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Degradation of Flax Polysaccharides with Purified Endo-Polygalacturonase

Claudine Morvan, Alain Jauneau, Armelle Flaman, Jacques Millet & Maurice Demarty

URA-CNRS D 0203, EquippeParois et Polysaccharides Pariétaux, Scueor, Faculté des Sciences de Rouen, BP 118, 76134 Mont-Saint-Aignan, France

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ABSTRACT

An endo-polygalacturonase was purified from a commercial preparation of Aspergillus *by cation exchange and size exclusion chromatographies. It degraded a polysaccharide extracted from under-retted flax into three general size classes. The first product was a polysaccharide of high molecular weight (close to 100 000), enriched in galactose and characterized by a ratio of galacturonic acid:rhamnose 2:1. The second product was an acidic polysaccharide (the molecular weight of which was close to 10 000) enriched in glucose and rhamnose, which reacted little with the usual colorimetric methods for uronic acids and gave a negative circular dicroism bond. The last and major products were oligomers rich in galacturonic acids.*

INTRODUCTION

Cellulosic fibres prepared from flax are used in the textile industry. In the flax plant these cellulosic fibres are arranged in bundles which, throughout the plant's growth become progressively more differentiated from the phloem parenchyma by synthesizing successive layers of cellulose and pectins (Morvan *et al.*, 1989*a*). Retting is the process of dissociating cellulose fibres from the cortex by degradation of pectic substances. Pectins from cortical cell walls have been previously studied (Morvan *et al.*, 1984, 1988). Due to their digestion by microorganisms during the first steps of retting, pectins from the fibre bundles could be easily studied. Thus, assays were run on pectins solubilized from long fibres

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obtained by scutching dried stems of flax. The flax fibres were defined as under-retted as the stems were kept for 17 days on the field. At this stage of retting the remaining fibres were constituted of 69·2% cellulose, 15·4% hemicellulose and 15·4% pectins (Morvan *et al.*, 1989*b*).

The work carried out shows that all the pectins from under-retted flax, except the polysaccharides designated P_0, were resistant to the purified endo-polygalacturonase (endo-PGH). Such a specificity in the hydrolysis has to be discussed in terms of structure and function and finally in terms of the choice of conditions and enzymes for the enzymic retting process.

MATERIALS AND METHODS

Purification of polygalacturonase (PGH)

Flaxzyme, a commercial solution of enzymes synthesized by an *Aspergillus*, sp., was used. This preparation was a gift from Novo industries (Denmark).

Assay procedures

Proteins were measured by the method described by Bradford (1976) with bovine serum albumin as a standard.

The hydrolysis of polygalacturonic acid (PGA from citrus, Sigma, La Verpillière, France) by the endo-PGH was measured by the formation of reducing groups or by the decrease in viscosity. The reaction mixture (9 ml) comprised PGA (0·4% w/v) in 50 mM acetate buffer, pH 5·0 and enzyme solution (1 ml, suitably diluted). The reducing groups formed were measured by the method of Nelson (1944) using GA as a standard. The activity was expressed in katals. One katal is defined as the liberation of one mole of the reducing groups per second at 30°C. The activity was linearly related to the concentration of enzyme used. Viscosity measurements were performed at 30°C (Ostwald viscometer, AMTEC, Villeneuve-Loubet, France). The specific viscosity of the reaction mixture (η_{sp}) was plotted versus the reaction time. The percentage of split α(1-4) linkages could be calculated for a decrease of 50% in the specific viscosity (Thibault, 1983).

Pectate lyase activity was measured at 232 nm with PGA as the substrate, in tris HCl 0·05 M, pH 8 10^{-3} M $CaCl_2$ buffer solution (Konno & Yamasaki, 1982). Pectin lyase activity was measured with citrus pectin as the substrate in the same buffer without calcium. In both cases a blank was performed with inactivated enzymes to correct for β-elimination. Glucanase and galactanase were detected by the Nelson method with

carboxymethyl cellulose (Sigma) and arabinogalactan (Sigma) or galactan from flax hemicellulose in sodium acetate 0·05 M, pH5 buffer solution.

