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Chemically methylated and reduced pectins: preparation, characterisation by ^{1}H NMR spectroscopy, enzymatic degradation, and gelling properties

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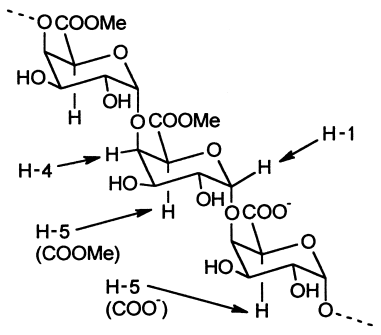
Abstract

The gelling properties of pectins are known to be closely related to the degree of methylation (DM) and the distribution of the ester groups. In order to investigate this dependency, a natural citrus pectin (DM 64%) has been methylated to pectins with higher DM or saponified to achieve pectins with lower DM. A simple method for determination of DM by ^{1}H NMR spectroscopy is presented. New modified pectins have been prepared by treatment of pectins having different DM with $NaBH_4$ to reduce selectively the methyl esters to primary alcohols in the presence of free acids. The degree of reduction (DR) and the DM of the remaining carboxylic acids could likewise be determined by ^{1}H NMR spectroscopy. The new reduced pectins are recognized by the pectin degrading enzymes polygalacturonase PGI and PGII as well as by pectin lyase, all from *Aspergillus niger*, but the enzymes exhibit lower specific activities as compared with unmodified pectin. The new reduced pectins exhibit high gelling properties.

Keywords: Pectin; Methylation; Deesterification; Reduction; DM and DR by ^{1}H NMR spectroscopy; Reduced Pectin; Pectinases; Gelling properties

1. Introduction

Pectin is a complex heterogeneous polysaccharide found in the primary cell wall of most plants, where it gives mechanical strength and flexibility due to its interaction with other wall components. An exception concerns the grasses (*Graminea*) which contain pectins with less heterogenous structures, but in very small quantity.[1] The dominant structural feature of pectin is a linear chain of poly-α-(1→4)-D-galacturonic acid with varying degree of methylation of the carboxylic acid residues. This backbone, 'the smooth region' Scheme 1, is occasionally interrupted by stretches with side chains, which are rich in neutral sugars, mainly arabinose, galactose, and rhamnose, 'the hairy region'. The backbone in the hairy region is composed of alternating α-D-galacturonic acid and α-L-rhamnose with the arabinan and galactan side chains attached mainly to the 4-position of rhamnose. The composition of pectin varies with the source from which it is isolated, as well



Scheme 1. Structure of a pectin fragment (smooth region).

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as with the conditions used during isolation and purification.[2] Pectins isolated from citrus and apple have been used in the food industry as gelling agents. The industrial demand for pectins with varying ability to gel or stabilise fruit and dairy products increases the need for accessing pectins of different types or pectin derivatives with tailored properties.

Pectins with a low degree of methylation forms gels in the presence of calcium ions whereas pectins with a high degree of methylation forms gels in acidic media with the addition of different sugars, e.g., sucrose or glucose.[3] In order to obtain knowledge about the structural requirements on a molecular basis for the gelling properties of pectin, series of pectins with different distribution of methylester groups were prepared by de-esterification using pectin methyl esterases (PME) followed by digestion with pectin degrading enzymes.[4,5] In this way, high ester pectins (DM > 50%) containing regions with a series of adjacent free galacturonic acid units on the backbone of the smooth region, have been identified.[4] Such pectins showed increased calcium sensitivity, i.e., gelling properties.[4] In contrast, chemical de-esterification produces a random methylation pattern. The ability of calcium to crosslink pectin chains is believed to follow the egg box type interaction in which the backbone with the free galacturonic acid units coordinate to a middle layer of calcium ions.[6] The exact number of free consecutive acids necessary for these properties is not known, but numbers between 8 and 12 have been suggested.[7] The 'egg box model' is, however, still a subject of debate.[8]

A thorough knowledge of catalytic activity and the preferred cleavage site of pectin degrading enzymes was obtained,[4,5,9] which is a prerequisite for their use in the structural analysis of pectins. Such methods for structural analysis have also been complemented with immunochemical techniques,[10] as well as with the use of chemically synthesised well defined small oligogalacturonates having methyl ester groups at specified positions.[11]

The different properties of pectin results from variations in the structures at the molecular level, which may often be deduced by spectroscopic methods. Thus, recent advances in MALDI MS[12] and ESI MS[13] allow analysis of complex mixtures of galacturonic acid oligomers and the methyl ester distribution pattern, respectively. ^{1}H NMR spectroscopy has proven itself a valuble tool in the study of pectins and the complete structural and conformational assignment of oligosaccharides obtained by degradation of pectins, e.g., oligogalactopyranuric acids with different methylation patterns[14,15] and rhamnogalacturonan I and II,[16,17] have been determined.

When searching for pectin derivatives with new or improved properties, e.g., as gelling agents, it is important to be able to rely on a spectroscopic method which, on a macroscopic level, can quantify the degree of methylation and the degree of acetylation, factors which both greatly influence the behavior of the pectin.[3] ^{1}H NMR spectroscopy is a very attractive method for such studies[18] since it requires only a small amount of material compared to the titration method normally used. We describe here the preparation of pectins with different degrees of methylation (DM) and the quantification of DM by ^{1}H NMR spectroscopy.[18]

Selective reduction of the methyl ester groups in pectins was performed with sodium borohydride at pH 7. Thereby new pectin derivatives were prepared, in which the smooth region consists of galacturonic acids and galactose units. By esterification of these pectins, new derivatives with esterified galacturonic acids and galactose units were also prepared. The degree of reduction (DR), corresponding to the amount of galactose units present, has likewise been quantified by ^{1}H NMR spectroscopy.

The reduced pectins have been investigated as substrates for pectin degrading enzymes, the polygalacturonases PGI and PGII as well as pectin lyase, all from *Aspergillus niger*. The properties as gelling agents of the new reduced pectins have been investigated by rheology.

2. Results and discussion

Citrus pectin from Danisco (DM 64%) was used as starting material for the methylation experiments under different conditions in order to increase the DM. A fully methylated pectin (DM 100%) was subsequently saponified, decreasing the DM to any desired amount. The DM was determined by ^{1}H NMR spectroscopy and controlled by a standard titration method[19] (Table 1).

A wide range of different methods for the alkylation of carboxylic acids, and in particular for the methylation, is available. These methods might roughly be divided into two groups: basic, i.e., nucleophilic attack by the carboxylate at an electrophile and acidic, i.e., nucleophilic attack at the carbonyl oxygen within the carboxylic acid group.[20] Different basic methylation conditions were explored (Table 1) and the molecular weight (MW) of the products obtained was determined. Reaction of tetrabutylammonium pectinates with methyl iodide as the electrophile (Entry 1) has previously been used for the alkylation and cross-linking of galacturonans.[21–23] We were, however, unable to achieve high degrees of methylation without extensive depolymerisation. No comments upon the MW or the degree of degradation were made.[21–23] Likewise, depolymerisation and no methylation was observed when the pectin was treated with cesium fluoride and methyl iodide in *N*,*N*-dimethylformamide[24] (Entry 2). Under

Table 1
Methylation of raw pectin (Grindsted®Pectin R 450)

	Methylation conditions	Temp.	Time	DM by NMR	DM [a]	Mw (kDa) [b]
1	TBA·OH, MeI, DMSO	rt	2 days	84%	84%	[c]
2	CsF, MeI, DMF	rt	5 days	61%	–	[c]
3	MeOH, HCl (36%) [d]	reflux	16 h	96%	–	[c]
4	MeOH, HCl (36%) [d]	35 °C	6 h	84%	82%	51
5	MeOH, H_2SO_4 (concd) [d]	rt	6 days	92%	94%	13
6	MeOH, H_2SO_4 (concd) [d]	5 °C	24 days	92%	95%	32
7	MeOH, AcCl [e]	5 °C	5 days	~100%	~100%	31

[a] Determined by the standard titration method.[19]
[b] Starting pectin: Mw = 93 kDa, determined by viscosity measurement.
[c] The product was soluble in MeOH indicating a very low Mw.
[d] 2N.
[e] 1.8N.

