activity of rhamnogalacturonan *O*-acetyl-transferase characterised during the linear growth phase of potato suspension-cultured cells (Pauly & Scheller, 2000). However, in the green pods of bean, the DA falls down during elongation and rises again just before senescence (Stolle-Smits et al., 1999). The location of the *O*-acetyl esters on the flax pectins was not investigated in this report. Nature of the acetylated sugars was not identified. Indeed, acetylation of cell wall polysaccharides is not specific of pectins. Thus, in the retted flax, a β-(1→4)-linked xylan was *O*-acetylated on the C-2 and C-3 positions of the xylose residues, whereas a β-(1→4)-linked glucomannan contained acetylated mannose residues (Van Hazendonk, Reinerink, de Waard, & van Dam, 1996). More interestingly, in the olive cell wall, *O*-acetylated arabinose residues were identified in the side chains of xyloglucan oligosaccharides (Vierhuis et al., 2001).

Acetylation of pectins and its relationships with the development of the plant is not clear until now. It was stated that acetylation of cell wall pectins can be correlated to the cell size in cultured sugar beet cells. The *O*-acetyl substituents could affect the ability of pectins to modulate or control cell elongation call (Ishii, 1997). On the other hand, the protective function of acetyl esters were clearly demonstrated (Schols, Geraeds, Searle-van Leeuwen, Kormelink, & Voragen, 1990). The acetyl esters are a major hindrance to the effect of rhamnogalacturonases on depolymerisation of RG-I polysaccharides. The removal of acetyl-esters either with a rhamnogalacturonan acetylesterase, or by chemical saponification, is a prerequisite for the degradation of the polysaccharide core (Kauppinen et al., 1995). The acetyl-groups linked to homogalacturonans and rhamnogalacturonans are also known to reduce pectin methyl esterase activity (Oosterveld, Beldman, Searle-van Leeuwen, & Voragen, 2000).

Data obtained in this study indicated that a mild acid incubation led to the specific extraction of highly esterified pectins from stems and leaves of flax along the development. However, only a small part of pectins was extracted since a mature flax cell wall consists of around 26% of pectic material (Morvan et al., 1989), whereas at the senescence period, only 2% of pectic substances were solubilised in our study. Absence of hemicellulose in the extracts together with the low degradation of control arabinogalactans seemed to exclude the extraction of heavy cross-linked pectins. The conditions used seemed to extract young pectins from tissues. A weak bridging with other parietal materials could occurred, making pectins highly soluble suggesting that the synthesis of methylated and acetylated pectins occurred in stems and leaves up to the late stages of flax development. For one particular period of development, flax plants synthesised pectins with different features concerning principally the size and the monosaccharides composition, whereas the differences in the levels of methylation and acetylation were more reduced.

Acknowledgements

The authors are indebted to the 'Conseil Régional de Picardie' for his financial help, to Daniel Dorvillez for flax cultivation, and to Dominique Cailleu for advices on the NMR experiments.

References

Bédouet, L., Courtois, B., & Courtois, J. (2003). Rapid quantification of *O*-acetyl and *O*-methyl residues in pectin extracts. *Carbohydrate Research*, *338*, 379–383.

Davis, E. A., Derouet, C., & Hervé Du Penhoat, C. (1990). Isolation and NMR study of pectins from flax (*Linum usitatissimum* L). *Carbohydrate Research*, *197*, 205–215.

Gorshkova, T. A., Chemikosova, S. B., Sal'nikov, V. V., Pavlencheva, N. V., Gur'janov, O. P., Stolle-Smits, T., et al. (2004). Occurrence of cell-specific galactan is coinciding with bast fiber developmental transition in flax. *Industrial Crops and Products*, *19*, 217–224.

Grasdalen, H., BakØy, O. E., & Larsen, B. (1988). Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectin by ^{1}H-NMR spectroscopy. *Carbohydrate Research*, *184*, 183–191.

Ishii, T. (1997). *O*-acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiology*, *113*, 1265–1272.

Jauneau, A., Cabin-Flaman, A., Morvan, C., Pariot, C., Ripoll, C., & Demarty, M. (1994). Polysaccharide distribution in the cellular junctions of immature fibre cells of flax seedlings. *Histochemical Journal*, *26*(3), 226–232.