Chromatography procedures

The crude enzyme solution was first subjected to size exclusion chromatography on a Sephadex G25 column (2·6 by 14·5 cm) equilibrated with 0·05 M acetate buffer, pH 4·0. Ion-exchange chromatography was performed on a CM-Sepharose CL6B column (2·6 by 14·5 cm). Absorbed proteins were eluted with a linear gradient of 0–0·4 M NaCl (250 ml total volume) in 0·05 M acetate buffer, pH 4·0. Fractions (4·5 ml) were collected and analysed for total protein and PGH activity. Fractions showing the highest PGH activity were combined and lyophilized. The CM extract, solubilized in distilled water, was subjected to size exclusion chromatography on a Sephacryl S200 column (1·1 by 33 cm) previously equilibrated with distilled water. Fractions with PGH activity were collected and designated as the S200 extract.

Electrophoretic procedures

Electrofocusing was performed with ultra thin (0·3 mm) polyacrylamide slab gels in ampholines pH range 4–6.5 (Pharmacia). LKB (Paris, France) apparatus (Multiphor 2117 and 2297 power supply) was used according to the procedure described by the manufacturer. Polygalacturonase active bands were revealed by the PGA–agar sandwich technique (Bertheau *et al.*, 1984).

Electrophoresis was carried out in a discontinuous system buffer without sodium dodecyl sulphate. Hoeffer (San Francisco, USA) apparatus (S600 vertical slab unit and PS 250 power supply) was used according to the procedure described by the manufacturer. Proteins were stained with coomassie blue R250 (Merk), and the gels were scanned in a Shimadzu (Kyoto, Japan) apparatus (optical density of 590 nm).

Flax polysaccharides

Pectic substances were extracted at 20°C with water (p_f) and at 100°C with water (p_c), 0·5% ammonium oxalate (p_o) and 0·5% EDTA-Na_2 (p_e) from flax fibres (*Linum usitatissimum* L.) which had been spread out for 17 days in the field. The composition of neutral sugars and uronic acids, and the equivalent molar weight (MM_{eq}) previously measured, showed that the galactose (gal):arabinose (ara) ratio increased from 2·9 (p_f) to 25 (p_e) in the sequential extracts (Morvan *et al.*, 1989*b*). Moreover two ratios of rhamnose (rha):GA were measured; a value of 1:5 characterized p_f and p_o whereas a ratio of 1:1 was estimated for p_c and p_e.

Whatever the fraction, the pectins had a low degree of esterification ($\leqslant 0{\cdot}22$).

Enzymic degradation of flax polysaccharides

Enzymic degradations were carried out under standardized conditions. Flax polysaccharide solutions (0·4% in Na-acetate 0·05 M, w/v, pH 5) were incubated at 35°C with the purified PGH (1 nkatal ml^{-1}). The efficiency of the hydrolyses was estimated by comparing the amount of reducing sugars produced with an assay performed concurrently with PGA. The digestion products were analysed by three methods: size exclusion chromatography (Sephacryl S200; Bio-gel (Biorad)), fractionation on thin layer aluminia sheet silica-gel 60 (Merk) with butanol:acetic acid:H_2O (2:1:1) and neutral sugar release (gas chromatography).

A quantitative experiment was run with the pectins p_o (375 mg) where the reaction (100 nkatal) was followed for 1 h in the viscometer. To maximize the reaction, 200 nkatal were then added and the reaction kept going for 4 h at 40°C. The digestion products were combined from two successive separations on a column Sephacryl S200 (2·5 by 600 cm) equilibrated and eluted (120 ml h^{-1}) with NaCl 0·2 M to minimize the electrostatic repulsion of the charged polymers. The void volume (V_o) and included volume (V_i) were determined with blue dextran and PGA. The chromatography profile was followed by absorbance at 214 nm. The molecular weights of the polysaccharide fractions were estimated from a calibration curve previously established with flax pectins (Hourdet, 1989). The fractions of higher molecular weight were ultrafiltered on Pellicon membranes (PTGC 00005, Millipore) with a 10 kDa cut off, and lyophilized. The digestion products of lower molecular weight were applied to an LKB column (1·5 by 90 cm) of Bio-gel P2 equilibrated and eluted (36 ml h^{-1}) with ammonium acetate 0·1 M, pH 3·6 at 20°C. The degree of polymerization (DP 1–7) of oligouronides was estimated as described by Thibault (1980).

