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Enzymatic modifications of pectins and the impact on their rheological properties

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Abstract

The interaction of new pectin-modifying enzymes (Novo) with three commercial pectins and their chemically de-*O*-acetylated and de-esterified counterparts is investigated. The study focuses not only on the ability of these pectins to act as substrates, but also on the consequences of modifications on their rheology and molecular weight. We present results that demonstrate that highly specific modifications of pectins are attainable. Enzymatic alterations of the side chain regions yielded polymers having a significantly lower viscosity, in the absence of calcium. In contrast, the viscosity of calcium-free pectin samples was increased after de-esterification of the backbone with pectin methylesterase. If the rheological properties of de-methoxylated pectin were studied in the presence of calcium, G'-values were increased 35-fold and the gel-like properties were markedly enhanced.These preliminary experiments emphasise the high application potential of enzyme-treated pectins. However, further investigations are required to custom-tailor pectins for utilisation in various food or other products.

Keywords: Pectin; De-esterification; Xylanase; Rhamnoglucanase; Endoglucanase

1. Introduction

Pectins are complex heteropolysaccharides consisting of homogalacturonan ('smooth', α(1,4)-linked galacturonic acid) and rhamnogalacturonan ('hairy', galA*p*α(1,2)-rha*p*α(1,4)-galA*p*α(1,2)-rha*p*) regions. In the latter, neutral sugar side chains containing mainly L-arabinose, D-galactose and D-xylose are covalently attached to the rhamnosyl residues of the backbone (DeVries, Rombouts, Voragen & Pilnik, 1982; DeVries, Den Vijl, Voragen, Rombouts & Pilnik, 1983). The galacturonic acid residues may be *O*-acetylated at HO-2 and/or HO-3 or methyl esterified at C-6 (Nelson, Smit & Wiles, 1977). Depending on the degree of methoxylation, pectins are classified into low-methoxyl (25–50%) or high-methoxyl (50–80%) pectins. Gelation in low-methoxyl pectin is mediated by calcium bridges between two carboxyl groups belonging to two different chains in close contact (Axelos & Thibault, 1991). Previous studies have shown that the gel properties of pectin gels depend on the molecular properties of the polymer. For example, Powell, Morris, Gidley and Rees (1982)demonstrated that the pattern of esterification, block-wise or random, has an impact on the aggregation and network properties. Moreover, the gelation properties of pectins are influenced by the length of their side branches and the degree of acetylation (Matthew, Howson, Keenan & Belton, 1990). The contribution of the side chains to the rheological properties of pectins has been scarcely studied (Hwang & Kokini, 1991, 1992).

Due to the widespread application of pectins in food products as gelling agents and stabilisers, they are of significant importance to the food industry. New pectin-modifying enzymes are now available in sufficient amounts to effectively study changes of the molecular properties of these polymers. This prompted us to investigate the effect of side chain and backbone-modifying enzymes on the viscosity of pectins and the viscoelastic properties of the corresponding gels.

2. Experimental

2.1. Materials

All enzymes obtained from Novo Nordisk (Denmark)

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were cloned from an *Aspergillus* species and expressed in *Aspergillus oryzae*, with the exception of polygalacturonase which was expressed in another *Aspergillus* species. Pectins were purchased from Fluka or Sigma. The pectins differ in their monosaccharide composition as well as in their degree of methoxylation and acetylation. The degrees of methoxylation are as follows: apple pectin from Fluka, 70–75%; apple pectin from Sigma, 70%; and citrus pectin from Sigma, 80%. Pectin methylesterase (from orange peel and tomato) were purchased from Sigma. D(+)-glucono-δ-lactone and calcium chloride dihydrate were obtained from Fluka and Merck, respectively.

2.2. Enzyme assays

In a standard procedure, 10 ml of a 2% solution of pectin in 50 mM ammonium acetate was used as a substrate for the enzyme reactions. Incubations with enzymes (10 mg) were performed at the pH values and temperatures indicated below. The reactions were terminated by heating at 90°C for 10 min.

Reactions with arabinanase were performed at pH 5.5 and a temperature of 55°C. The amount of arabinose released was estimated by using a commercially available enzymatic assay (Boehringer Mannheim, Germany).