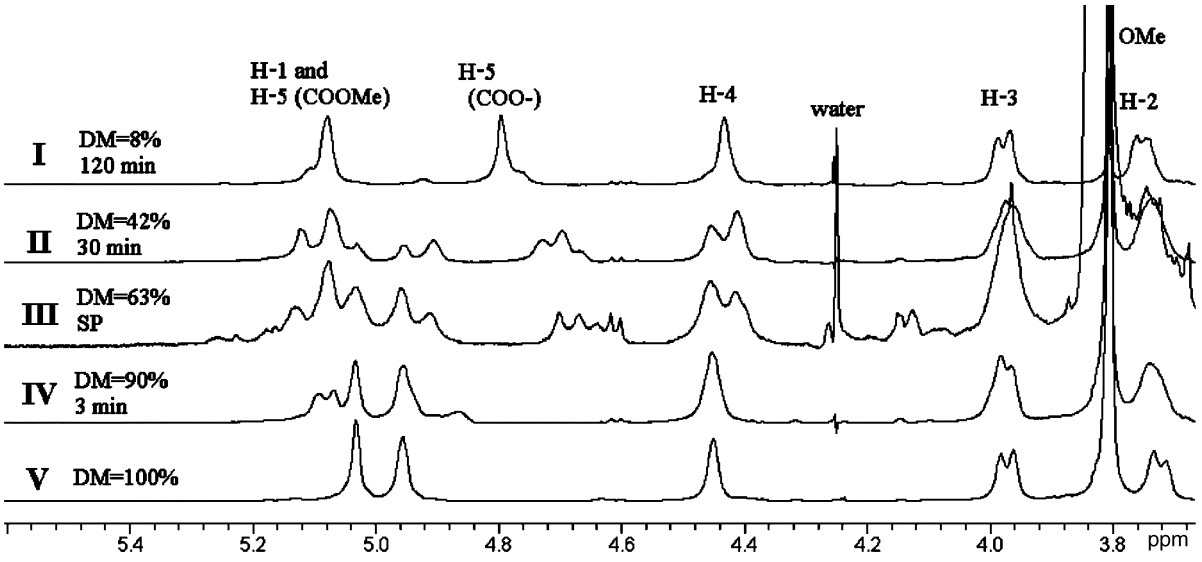


Fig. 1. ^{1}H NMR spectra (D_2O) of pectins with different degree of methylation (DM). The original-pectin (SP, **III**) was first esterified to DM 100% (**V**) and then saponified for the times shown to give the products **I**, **II** and **IV**).

these basic conditions, partly methylated pectin underwent significant degradation of the polymer chain, due to backbone cleavages caused by β-eliminations.[25] The most commonly used method for methylation of pectin has been treatment with methanol acidified with either sulfuric or hydrochloric acid.[26,27] These are heterogeneous reactions due to the lack of solubility of pectin in organic solvents. Furthermore, the reactions have to be carried out at 0–5 °C in order to minimise the cleavage of the glycosidic linkages. This means that very long reaction times, typically in the order of weeks, are required to obtain fully methylated material.

In order to shorten the reaction times, treatment of pectin with acidic methanol at elevated temperatures was investigated (Entries 3–5). These conditions gave products with a DM around 90% but degradation also occurred. In order to minimise the hydrolysis of the glycosidic bonds and to eliminate the possible hydrolysis of the already formed methyl esters, the use of anhydrous conditions was investigated. Methylation using methanol acidified with concd H_2SO_4 at 5 °C[28] proceeded with some degradation and at a fairly low rate (Entry 6). We found that the most efficient method, which proceeded with the least degradation (when accounting for the achieved DM) and with a reasonable reaction rate, was the use of anhydrous methanol acidified with acetyl chloride at 5 °C (Entry 7). Under these conditions, pectin with DM ~ 100% was prepared.

Pectin with lower DM can easily be obtained by simple treatment with aqueous base. The degree of methylation can be tailored to any desired value by controlling the reaction time. The DM of these products has also been determined by ^{1}H NMR spectroscopy (Fig. 1).

2.1. NMR analysis of the pectins: determination of DM

The NMR spectra were measured at 80 °C with dilute samples of pectin in D_2O in order to diminish the viscosity of the sample.[18] Measuring the spectra at 80 °C had the additional advantage to shift the signal from residual water upfield to approximately 4.2 ppm, where it did not interfere with any of the signals from pectin. Even when the pectin samples were carefully dried and coevaporated several times with D_2O, the DOH peak could not be avoided completely. It could, however, be suppressed by presaturation of the signal with a selective soft pulse. The spectra were recorded at a pD around 7. The integral limits have been defined manually for each spectrum according to its appearance.

^{1}H NMR spectra of pectin samples with a high DM (**V**) and a low DM (**I**) (Fig. 1) allowed the assignment of the anomeric protons (H-1) and the H-5 protons in fully and low methylated pectin samples. In **V** these protons are all found as two peaks around 5 ppm, whereas H-5 in samples having free carboxylic acid groups (**I**) are found around 4.8 ppm.[18] Measuring the samples at a pD around 7 ensured full deprotonation of the carboxylic acid groups. The pD was adjusted by addition of sodium triethylene tetraamino hexaacetate until pD 7. This salt will also chelate with divalent cations present in the sample, especially Ca^{2+}, thereby preventing possible interactions between metal ions and pectin, which could cause an increased viscosity.

The five spectra shown in Fig. 1 are obtained from pectin samples with different degrees of methylation (DM). Spectrum **III** is obtained from pectin (isolated from lemon peels) having a DM 64% (titration). The H-5 protons adjacent to the free carboxylate groups are clearly seen at 4.6–4.8 ppm whereas the signals for H-5 protons adjacent to the ester groups have shifted downfield to about 5.0 ppm, as seen by comparing spectrum **I** and **V**. The signal from H-1 protons is found in the lower region. The H-4 protons absorb around 4.4 ppm and are shifted slightly downfield upon methylation (compare **I** and **V**). In the intermediate range of DM the H-4 protons are usually seen as two adjacent peaks without baseline separation. The H-2 and H-3 protons are found around 3.7 ppm and 4.0 ppm, respectively, and are usually unaffected by methylation of the carboxylic acid residue.