Carbohydrate analyses

The content of total sugars (including the GAs) and GAs in each sample were estimated by the sulphuric orcinol (Montreuil *et al.*, 1981) and *meta*-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973) methods.

Samples were hydrolysed for 2 h at 100°C with 2×10^{-3} mol m^{-3} (2N) trifluoroacetic acid (TFA) and the neutral sugars from the hydrolysates

were analysed by gas–liquid chromatography as their alditol acetate derivatives (Albersheim *et al.*, 1967). Gas–liquid chromatography was performed with a Girdel instrument (model) with a capillary column CP Sil 5CB.

Potentiometric measurements were carried out as previously described (Goldberg *et al.*, 1986; Morvan *et al.*, 1989*a*). Four parameters have been defined: (1) the MM_{eq}, (2) the degree of esterification (DE), (3) the ratio of neutral residues to acid groups (SN) and (4) the charge density parameter (ξ) (Morvan *et al.*, 1985).

RESULTS

Purification of the endo-PGH

Dialysis has often been used as a first step in the purification of PGHs from the crude flaxzyme solution. In the present work this procedure resulted in a substantial loss (82%) of enzyme activity. Hence Sephadex G25 chromatography was performed. In this way, 83% of the total activity was recovered and the G25 extract (total volume: 127 ml) was applied to the cation exchange gel. The PGH-rich CM extract, localized in a major peak eluted at 0·1 M NaCl, contained 51% of the injected endo-PGH activity and 10% of the protein. This extract was lyophilized. An aliquot (150 μl) of the aqueous enzyme solution was then fractionated by gel filtration on Sephacryl S200. The proteins were separated into at least two peaks, one of which was coincident with the peak of enzyme activity. The purified enzyme (S200 extract) gave a single protein band on polyacrylamide gel electrophoresis. The data in Table 1, summarizing a typical purification of endo-PGH, show that a 263 fold increase in specific activity was achieved. The preparation had

TABLE 1
Scheme of the Purification of an endo-PGH from Flaxzyme

	Crude solution	*G25*	*CM*	*S200*
Proteins (mg)	198·45	91	9·2	0·4
Protein yield (%)	100	46	4·6	0·2
Total activity (μkatal)	251·7	211	1 085	133
Activity yield (%)	100	83	43	53
Specific activity (μkatal mg^{-1})	1·27	2·3	11·8	333
Degree of purification	1	1·8	9·3	263

no glucanase, galactanase, pectin methylesterase, pectate lyase or pectin lyase activity.

Characterization of the purified PGH

The endo-PGH caused a rapid decrease in PGA viscosity and resulted in only a slow liberation of reducing sugar (Fig. 1). For a 50% reduction in the viscosity of a 0·4% PGA solution (0·2 M NaCl), only 2·6% of the glycosidic bonds were cleaved, indicating that random cleavage occurred in the pectate chain.

The molecular weight was estimated to be 39 kDa by gel filtration and 33 kDa by electrophoresis. The isoelectric point of the enzyme was estimated between 4·40 and 4·50. The final enzyme preparation showed one major band on isoelectric focusing.

The optimum pH of endo-PGH for PGA was close to 5·0 with acetate as the buffer. It gave no detectable activity on PGA at pH 8·0. No significant differences in endo-PGH activity were observed when acetate buffer was used in reaction mixtures at 10–50 mM, but activity was slightly reduced at 100 mM. The same was true for citrate NaOH buffer up to 30 mM. Activity in 0·1 M NaCl was approximately 18% higher than without NaCl. It was constant up to 0·2 M at which the substrate precipitated. Activity on pectin from apple (DE = 45%) or citrus (DE = 63%) was lower than with PGA due to their higher degree of methylation.