Incubations with galactanase were conducted under the same conditions, with the exception that the pH was 4.0 and the temperature 60°C. The amount of galactose liberated was assessed by employing an enzymatic assay (Boehringer Mannheim, Germany).

The reactions with endoglucanase and xylanase were carried out at 50°C at a pH of either 5.0 (endoglucanase) or 6.0 (xylanase). The activities of both enzymes were estimated by measuring the increase in reducing groups using the Nelson–Somogyi assay (Collmer, Ried, & Mount, 1988).

The reactions with rhamnogalacturonase A and B were performed and monitored as described for endoglucanase and xylanase. The pH was adjusted to either 4.0 or 6.0 for rhamnogalacturonase A or rhamnogalacturonase B, respectively.

The polygalacturonase assay was conducted at 60°C, pH 6.0. The cleavage of the backbone was estimated by measuring the increase in reducing groups using the Nelson–Somogyi assay (Collmer et al., 1988).

Incubations with pectin lyase were carried out at 55°C, pH 6.5. The reaction was monitored by measuring the increasing absorbance of unsaturated products at 240 nm (Wijesundera, Bailey & Byrde, 1984).

Pectins were reacted with rhamnogalacturonan acetylesterase at pH 5.5 at 50°C. The reaction was followed by using an enzymatic assay (Boehringer Mannheim, Germany).

The pectin methylesterase assay was conducted at 50°C, pH 4.5. The release of methanol was followed according to the procedure of Wood and Siddiqui (1971).

2.3. Saponification of pectins

The acetyl groups and methyl esters were saponified by treating the pectins with 50 mM NaOH for 24 h at 0°C. The reaction mixtures were subsequently dialysed and lyophilised (Schols & Voragen, 1994).

2.4. Isoelectric point determination

The isoelectric point of the pectin methylesterase was estimated using the Pharmacia LKB Phast System. About 10 μg of protein sample and standards (pH 3.5–9.3) were applied to a phast gel, pre-focused between pH 3–9. The gel was stained with Coomassie Blue.

2.5. Rheological measurements

2.5.1. Measurement of the viscosity of pectins

10 ml of 2% solutions of pectins in 50 mM ammonium acetate, at the pH indicated above, were incubated with 10 mg of enzyme under the conditions described in enzyme assays (vide supra). Pectin samples, that were subjected to the same conditions in absence of enzyme, served as controls. The viscosities of the pectins were determined using a controlled stress Carri-Med Rheometer CSL2-50 equipped with a cone-plate device (6 cm diameter, 2°). Flow curves with increasing shear rates (10–300 s^{-1}) were measured at 20°C.

2.5.2. Characterisation of pectin gels

10 ml of 2% solutions of citrus pectin were incubated with enzymes as described above. Gelation was induced on the rheometer plate by first adding 200 mg of D(+)-glucono-δ-lactone followed by the addition of slowly dissolving anhydrous monobasic calciumphosphate to a final concentration of 0.8 mM. Samples were mixed by preshearing for 30 s at 5 Pa. Under these circumstances, homogeneous gels were obtained. Gel formation was also performed, under the same conditions, with unmodified citrus pectin. The same rheometer was employed, as described above, with a cone-plate device having a diameter of 4 cm (2°). Gel formation was followed by recording the storage modulus G' and loss modulus G'' up to maximal 1500 s at an oscillation frequency of 1 Hz. The stress applied was 1 Pa which was in the linear regime. The gels were further characterised by measuring the mechanical spectrum between 0.1 and 10 Hz at 1 Pa. The rheological experiments were conducted at 20°C.

2.6. Penetration test

Penetration tests were performed at 20°C using a Stevens-LFRA Texture Analyser (Stevens and Son) equipped with a cylindrical rod having a diameter of 4 mm. The rod was moved 20 mm below the surface of the gel at a rate of 1 mms^{-1}. The resistance against penetration of the rod was measured giving an indication of the gel strength

(Stevens value). The gels were prepared using one of the following procedures.

Procedure A. To 20 ml of a 2% pectin solution in 50 mM ammonium acetate (pH 4.5) the following was added: 50 mg rhamnogalacturonan acetylesterase and 10 mg methylesterase or 30 mg methylesterase without acetylesterase. The solutions were incubated overnight at 50°C, followed by addition of calcium chloride dihydrate to a final concentration of 50 mM. The incubation was continued for another 15 min at 50°C and the samples were then kept at room temperature for 6 h. Penetration tests were performed and the data were compared to those obtained after 7 d at 4°C. All penetration tests were conducted at 20°C.