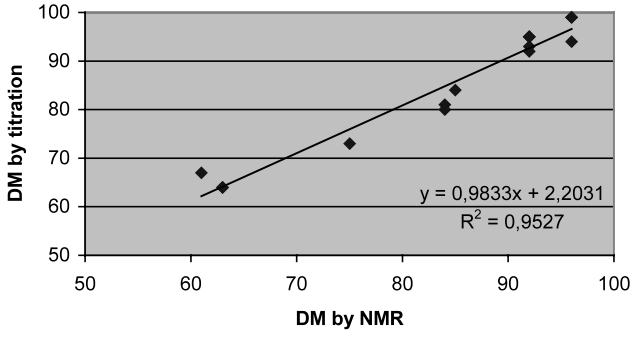


Fig. 2. DM by NMR vs DM by titration. $R^2 = 0.95$.

The degree of methylation (DM) is defined as the amount of ester groups compared to the total amount of carboxylic acid and ester groups. For determination of DM, the integrals of H-5 adjacent to esters (I_{COOMe}) are compared to the sum of the integrals of H-5 adjacent to esters (I_{COOMe}) and H-5 adjacent to carboxylates (I_{COO^-}). Due to the close proximity (or overlap) of the signals for H-1 and H-5_{COOMe}, it is only possible to determine the combined integrals for H-1 and H-5_{COOMe} ($I_{H1} + I_{COOMe}$). Fortunately, this value can be introduced into the equation for the DM since the total amount of the H-5 protons are equal to the sum of the anomeric protons H-1: $I_{COOMe} + I_{COO^-} = I_{H1}$. After some simple mathematical manipulation, the DM can be determined from Eq (1):

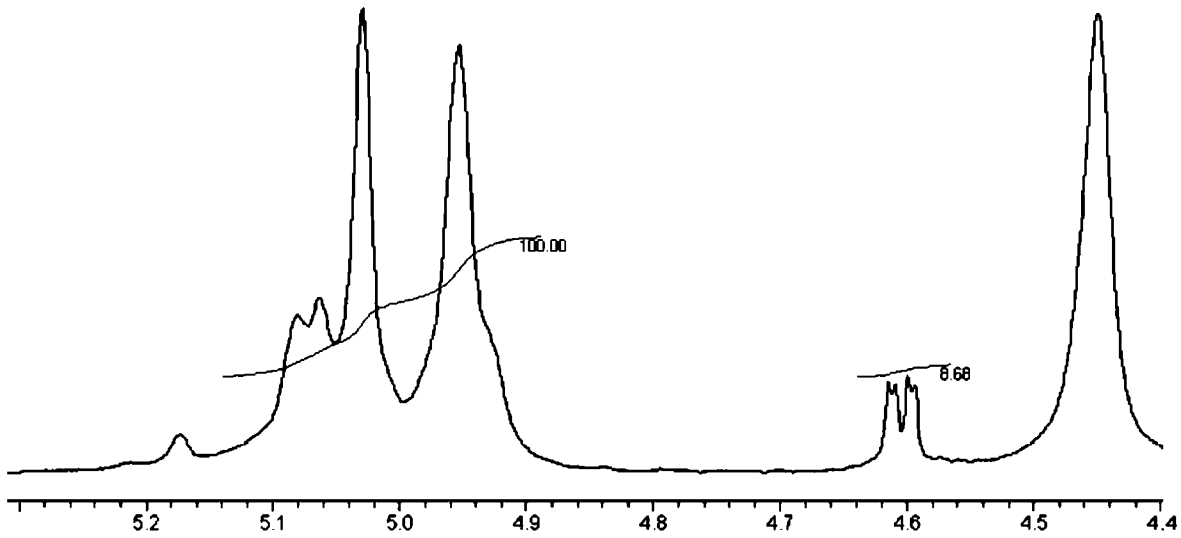
$$\begin{aligned} DM &= \frac{I_{COOMe}}{I_{COOMe} + I_{COO^-}} = \frac{2I_{COOMe}}{2(I_{COOMe} + I_{COO^-})} \\ &= \frac{I_{COOMe} + I_{COOMe} + (I_{COO^-} - I_{COO^-})}{I_{COOMe} + I_{COOMe} + I_{COO^-} + I_{COO^-}} \\ &= \frac{(I_{COOMe} + I_{H1}) - I_{COO^-}}{(I_{COOMe} + I_{H1}) + I_{COO^-}} \end{aligned}$$

Determination of the DM (1)

The determination of DM by ^{1}H NMR spectroscopy shows very good agreement with the results obtained by the standard titration method[19] (Table 1) as is also seen clearly from the close correlation (R^2 0.95 by least squares fit) between the values obtained from ^{1}H NMR measurements and those obtained by titrations (Fig. 2).

Determination of DM in pectins by NMR spectroscopy has been pioneered by Grasdalen[18] who performed detailed analysis of the sequence of free galacturonic acid and the methyl esters in tri- and tetrameric pectin fragments. Our goal was to establish a convenient method for analysis of the DM of high molecular pectins with different degree of methylation, compounds which have been prepared by modification of pectin by chemical means. An illustrative example is shown in Fig. 3 of a methylated pectin resulting in a product with DM 84%, determined by NMR spectroscopy as well as by titration.

By using our simple method, it will now be possible to prepare pectins having a specific DM, and thus with specific gelling properties which are dependent on the DM.[29,30] The procedure is to convert raw pectin to the fully methylated derivative, followed by partial demethylation for desired degree of DM, and determination hereof by NMR spectroscopy.



$$DM = \frac{(I_{COOMe}+I_{H1}) - I_{COO-}}{(I_{COOMe}+I_{H1}) + I_{COO-}} = \frac{100 - 8.68}{100 + 8.68} = 84\ \%$$

Fig. 3. Determination of the degree of methylation (DM) from the ^{1}H NMR spectrum (D_2O) of a methylated pectin.

2.2. Reduction of pectin

As discussed above, the degree of esterification of pectin is responsible for the gelling properties due to variation of the interactions between pectin molecules and calcium-ions[4,29,30] and with other molecules, e.g., sucrose[29,30] as discussed above. In order to obtain pectin with new properties, it was desirable to transform the galacturonate residues in a pectin polymer into galactose residues. In this way, modified pectins could be prepared thereby changing the ability to participate in the natural gelling processes. Since a carboxylic ester can be reduced to an alcohol group in the presence of a free carboxylic acid group, an investigation of the reduction of pectins with different DM was undertaken.

The major problem with chemical modifications of pectin is the lack of solubility in organic solvents. It is, however, well known that methyl esters of carboxylic acids, and especially methyl esters of amino acids[31,32] can be reduced using an excess of sodium borohydride in solvents like water or water/ethanol mixtures. Furthermore, these conditions are selective for the reduction of the methyl ester to alcohol groups in the presence of carboxylic acids,[33] and will finally result in an alkaline reaction mixture. Thus, in order to avoid the base induced β-elimination reaction and a potential hydrolysis of the esters, it was necessary to keep the pH around 7 using imidazole/HCl as a buffer and to perform the reactions at 5 °C[34] (Scheme 2, reaction a).

The introduction of primary alcohol groups instead of carboxylic acid functions can be tailored by choosing the DM of the starting pectin. When a pectin with DM 100% was treated under these conditions, complete reduction was obtained to give compound **C** (Scheme 1), while a pectin with DM 64% will be converted into product **A** (Scheme 2), where 2 out of 3 galacturonic units have been reduced, corresponding to complete reduction of the esters in this pectin. Reduction of ester groups in pectin on a preparative scale has never been described and the compounds prepared in this work are new modified pectins.