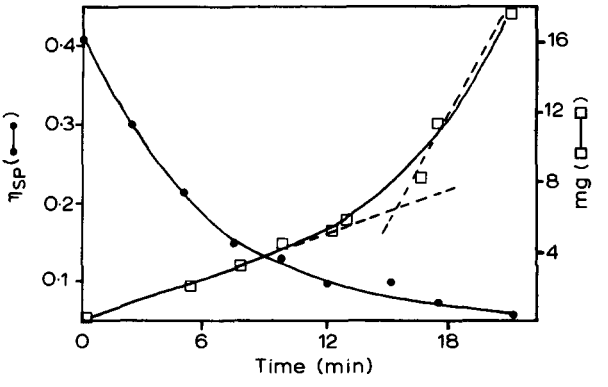

Fig. 1. Viscosity changes of PGA and reducing sugars released by endo-PGH as a function of time. A 2 ml aliquot of S200 extract (diluted 1/500, v/v:35 nkatal) was added to the reaction mixture (PGA 0·4%, w/v, in 50 mM acetate buffer, pH 5 with NaCl 0·2 M) at 30°C. Specific viscosity η_{sp} (●) and reducing sugars (□) in milligrams were reported versus the time (min).

Polygalacturonase digestion of flax polysaccharides

The release of reducing sugars from flax pectic polymers by endo-PGH (Table 2) made it clear that p_f, p_c and p_e cannot be hydrolysed. The high ratio of rha to GA (1:1) of p_c and p_e which may be characteristic of rhamnogalacturonan I (RGI)-like polysaccharides could explain why the enzyme failed to hydrolyse these pectic fractions. The release of reducing sugars by PGH from polymer p_o was as large as the substrate PGA. However, while the viscosity of the digest with p_o decreased very rapidly, the flow time measured at the end of the reaction never reached that of the solvent as PGA did.

Characterization of the pectic substance p_o

Potentiometric measurements indicate (Morvan *et al.*, 1989*b*) that p_o acted as a polyelectrolyte, the MM_{eq} of which was 375, and the DE less than 10%.

As shown by chromatography on Sephacryl S200 (Fig. 2, curve a) the crude polymer p_o was polydisperse, the molecular weight of the major fraction p_o 2–5 (72·5%) being between 6800 and 33 500. Fraction P_o 1 (20%) eluted very close to V_0 was characterized by a higher molecular weight (70 000), while a last fraction p_o 6 (7·5%) with lower molecular weight ($\leqslant 1000$) would include oligomers (Table 3).

The MM_{eq} of the pectins was calculated from colorimetric assay using the relationship m/N; m being the weight (grams) and N the number of uronic acids (equivalent) estimated by the *meta*-hydroxydiphenyl method. The MM_{eq} of the different fractions p_o 1–6 which decreased with their molecular weight, indicated, besides the polydispersity, a heteromolecularity of p_o. the MM_{eq} of the major fraction p_o 3 was close to that

TABLE 2
Reducing Sugars Released by Digestion of Flax Polysaccharides by Purified endo-PGH[a]

Fraction	p_f	p_c	p_o	p_e	*PGA*
Reducing sugars (meq$*10^3$)	0	0·08	3·77	0·07	3·8
Efficiency of hydrolysis (%)	0	2	99	2	100

[a]The reaction mixture comprised flax polysaccharides or PGA (0·4% w/v) in 50 mM acetate buffer (pH 5, 0·9 ml, 30°C) and solution of the purified endo-PGH(0·1 ml). Fractions p_f, p_c, p_o, p_e were extracted from under-retted flax successively with water at 20 and 100°C, 0·5% ammonium oxalate and 0·5% EDTA-Na_2. The efficiency of the flax polysaccharide digestion was calculated relative to PGA.

of the crude polymer, as determined potentiometrically. The oligomers had the lowest MM_{eq} (200) close to that of the galacturonic acid. For the fraction p_o 4–5 the MM_{eq}, determined colorimetrically, was higher than the MM_{eq}, measured potentiometrically (Hourdet & Muller, 1987).