Kauppinen, S., Christgau, S., Kofod, L. V., Halkier, T., Dörreich, K., & Dalbøge, H. (1995). Molecular cloning and characterization of a rhamnogalacturonan acetylesterase from *Aspergillus aculeatus*. *Journal of Biological Chemistry*, *270*(45), 27172–27178.

Mannes, N. O., Ryan, J. D., & Mort, A. J. (1990). Determination of the degree of methyl esterification of pectins in small samples by selective reduction of esterified galacturonic acid to galactose. *Analytical Biochemistry*, *185*, 346–352.

Micheli, F. (2001). Pectin methylesterases: Cell wall enzymes with important roles in plant physiology. *Trends in Plant Science*, *6*, 414–419.

Monsigny, M., Petit, C., & Roche, A. C. (1988). Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Analytical Biochemistry*, *175*, 525–530.

Morvan, C., Abdul Hafez, A., Morvan, O., Jauneau, A., & Demarty, M. (1989). Etude physicochimique et biochimique de polysaccharides extraits de lin sous-roui. *Plant Physiology and Biochemistry*, *27*, 451–459.

Morvan, C., Jauneau, A., Morvan, C., Voreux, H., & Demarty, M. (1989). Biosynthèse des pectines et différenciation des fibres cellulosiques au cours de la croissance du lin. *Canadian Journal of Botanic*, *67*, 135–139.

Oosterveld, A., Beldman, G., Searle-van Leeuwen, M. J. F., & Voragen, A. G. J. (2000). Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium. *Carbohydrate Research*, *43*, 249–256.

Pauly, M., & Scheller, H. V. (2000). *O*-acetylation of plant cell wall polysaccharides: Identification and partial characterization of a rhamnogalacturonan *O*-acetyl-transferase from potato suspension-cultured cells. *Planta*, *210*, 659–667.

Quemener, B., & Thibault, J. F. (1990). Assessment of methanolysis for the determination of sugars in pectins. *Carbohydrate Research*, *106*, 277–287.

Renard, C. M. G. C., Voragen, A. G. J., Thibault, J. F., & Pilnik, W. (1990). Studies on apple protopectin: I. Extraction of insoluble pectin by chemical means. *Carbohydrate Polymers*, *12*, 9–25.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, *57*, 929–967.

Rihouey, C., Morvan, C., Borissova, I., Jauneau, A., Demarty, M., & Jarvis, M. (1995). Structural features of CDTA-soluble pectins from flax hypocotyls. *Carbohydrate Polymers*, *28*, 159–166.

Rose, J. K. C., Hadfield, K. A., Labavitch, J. M., & Bennet, A. B. (1998). Temporal sequence of cell wall disassembly in rapidly ripening melon fruit. *Plant Physiology*, *117*, 345–361.

Schols, H. A., Geraeds, C. C. J. M., Searle-van Leeuwen, M. F., Kormelink, F. J. M., & Voragen, A. G. J. (1990). Rhamnogalacturonase: A novel enzyme that degrades the hairy regions of pectins. *Carbohydrate Research*, *206*, 105–115.

Stolle-Smits, T., Beekhuizen, J. G., Kok, M. T., Pijnenburg, M., Recourt, K., Derksen, J., et al. (1999). Changes in cell wall polysaccharides of green bean pods during development. *Plant Physiology*, *121*, 363–372.

Van den Hoogen, B. M., Van Weeren, P. M., Lopes-Cardozo, M., Van Golde, L. M. G., Barneveld, A., & Van de Lest, C. H. A. (1998). A microtiter plate assay for the determination of uronic acids. *Analytical Biochemistry*, *257*, 107–111.

Van Hazendonk, J. M., Reinerink, E. J. M., de Waard, P., & van Dam, J. E. G. (1996). Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (*Linum usitatissimum* L). *Carbohydrate Research*, *291*, 141–154.

Vierhuis, E., York, W. S., Kumar Kolli, V. S., Vincken, J. P., Schols, H. A., Van Alebeek, G. J. W. M., et al. (2001). Structural analyses of the two arabinose containing oligosaccharides derived from olive fruit xyloglucan: XXSG and XLSG. *Carbohydrate Research*, *332*, 285–297.

Willats, W. G. T., Steele-King, C. G., Marcus, S. E., & Knox, J. P. (1999). Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. *Plant Journal*, *20*, 619–628.