Further modification can be obtained by a subsequent methylation of the remaining acids in **A** to give product **B** (Scheme 2). By these reactions, three new types of pectin analogues have been prepared: **A** containing free carboxylic acid groups and primary alcohols, **B** containing methyl esters and primary alcohols, and **C** in which only primary alcohol groups are present (Scheme 2). It should however be noted, that methylation under acidic conditions of compounds like **A**, gave significant amounts of depolymerisation, as measured by the reduction in molecular weight. This is consistent with the fact that the glycosidic linkage between galactose residues are more readily hydrolysed than the glycosidic linkage between galacturonic acid residues.

2.3. NMR analysis of the reduced pectins A, B and C

The partly reduced compounds **A** and **B** and the fully reduced compound **C** were investigated by ^{1}H NMR spectroscopy. In the spectrum of compound **C**, which only contains galactose units, the signal at $\sim$5 ppm corresponds exclusively to the anomeric protons, H-1_{OH} (Fig. 4), while the H-5 protons appear as a new signal, H-5_{OH}, at 4.1 ppm. In the ^{1}H NMR spectrum of **A**,

signals from galactose (e.g., H-5_{OH} at 4.1 ppm) as well as from galacturonic acid ring protons (e.g., H-5_{COO-} at 4.7 ppm) are found. The 2:1 ratio between these integrals is in accordance with complete reduction of the ester groups in a pectin with DM 64%, corresponding to a 2:1 ratio of galactose units and free uronic acids. No signals from OCH_3 groups were observed. When compound **A** was treated with acidic methanol, the galacturonic acids were methylated as shown by the downfield shift of the H-5 protons and the disappearance of the H-5_{COO-} (**B**, Fig. 4).

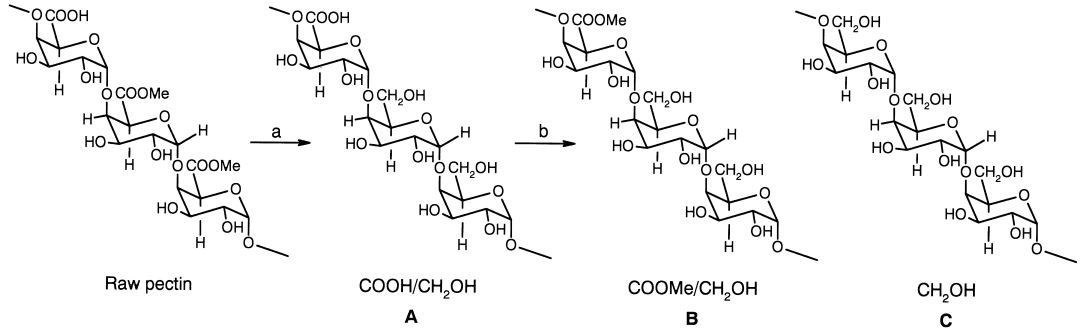
The degree of reduction DR was defined as the amount of alcohol groups compared to the total amount of carbohydrate residues. For the determination of DR, the integral of H-4 in galactose ($I_{H4(OH)}$) was compared to the sum of the integrals of all H-4 protons ($I_{H\text{-}4(OH)} + I_{H\text{-}4(COOMe\&COO^-)}$) (Eq (2)):

$$DR = \frac{I_{H\text{-}4(OH)}}{I_{H\text{-}4(OH)} + I_{H\text{-}4(COOMe\&COO^-)}} \quad \text{Determination of the DR} \qquad (2)$$

The DR-value can be calculated fairly accurately due to the summation of the integrals in the denominator. It is, of course, important to have the signal from residual water suppressed due to its close proximity.

To ensure complete reduction of the ester groups, a reaction time of 16 h was required. Shorter reaction times lead to incomplete reductions, yielding pectin derivatives containing both ester, acid and alcohol groups (Fig. 5).

Compounds obtained from incomplete reduction gave ^{1}H NMR spectra from which it was possible to determine the degree of reduction DR as well as the degree of methylation of the non-reduced carboxylic groups, DM_{NR}. The degree of methylation of the remaining carboxylic groups, DM_{NR}, was defined as the amount of methyl esters compared to the total amount of carboxylic acid groups present. For the determination of DM_{NR} the integral of any proton in the esterified galacturonic acids can be used (Fig. 4). The amount of ester groups was in this case determined as the difference between the integrals of all carboxylic groups and the free acids present (Eq (3)):



Scheme 2. (a) $NaBH_4$, imidazole, HCl, pH 7, H_2O, 16 h, 0 °C; (b) MeOH, AcCl, 2 days, 50 °C.

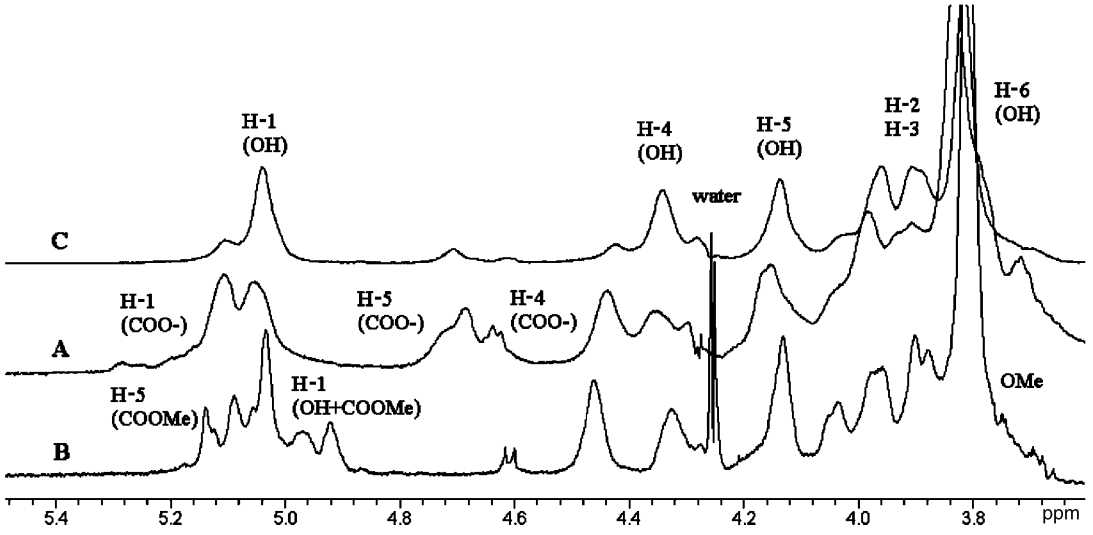


Fig. 4. ^{1}H NMR spectra (D_2O) of reduced pectins. **A**: 2/3 alcohol and 1/3 acid groups; **B**: 2/3 alcohol and 1/3 ester groups; **C**: only alcohol groups, i.e., 100% galactose.