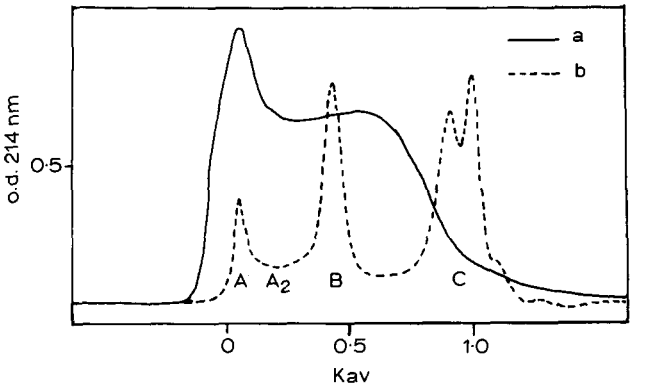

Fig. 2. Size exclusion chromatography of the flax polysaccharide p_o on Sephacryl S200. The column (2·4 × 60 cm) was equilibrated and eluted (flow rate 120 ml h^{-1}) with NaCl 0·2 M. The chromatography profile was followed by absorbance at 214 nm. Curve a, fractionation of the crude polymer p_o; curve b, fractionation of the polymer p_o after its digestion by the purified endo-PGH. The products A, B, and C were characterized respectively by a K_{av} of 0·05, 0·45 and 1.

TABLE 3
Characterization of the Pectic Fractions p_o 1–6 after their Separation on a Column Sephacryl S200

Fraction	*Relative weight (%)*	MM_{eq}	K_{av}	*Molecular weight*
p_o 1	20·5	540	0·05	70 000
p_o 2	11·4	525	0·15	33 500
p_o 3	28·6	400	0·25	26 000
p_o 4	17·8	495	0·45	10 500
p_o 5	14·7	600	0·55	6 800
p_o 6	6·9	210	1	1 000

The pectic fractions p_o 1–6 were collected from size exclusion chromatography of the crude polysaccharide p_o (Fig. 2, curve a). The MM_{eq} was calculated from the relation m/N, m being the weight (g) of the polysaccharide and N the number of uronic acids (equivalent) estimated by the *meta*-hydroxydiphenyl method. The molecular weight was estimated from a calibration curve (Hourdet, pers. comm.).

Chromatography of the products of PGH on p_o

Pectin fraction p_o digested with the purified endo-PGH showed a separation pattern on Sephacryl S200, which was much better resolved than the crude polymer (Fig. 2, curve b). The carbohydrates were eluted in three main peaks, A, B and C, the k_{av} of which were respectively 0·05, 0·45 and 1 and molecular weights 70 000, 10 500 and 1000. The relative areas of the peaks A, B and C were respectively 10, 27·6 and 42·9%, showing that the major products were oligomers (Table 4). Peak A, as well as peak B were found to be resistant to further attack by the polygalacturonase.

Digestion of fraction p_o 1–2 gave separations very similar to the crude one, except for peak C which was smaller. In contrast the subsequent fraction p_o 3–6 released more reducing sugars and gave mainly peak B (25–35%) and C (50–55%). Comparing the results of digestion by endo-PGH of crude and fractions of p_o showed that more than 60% of the peaks B and C came from the fractions p_o (3–5) (Table 5).

Physicochemical characterization of the degradation products

The behaviour of the degradation products was characterized from the quantitative experiment, where 375 mg of p_o were treated with 300 nkatal of PGH. The three main peaks were collected by chromatography on a Sephacryl S200 (Table 4). Colorimetric estimation of the total sugars of peak A gave a quantity very close to its real weight after ultra-

TABLE 4
Quantitative Digestion of the Flax Polysaccharide p_o by the Purified endo-PGH[a]

Fraction	*Relative area (%)*	*Theoretical weight (mg)*	*Weight (mg)*	*Total sugars (mg)*	*GalU (mg)*
A	10	37·6	47	44	12
A2	19·5	73	56	37	13
B	27·6	103·7	22·5	12	7
C	42·9	160·6	—	76	99
Total	100	375	125·5	169	131

[a]The digest products of p_o (theoretical weight = 375 mg) were separated by size exclusion chromatography (Fig. 2, curve a) and characterized colorimetrically before the ultrafiltration by *meta*-hydroxydiphenyl and orcinol methods. The weights of A, A_2 and C were measured after the ultrafiltration.

filtration, and to the theoretical weight calculated from its relative area (10%).

There was a large discrepancy between the area of peak B and its yield estimated colorimetrically or by weight. The low yield in weight (22%) could be explained by the molecular weight of peak B, very close to the cut off of the ultrafiltration membrane. The low yield obtained from colorimetric measurements could not be explained by the ultrafiltration losses since the assays were run first. Peak B would hence contain the same products from the fraction p_o 4–5, which did not react colorimetrically.

Finally the oligomers of peak C are mainly oligouronides since the total sugars were found very close the uronic acids. The recovery relative to the theoretical weight was more than 60%. The discrepancy probably came from an overestimation of the theoretical weight, due to the high value of the extinction coefficient at 214 nm of the oligomers (Hourdet & Muller, 1987). The chromatography of these oligouronides on Bio-gel P2 (Fig. 3) showed that their degree of polymerisation (DP)

TABLE 5
Reducing Sugars Released by Digestion of Flax Polysaccharides p_o by Purified endo-PGH[a]

Fraction	*Number of uronic acids,*[b] *N*[b] *(10^6eq)*	*Number of reducing sugars, RS*[c] *(10^6eq)*	*Hydrolysis*[d] *(%)*	*Contribution*[e]		
				Peak A	*Peak B*	*Peak C*
p_o^{2f}	18	0·13	0·7	73·8	19·0	7·4
p_o^2	18	0·18	1	20·5	10·6	6·9
p_o^3	24	0·96	4	2·6	26·5	36·5
p_o^4	21	1·10	5·2	1·6	18·5	21·4
p_o^5	17	1·81	10·6	1·0	16·4	19·5
p_o^6	48	4·72	9·8	0·5	9·0	8·3

[a]The reaction mixture comprised flax polysaccharides (0·4% w/v) in 50 mM acetate buffer (pH 5, 30°C) and solution of the purified endo-PGH (1 nkatal ml^{-1}).
[b]Estimated from the *meta*-hydroxydiphenyl method.
[c]Calculated from the Nelson method.
[d]Calculated from the relation *RS/N*.
[e]The contribution of each digested fraction p_o (1–6) to the peaks A, B and C was calculated from their relative areas after separation on S200 (data not shown) and from their relative weight in the crude polymer (Table 3).
[f]The pectic fractions p_o 1–6 were collected from size exclusion chromatography of the crude polysaccharide p_o (Fig. 2, curve a).

were mainly DP2–4. These results were confirmed by chromatography on thin layer silica gel where DP1–3 have been detected.

The physicochemical behaviour of peaks A, A_2 and B was investigated after ultrafiltration by potentiometric measurements. Titration curves showed that peak A behaved similarly to classical pectic acids, the pK of which were close to three (Fig. 4). The variation of pK with the degree of ionization (α) compared to reference curves (Morvan *et al.*, 1985) indicated a charge density parameter 0·5–0·75. From the relationship $b = 7{\cdot}14/\xi$, a mean distance (b) between two charged groups was estimated at 9·5–14 Å, which means that two charged groups were separated by 1–2 neutral sugar residues. The MM_{eq} of peak A was between 850 and 1000 depending on whether it was estimated colorimetrically, (m/N_c) or by potentiometric titration (m/N_p). The ratio of neutral sugars to uronic acid was estimated as 4–5. The analysis of the neutral sugars of peak A showed that 73% of them were galactose (gal) (Table 6). The relative content of gal:glu:rha:ara were 1:0·11:0·13:0·09. The ratio between the GAs and the rha was estimated to be between 2·5 and 3.