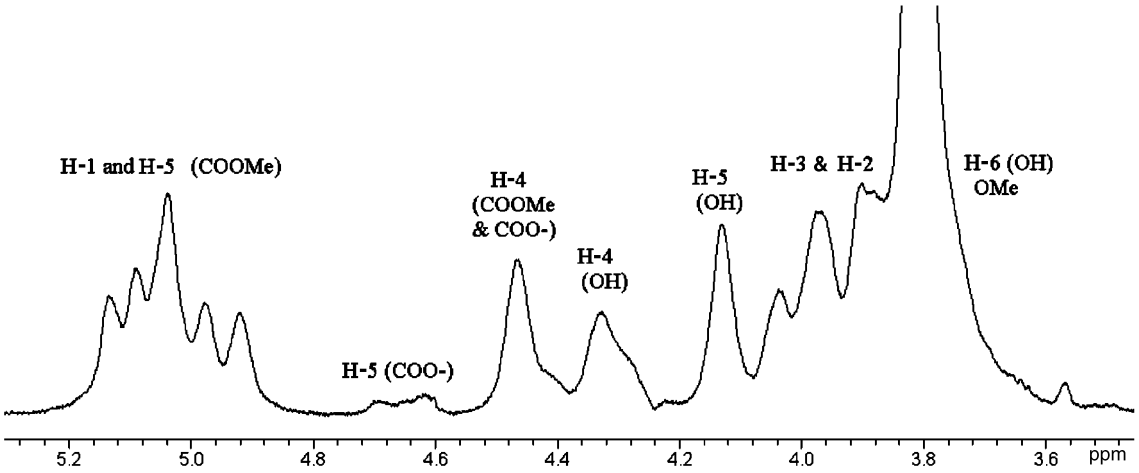


Fig. 5. Partly reduction of pectin with DM = 96%. The degree of reduction (DR) is 47%, and the degree of methylation of the remaining acids is 90%.

$$DM_{NR} = \frac{I_{H\text{-}4(COOMe\&COO^-)} - I_{H\text{-}5(COO^-)}}{I_{H\text{-}4(COOMe\&COO^-)}} = 1 - \frac{I_{H\text{-}5(COO^-)}}{I_{H\text{-}4(COOMe\&COO^-)}}$$

Determination of the DM_{NR} (3)

By comparison of DM and DM_{NR} values for a given reduction reaction, it was seen that no significant amount of the ester groups were hydrolysed in competition with the reduction.

Using Eq (3), it was established that the DM_{NR} could not be tailored in a simple way by varying the reaction time, DM of the starting pectin and the amount of $NaBH_4$ used. Thus, when pectin derivatives having both acid and ester groups in addition to primary alcohols should be prepared, the most efficient approach would be to perform a complete reduction of the esters followed by a partial methylation of the carboxylic acid residues.

2.4. Reduced pectins as substrates for pectinases

The activities of polygalacturonases from *Aspergillus niger*, PGI and PGII, and pectin lyase (PL) from the same species, were investigated using the reduced pectins **A**, **B** and **C** as substrates. The initial specific activities of PGI, PGII and PL are shown in Table 2, and are expressed as relative activities.

Polygalacturonase cleaves in non-methylated galacturonic acid regions. For polygalacturonases, the specific activity is therefore calculated relative to the activity obtained with polygalacturonic acid (**PGA**) as substrate (Table 2). The presence of methylester groups reduces the specific activity of polygalacturonases. The activity of PGI and PGII with pectin (DM 64%) as substrate is 55% and 32%, respectively, relative to the activity with polygalacturonic acid. Reduction of methyl ester groups to alcohol groups (sample **A**) decreased the activity further, whereas sample **B**, containing no carboxylic acid groups, exhibited only very low activity. The completely reduced sample **C** was neither a substrate for PGI, nor for PGII.

Both polygalacturonases, PGI and PGII, exhibit lower specific activity using the reduced pectins **A** and **B** as substrates. PGI is a processive enzyme while PGII is a non-processive enzyme.[35] PGI has a slightly higher activity with high methylated pectin than PGII, a feature which is also recognised with the reduced pectins where PGI showed slightly higher specific activity with the reduced pectins **A** and **B** than PGII. The results also indicates that there is no difference in affinity for alcohol groups between processive and non-processive PG enzymes.

The degradation products obtained from total digestion of sample **A** with PGII were characterised by MALDI TOF MS. The analysis showed that the prod-

Table 2
Initial specific activities *expressed as relative activities* in comparison of reduced pectins (samples **A**, **B** and **C**, Scheme 2) with Grindsted®Pectin RS 450 and polygalacturonic acid as substrates for the polygalacturonases from *Aspergillus niger*, PGI and PGII, and for pectin lyase, PL, from the same species

Sample	Structure	PGI	PGII	PL
A	2/3 alcohol, 1/3 acid	29	22	4
B	2/3 alcohol, 1/3 methyl ester	9	4	46
C	100% alcohol groups	0	0	0
Pectin RS 450	DM = 64%	55	32	100
PGA	DM ≈ 0–1%	100	100	0