The neutralization curves of peak B appeared quite different from those of peak A especially in relation to the autodissociation of the polyelectrolyte (α_0); even when the neutralization degree (α') was zero, α was higher than 0·6, due to free protons in the solution. This behaviour was incompatible with a pectic polymer which would classically show (as for

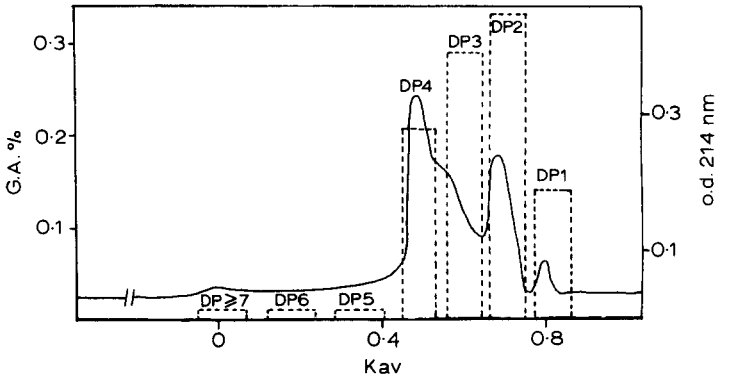

Fig. 3. Size exclusion chromatography of the peak C of the flax polysaccharide p_0 digested by the purified PGH on a column Bio-gel P2. The column (1·5 × 90 cm) was equilibrated and eluted (flow rate 36 ml h^{-1}) with ammonium acetate 0·1 M, pH 3·6 at 20°C. The chromatography profile was followed by absorbance at 214 nm. The DP (1–7) of the oligouronides were calculated from Thibault (1980) and the histogram represents their relative content in uronic acids (measured with the *meta*-hydroxydiphenyl method).

peak A) an autodissociation close to 0·3. Although the titration curve (pH = $f(\alpha')$) seemed regular (not shown), the curve pH = $f(\alpha)$ suggested the presence of two kinds of acids: the first one (30–60%) totally dissociated like a strong acid and the second one (40–70%) behaved like a classical polyuronic acid. The presence of these two acids was confirmed by the colorimetric assay which showed that only a part of the measured acids were uronic acids (Table 4). This point could explain why the peak B as well as the fractions p_o 4 and p_o 5 (the molecular weights of which were close to that of peak B) had a higher MM_{eq} when it was calculated from colorimetric measurement (730, 495 and 600 respectively). On the contrary, the MM_{eq} of peak B was lower (510) than that of peak A when it was measured potentiometrically. The number of neutral sugars per acid was estimated to be between 2 and 3.

The analysis of the neutral sugars of peak B showed that 53% of them were gal (Table 6). The relative contents of gal:glu:rha:ara were 1:0·46:0·23:0·13. The ratio between the GAs and the rha was estimated between 1·5 and 2·4. The neutral sugar composition of peak B was different from that of peak A especially with regard to its higher ratio of glu, thus the ratio rh:glu decreased from peak A (1·1) to peak B (0·5).

The difference in the behaviour between peaks A and B was also confirmed simply by the absorption spectra between 350 and 190 nm where

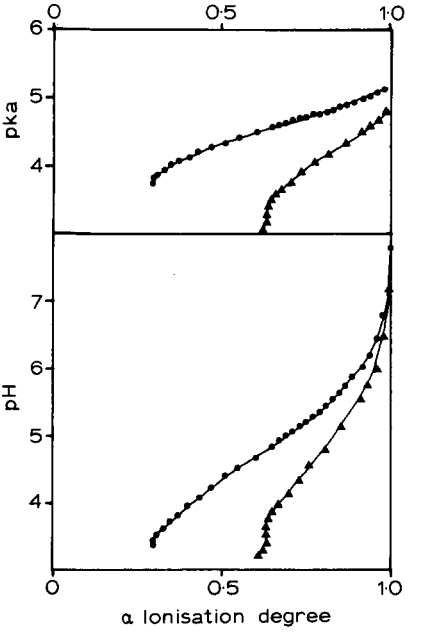

Fig. 4. Titration curves of peaks A (●) and B (▲) fractionated on S200 after digestion of the polymer p_o. The polymers were ultrafiltrated, protonated by passing through an exchange resin (amberlite IR 120) and then neutralized with KOH (N/10). The apparent pK was calculated from the relation: $pKa = pH - \log(\alpha/(1-\alpha))$, where a was the ionisation degree.

an absorption maximum was observed at 250–260 nm for peak B. Moreover, the circular dicroism give spectra of opposite sign for peaks A and B. Surprisingly the optical rotation appeared very similar for peaks A and B, probably resulting from the major sugars i.e. gal, glu, rha and galacturonic acids as shown in Table 6.