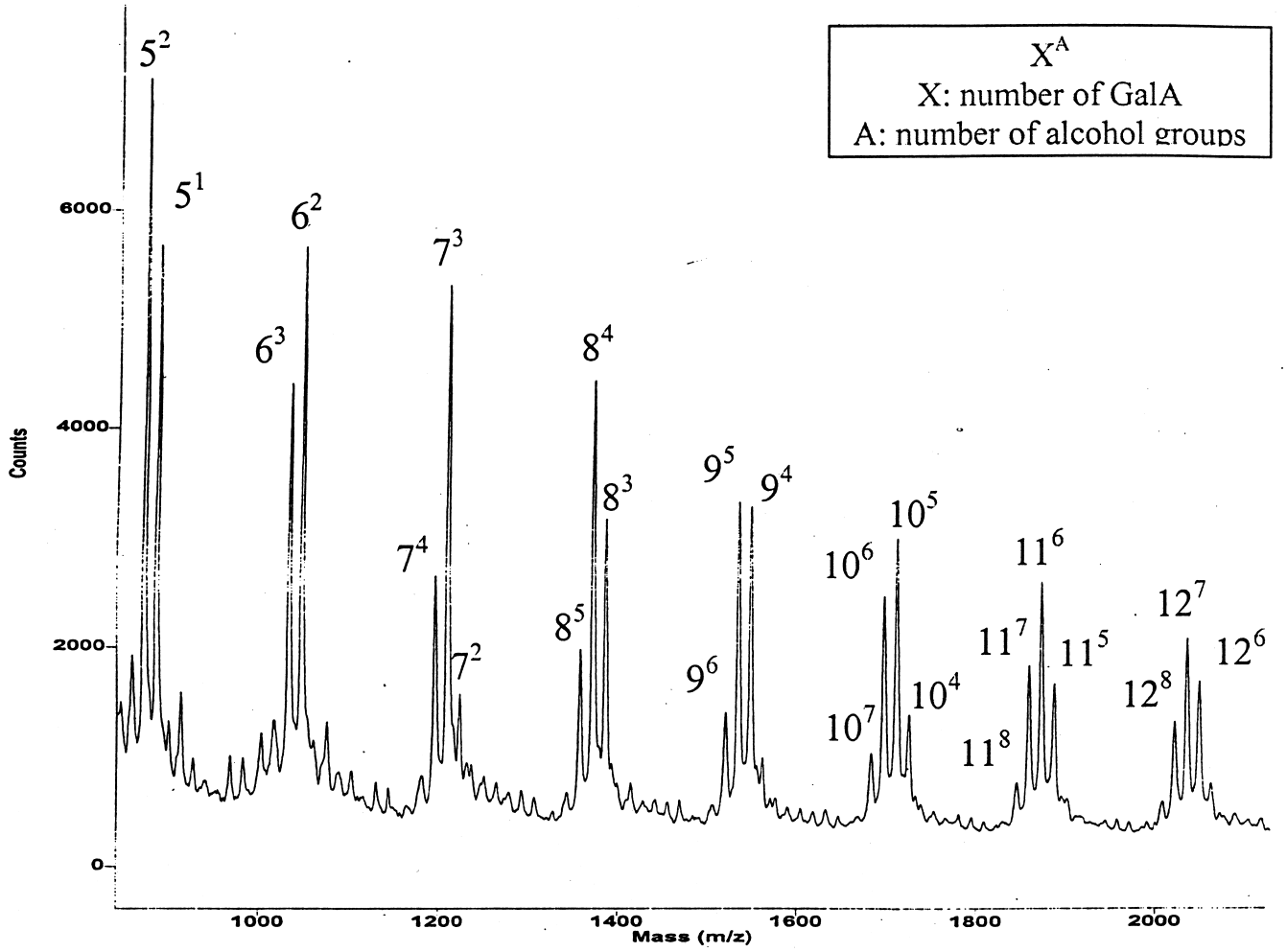


Fig. 6. Digestion (24 h) of sample **A** with PGII analysed by MALDI TOF MS in negative ion mode. The peaks are labeled by the DP of the generated fragment ion and the number of alcohol groups on then oligomer in subscript.

ucts have varying degree of polymerisation (DP) and varying amount of alcohol groups, e.g., pentamers with 1 or 2 alcohol groups (5^1 and 5^2), hexamers with 2 and 3 alcohol groups (6^2 and 6^3), heptamers with 3 alcohol groups (7^3) (Fig. 6). Only smaller oligomers (DP < 4) were detected without alcohol groups.

To determine the location of the methyl ester and alcohol groups, specific oligomer products were isolated and systematically fragmented by collision induced dissociation. In order to distinguish oligomer products possessing the reducing end from those possessing the non-reducing end, digests were labelled with ^{18}O after which tandem mass spectrometry was carried out.[5] The sequence analysis using ESI MS/MS of sample A digested with PGII revealed that both the reducing end and the non-reducing end contained a non-substituted galacturonic acid as the first unit (Fig. 7). PGII is therefore restricted to cleave between two non-substituted galacturonic acid. Furthermore, in many oligomers the second unit from the reducing end was

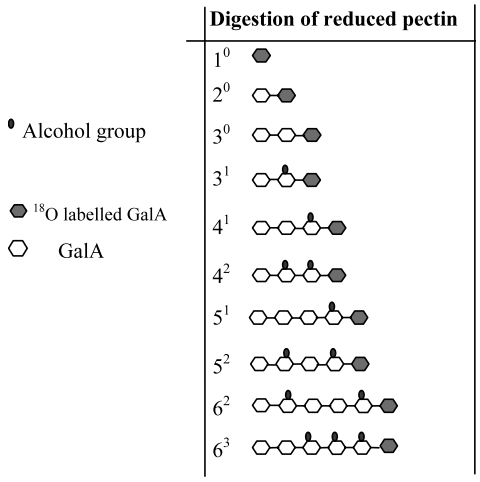


Fig. 7. Major degradation products from pectin sample **B** digested with PGII analysed by ESI MS/MS.

Table 3
Rheological data from the dynamic oscillatory experiments carried out at 25 °C at pH 3.9 and a sugar level at 50% on the unmodified (Grindsted®Pectin R 450) and the reduced pectin (sample **A**) at three different concentrations

Samples concentrations	Dynamic viscosity (Pas)	G' (Pa)	G'' (Pa)	Tan delta	Phase angle (°)
Pectin RS 450					
0.4%	0.125	–	0.580	–	90
0.6%	0.264	–	1.666	–	90
0.8%	0.517	0.168	3.249	19.33	86
Sample **A**					
0.4%	1.545	52.48	9.707	0.184	10.09
0.6%	5.236	354.6	32.95	0.0929	5.32
0.8%	9.943	847.9	62.48	0.0735	4.44

predominantly found to contain an alcohol group. Similar feature was also found for the methyl-esterified pectin RS 450 digested with PGII where the second unit from the reducing end was found to contain a methylester group (results not shown). Earlier results have shown that the methyl-esterification pattern found on single oligomer species were greatly conserved regardless of pectin.[5] With the present results, we found similar alcohol substitution pattern as found for methyl-esterification pattern. This clearly indicates that substitution of methylester groups with alcohol groups does not change the specificity of PGII and PGII can tolerate alcohol groups in the active site.

In contrast to the polygalacturonases, the pectin lyase (PL) requires highly methylated pectin as substrates, and the PL activities for the new compounds are thus calculated relative to the activity obtained with the unmodified pectin (Grindsted® Pectin RS 450). The results show that PL activity is significantly reduced when the methylester groups have been converted into alcohol groups (samples **A** and **C**). When the carboxylic acid groups in the reduced pectin **A** are methylated (sample **B**) the specific activity of PL is only reduced to 46% compared with the activity of highly methylated pectin.

The very low activity of PL with sample **A** as substrate, indicates that PL does not cleave regions of alcohol groups, whereas it can degrade the regions containing methylester groups in the cleavage site. PL can degrade the regions containing methylester groups since sample **B** is a substrate, but having only a PL activity of 46% relative to unmodified pectin (Table 2). This is in accordance with **B** having about half of the amount of ester groups compared to unmodified pectin.

The degradation products of total digestion of sample **B** by PL were analysed by MALDI TOF MS. The results clearly showed that degraded oligomers did not contain alcohol groups but only methylester and acid groups (results not shown). The liberated oligomers are varying in DP and degree of methylation. This result confirmed that PL cleaves in the methylated region of pectin and it demonstrates that PL is very specific for methyl esterified GalA residues in the pectin molecule for recognition.

Modification of pectin by chemical reduction of methylester groups to alcohol groups has a great influence on the specific activity of the two polygalacturonases and PL tested. The new substrates are accepted by the enzymes with the degrading pattern similar to the ones found with methyl substituted pectic substrates, but with a comparable reduced activity.

2.5. Reduced pectins as gelling agents. Rheological investigations

The rheological data for the unmodified (Grindsted® Pectin RS 450) and the reduced pectin (sample **A**) are presented in Table 3. The unmodified pectin shows no elasticity at concentrations of 0.4 and 0.6%, as evidenced from the phase angle value of 90, i.e., the response signal from the sample is totally out of phase with the applied signal from the rheometer and the lack of a value for G', the elastic modulus. Even at 0.8% pectin concentration, very little elasticity is observed in the sample. Here, the phase angle has dropped to 86 and the degree of elasticity, as indicated by the high tan delta value of 19.33 is low. Tan delta being given by G''/G'. Hence, the near total lack of elasticity suggests therefore, that the system would behave as a Newtonian liquid and any increase in viscosity can be assigned to increased intermolecular contact as concentration increases.

The results for the reduced pectin however, are significantly different, where, even for the lowest concentration, 0.4%, the G' value is greater than G'', indicating gel-like behaviour. Correspondingly, with a tan delta value of 0.184, a high degree of elasticity is indicated and the phase angle is 10, which shows the system's behaviour to be dominated by the elastic component. At the higher concentrations of 0.6 and 0.8% such differences are merely accentuated.

These differences can be clearly seen in Fig. 8, which shows dynamic viscosity as a function of pectin concentration, together with linear regression fits to the data. These fits give an indication as to the dependence on concentration of the viscosity. We observe that for the mother pectin, a gradient of 0.98 is given, suggesting that dilute solution type behaviour dominates. However, for the reduced pectin, the greater slope of the linear fit indicates that the viscosity changes much more rapidly with concentration compared to the mother pectin. This would tend to suggest that an overall different method of thickening, indeed one of gelation, is taking place. We can better appreciate this sol-gel transition by comparing the frequency sweep data for the two most concentrated samples, 0.8% as shown in Fig. 9.

The G' values for the mother pectin are less than G'' over the entire frequency range studied. The gaps in the data for G' at the low frequencies are due to phase angle results of 90°, hence no elasticity. This then characterises the mother pectin as rheologically behaving as a dilute solution. Further evidence of the lack of structure present within the mother pectin system can be seen from the value obtained for the relative gel strength, of 1.503. This is a dimensionless quantity

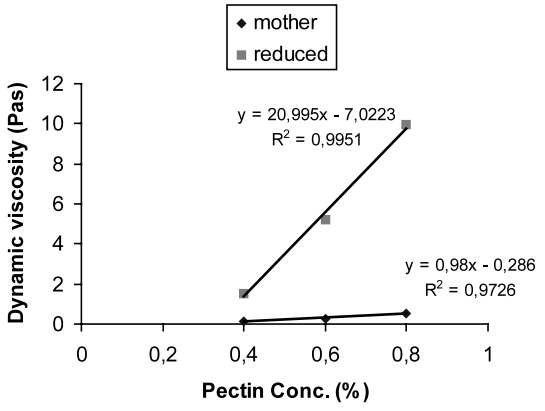


Fig. 8. Measurements of the dynamic viscosity versus pectin concentration for the unmodified (Pectin RS 450) and for the reduced citrus pectin (sample **A**).

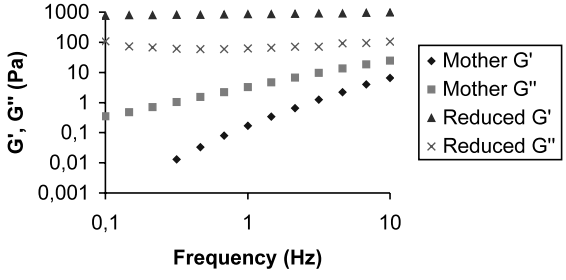


Fig. 9. The elastic and viscous modulii dependence on frequency for the unmodified and the reduced pectin (sample **A**) at a concentration of 0.8%.

described by Egelandsdal et al.[36] to quantify gel strength.

The data for the reduced pectin shows G' to be not only greater than G'' over the entire frequency, but generally independent of frequency. With relative gel strength of 0.0671, the value is very close to zero, the limit for totally elastic systems. Hence, the system can be characterised as a strong gel. If we compare the G' values for the mother and the reduced pectin at 1 Hz we see the extent of the sol-gel transition, resulting from the modification, being nearly 4 orders of magnitude.

Gelation mechanisms of pectin, both high ester (HE) and low ester (LE) have been extensively studied and reviewed.[37–39] HE pectin requires high soluble solids present as a co-solute in order to gel, while LE pectin can form gels in the absence of additional soluble solids, but requires the presence of calcium ions. Gelation and subsequent stability of HE gels depends on a complex mixture of hydrogen bonds and hydrophobic interactions, i.e., between the OH groups on the pectin backbone with sucrose and between the methyl groups, respectively.[40] In order for HE pectins to gel, the free energy of gel formation must be overcome.[41] These authors noted that both hydrophobic and hydrogen bonds are necessary for gelation, with hydrophobic interactions providing only half the free energy obtained from hydrogen bonding. The role of the sugar molecules is to stabilise the junction zones within the three-dimensional gel network.[40] Without the presence of the sugar, the hydrophobic interactions are too weak to form stable junction zones and no gel is formed.

In the systems described in this paper, the sucrose concentration was held at 50%, below the typical gel-forming threshold of 55%. Therefore, with insufficient sugar to adequately stabilise the junction zones, gel promotion is denied. Thus, our observed results for the mother pectin agree well with explained theory of HE pectin gelation, no gel is formed and the system behaves as a fluid.

In the modified pectin investigated (sample **A**) the methyl groups have been reduced into alcohol groups. Thus, **A** has two thirds of the carboxylic groups converted into primary alcohol groups and only one third remains as carboxylic groups. One effect of this modification can be considered as removal of hydrophobic acting groups in combination with addition of hydrogen bonding groups. With less methyl groups present, the tendency for hydrophobic interaction is reduced and consequently the amount of sugar required to help in the stabilisation of these weak junction zones may likewise be reduced, but the likelihood of a greater number of the relatively, much stronger hydrogen bonds being formed has increased.

We noted a definite transition from a 'dilute solution' to a 'week gel' between genuine and modified pectins, and in the absence of calcium, the most likely explana-

tion appears to be the increased OH groups promoting the formation of a greater number of hydrogen bonds. Adding this to the fact that less sugar is required to stabilise any remaining hydrophobic interaction and we have the basis for gelation within the modified samples under the same conditions that led to fluid behaviour from the mother pectin. We believe that difference seen between the mother and modified pectins are due solely to the chemical modifications described above, despite the fact that the mother pectin has not been subjected to the secondary dissolution and precipitation process undergone by the modified pectin.

3. Conclusion

Simple methodologies which allow customisation of the degree of methylation (DM) of pectin have been described. The methylations might be performed in anhydrous acidic methanol at low temperature to minimise depolymerisation (increasing the DM), or by saponification using aqueous base (lowering the DM). The DM can be tailored according to the saponification conditions in order to obtain pectins with specific gelling properties. The degree of methylation was easily determined by ^{1}H NMR spectroscopy, and a very good correlation with values obtained by the conventional titration method was achieved. The NMR method requires far less material (approximately 5 mg) than the titration method (100 mg to g amounts) and is thus more advantageous.

A new preparative method for making pectin derivatives, having primary alcohol groups at the C-6 position instead of/or in addition to the carboxylic acid and/or ester groups, has been described. Such modified pectins were obtained by reduction of pectins with different degree of methylation (DM). The degree of reduction (DR) and the degree of methylation of the remaining uronic acids could also be determined by ^{1}H NMR spectroscopy.

The new reduced pectins were tolerated as substrates for PGI and PGII and PL, and were cleaved accordingly to the preference of the enzymes for ester or carboxylic acid groups.

The reduced pectins showed an interesting, novel gelling ability compared to raw citrus pectin, and a method for preparation of pectins with other properties has now been demonstrated.