DISCUSSION

The digestion of the pectins p_o by a purified PGH releases a large amount of oligouronides. Peak A appeared more like a contamination than a release product since it behaved similarly to p_c and P_e. Peak B was the main product of the hydrolysis with the oligouronides in peak C. Its composition of neutral sugars was close to that of the crude polysaccharide p_o. However its properties ($MM_{eq} \geqslant 510$, molecular weight close to 10 000–12 000, two kinds of acidities, negative CD) were very different to that of p_o, due to hydrolysis of the GAs. This behaviour, as well as the separation profile of p_o products, which appeared very similar to the one described after action of a PGH on the cell walls of suspended culture cells (McNeil *et al.*, 1980) led to peak B being classified as a possible RGII-like polysaccharide. From a biotechnological point of view, further

TABLE 6
Characterization of the Digestion Products of the Polysaccharide p_o after their Separation on a Column S200[a]

Product	*Crude p_o*	*Peak A*	*Peak A 2*	*Peak B*
Acid microequivalents Np	1 000	47	74	44
MM_{eq}	375	1 000	755	510
Neutral sugar composition (%)				
Rhamnose (rha)	21·3	9·4	13·4	12·2
Fucose (fuc)	—	—	0·5	0·5
Arabinose (ara)	5·2	6·9	7·9	6·9
Xylose (xyl)	1·3	1	—	1
Mannose (man)	1·8	1·3	1·7	2·4
Glucose (glu)	22·5	8·3	15·4	24·1
Galactose (gal)	47·9	73·1	61·1	52·9

[a]The number of acids (N_p) was measured from the titration curves (Fig. 4) and the MM_{eq} was calculated from the relation $m/Np, m$ being the weight after lyophilisation. The polymers were hydrolysed with TFA 2 N (2 h 100°C) and the neutral sugars analysed by gas–liquid chromatography as their alditol acetate derivatives.

information will have to be derived from more sophisticated approaches such as NMR and methylation coupled with mass spectroscopy.

From the point of view of the physiology of flax, the cortical parenchyma is in contact with the phloem which is delimited by the pericycle: there is no endodermis to act as a barrier against propagation of microorganisms. The differentiated fibres with very thick cell walls (thicknesses up to 10–15 μm) and pectins totally different from classical ones (and resistant to pectinases usually secreted by microorganisms) could then be suggested as endotherm substituents for the protection of the stele from infection. According to the scheme of Fry (1988), these pectins could be interpreted as long complex chains: the enzyme has split the chains at sites of contiguous α1–4 galU residues, solubilising RGI and RGII and generating galU rich oligosaccharides from connecting homogalacturonan domains. These complex polysaccharides, which had been partially hydrolysed during the pre-retting would account at least for p_c, p_o and p_e. The fractions p_c and p_e would be enriched by RGI and p_o by RGII and the connecting block of homogalacturonan. The total amount of these complex polysaccharides could be estimated as 7% of the initial dry stem or 28% of the total pool of pectins present in the cortex and the fibres. This is lower than the 11% of RGI + RGII present in the cell walls of *Acer* (or 36% of the pectins). Nevertheless it might represent the totality of the pectins present in the cell walls of the fibre bundles.

From the biotechnological these results are important as a guide to the choice of enzymes as tools for controlled retting. Indeed, to ensure a minimum strength of the manufactured thread, only a small fraction of the pectins belonging to the fibre bundles has to be hydrolysed, without solubilizing the remaining pectins. Hence, it would be useful to compare the action of the purified enzymes on the extracted polysaccharides such as p_o to the enzymic action directly on the under-retted flax.

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