4. Experimental

4.1. General remarks

All reagents were obtained from commercial suppliers and were used without further purification. A commercial extracted slow set pectin (Grindsted®Pectin RS 450, DE 64%) from lemon peels was provided by Danisco. ^{1}H NMR spectra were recorded with a Varian Inova 500 spectrometer. Chemical shifts (δ) were measured in ppm using 2,2-dimethyl-2-silapentane-5-sulfonate as a reference. The *determination of molecular weight* (*MW*) was performed according to literature procedures.[4]

4.2. Enzyme assays

The polygalacturonases from *Aspergillus niger*, PGI and PGII, was a kind gift from Dr. Jacques Benen, Department Agrotechnology and Food Sciences, Laboratory of Microbiology, Wageningen University, The Netherlands. The initial activity of polygalacturonase (PG I and PG II, respectively) was measured by determining the increase in reducing ends[42] using galacturonic acid as a standard. Pectin dissolved in 50 mM NaOAc pH 4.2 (0.09% (w/w)) was mixed with 100 μL PG and incubated at 40 °C. Aliquots of 125 μL were withdrawn at time intervals and the reducing end was determined.[43] The initial activity was calculated from the rate of formation of new reducing ends over a time interval of 30 min. One U expresses the formation of 1 μmol reducing ends per min.

Pectin lyase (PL) from *Aspergillus niger* was purified as described by Limberg et al.[4]. PL cleaves pectin by β-elimination and generates non-saturated pectin oligomers containing a terminal non-reducing residue with a 4,5-double bond. The initial activity was followed continuously in a spectrophometer at 235 nm using a 1 mL solution of pectin (5 mg/ml) in 50 mM NaOAc pH 5.0 at 40 °C. The rates were calculated using 5500 M^{-1} cm^{-1} as the molar absorption coefficient.

For specificity determinations, the pectin samples were incubated with PL and PGII, respectively, for time intervals ranging from 0 to 48 h. After boiling for 5 min to stop the enzyme reaction, the digests were desalted over cation-exchange resin Dowex 50W X8 miniature columns before MS analysis.

4.3. Mass spectrometry

Matrix assisted laser desorption ionisation (MALDI) time-of-flight (TOF) spectra were acquired on a PerSeptive Biosystems Voyager-DE using delayed ion extraction (delay time 100 ns). To avoid saturation of detector gating (cut-off at 500 Da) was used. The instrument was calibrated externally in negative ion mode using PGII digested demethylated polygalacturonic acid oligomers.[5] Sample preparation was performed as previously described.[5]

ESI MS spectra were acquired on an Esquire-LC quadrupole ion trap mass spectrometer (Bruker Daltronik GmbH, Bremen, Germany). Analysis was performed at a fundamental radio frequency of 781 KHz.

The standard scan range was used at a scan speed of 13 000 $m/z\ s^{-1}$.

To determine the location of the alcohol groups and the methyl groups in the digest, specific oligomer products were isolated and systematically fragmented by collision induced dissociation. In order to distinguish oligomer products possessing the reducing end from those possessing the non-reducing end, digests were labelled with ^{18}O.[13]

4.4. Methylation of pectin

Anhydrous MeOH (800 mL) was cooled to 0 °C and acetyl chloride (40 mL) was added slowly. The mixture was stirred for 1 h and pectin (20 g) was added. The suspension was stirred at 5 °C for 27 days during which the acidic MeOH was changed twice. The methylated pectin was filtered off and washed carefully with 60% aq EtOH acidified with 5% concd aq HCl, followed by 60% aq EtOH until no more chloride was present in the washings. Finally, the methylated pectin was washed with abs EtOH and dried under diminished pressure. Yield: 19.2 g. The product had DM 100% (**V** Fig. 2). The yield corresponds to 94%, based on an increase of MW by 2.5% caused by the methylation.

4.5. Hydrolysis of methyl esters in pectin

Pectin (DM = 100%) was dissolved in water (10 mL) and the pH was adjusted to 11 with NaOH (0.1N). The pH was kept at 11 by further addition of base for the time desired. The reaction was stopped by lowering the pH to 5.5 with HCl (0.1N). The pectin was precipitated by addition of abs EtOH and filtered off, washed carefully with 60% aq EtOH acidified with 5% aq HCl, followed by 60% aq EtOH until no more chloride was washed out. Finally, the product was washed with abs EtOH and dried under diminished pressure.

4.6. Reduction of methyl esters in pectin

Pectin (2.0 g, DM 100%) was carefully dissolved in imidazole-HCl buffer (200 mL, 0.5 M, pH 7) and cooled to 0 °C. Use of a large beaker is recommended due to the possibility of excessive foaming in the subsequent reduction step. Sodium borohydride (10 g) was added in portions in the course of 2 h and stirring was continued at 5 °C for 16 h. The reaction was quenched by addition of AcOH (25 mL) while stirring was continued for 30 min. Addition of abs EtOH caused the product to precipitate. Filtration followed by careful washing of the product with 60% aq EtOH acidified with 5% aq HCl, followed by 60% aq EtOH until no more chloride was washed out. Finally, the reduced pectin was washed with abs EtOH and dried under diminished pressure. Yield: 1.48 g.

The degree of reduction was determined from a ^1H NMR spectroscopy (Fig. 4).

4.7. Preparation of NMR samples and NMR conditions

The pectin sample (5–7 mg) was dissolved in D_2O (1 mL) together with a trace of sodium 2,2-dimethyl-2-silapentane-5-sulfonate and the pH was adjusted to 7 with sodium triethylenetetraminehexaacetate. The sample was then coevaporated with D_2O twice and dried under diminished pressure overnight. It was redissolved in D_2O (0.7 mL).

NMR spectra were recorded at 80 °C with pre-saturation of the residual water signal.

In each case, the pulse width and intensity was optimised to achieve maximum water suppression with a minimum of disturbance of any other signal.

4.8. Rheological investigations

Rheological measurements were performed using a controlled stress rheometer, RS 150 from TermoHaake.

The natural pectin with a degree of esterification (DE) of $\sim 64\%$ was used as supplied. Reduced pectin, where one third of the carboxylic groups remained and two thirds has been substituted with primary alcohols (sample **A**) was ground with mortar and pestle such that it would pass a 0.25 mm sieve. Three pectin concentrations were chosen, 0.4, 0.6 and 0.8% for experimental examination. The sugar level within the system was 50% and pH was held at 3.9 ± 0.1 by addition of 0.1 M citric acid. All experimental systems ended with a final weight of 100 g. The experimental system was made up by first mixing a portion of the total sugar with the pectin to form a dry blend. This dry blend was dispersed in 50 mM Na acetate (40 mL) with stirring. Heat was then applied through a hot plate and stirring continued until boiling was achieved. Upon boiling, the remaining sugar was added and the suspension was stirred until dissolved, whereupon 12 g 0.1 M citric acid was added. The system was again stirred and heated until the weight reached 100 g before being left to cool. The sample was left to stand at room temperature overnight before measuring its rheological properties.

Dynamic oscillation experiments, were performed using a controlled stress rheometer, RS 150 from ThermoHaake. Two geometries were used, a cone-plate of 35 mm diameter and 2° cone angle, with a gap setting of 0.105 m and a double concentric cylinder, where the cup has an outer diameter of 43.5 mm and an inner diameter of 32 mm and the bob an outer diameter of 43 mm and inner diameter of 36 mm and a gap setting of 5.1 mm. Throughout all experiments, the temperature was held constant at 25 °C. For each sample, a frequency sweep from 0.1 to 10 Hz was run at the

constant stress of 1.0 Pa, deemed to be within the linear viscoelastic region. The results are depicted in Table 3, Figs. 8 and 9.

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