Procedure B. To 20 ml of a 2% citrus pectin solution in 50 mM ammonium acetate (pH 4.5) 30 mg pectin methylesterase was added. The enzyme was dissolved in the pectin solution at room temperature and the mixture was then kept overnight at 4°C.

2.6.1. Molecular weight estimation

Molecular weights of modified and unmodified citrus pectin were derived from the corresponding intrinsic viscosities. The intrinsic viscosity $[\eta]$ is a measure of the hydrodynamic volume of the polymer which depends on its molecular mass and conformation. The intrinsic viscosities were obtained by measuring the specific viscosity $(\eta - \eta_s)/\eta_s$ (where η_s and η are the viscosities of the solvent and the pectin solution, respectively) at various pectin concentrations and using the following virial expansion

$$\eta_{sp} = [\eta]c + h[\eta]^2c^2 + \ldots$$

In this equation c is the polymer concentration and h is the Huggins constant. This constant is a measure of pairwise hydrodynamic interactions between the macromolecules. Experimentally h is between 0.3 and 0.8. In practice the reduced viscosity $(\eta - \eta_s)/c\eta_s$ is plotted against the polymer concentration. $[\eta]$ is derived from extrapolation to $c = 0$, i.e. $[\eta] = (\eta_{red})_{c\to 0}$ and h can be determined from the slope. An Ubbelohde type viscometer (Schott) was used to measure η and η_s.

The molecular weights were determined by applying the Mark–Houwink equation: $[\eta] = kM^a$ dlg^{-1}, using values for the constants a (0.79) and k (216×10^{-6}) that are suitable for high-methoxyl pectins (Berth, Anger, & Linow, 1977; Lapasin & Pricl, 1995).

3. Results

3.1. Enzymatic modifications

Different pectins were treated with pectin-modifying enzymes. An overview of the results is given in Table 1. Since no sugar analysis of the pectins was available, it was not possible to calculate the relative amount of monosaccharides liberated from the total initially present. De-*O*-acetylated and de-methoxylated pectins were obtained by saponification under mild conditions (Schols & Voragen, 1994). Cleavage of the backbone due to β-elimination is negligible under these conditions.

Table 1 shows that each of the enzymes reacted with at least one of the pectins. Apple pectin from Fluka was the only pectin recognised as a substrate by endoglucanase. The activity of pectin lyase was about the same for all three non-saponified pectins. Saponification increased the activity of rhamnogalacturonase A and B (Table 1). Monitoring these reactions by means of HPAEC with PAD detection revealed the formation of oligosaccharides (data not shown). These degradation products were not further analysed. The results obtained with pectin methylesterase showed that the enzyme liberated about the same amount of methanol (10%) from each pectin. When incubated with rhamnogalacturonan acetylesterase, about twice the amount of *O*-acetyl groups was removed from citrus pectin compared with apple pectin from Sigma (Table 1). Arabinanase and galactanase liberated arabinose and galactose; they released between 2.5 and 4.4% of the total initial amount of pectin (Table 1).

3.2. Isoelectric point

A precise determination of the isoelectric point of the pectin methylesterase was not possible since its isoelectric point is below the lowest isoelectric point of the standards (3.5). However, it can be estimated to be in the range of pH 2–3 (data not shown).

3.3. Viscosities of modified pectins

Pectin solutions (2 wt%) were incubated with side chain modifying enzymes (galactase, arabinase, endoglucanase or xylanase). In the absence of calcium all modified pectins gave viscous solutions rather than gels. The viscosity was measured as a function of shear rate and compared to the viscosity of the corresponding unmodified pectin. As an example, Fig. 1A shows the effect of galactanase treatment on the viscosity of apple pectin (Sigma). The enzyme treatment resulted in a significant decrease of viscosity. The shear rate dependence of the viscosity was not affected by the enzyme treatment. Incubation with arabinase, endoglucanase or xylanase also resulted in a decrease of viscosity. In all cases, viscosity was almost independent of shear rate. Zero-shear viscosities were estimated from the flow curves. Results for the various pectin/enzyme combinations are summarised in Fig. 2.

Pectin solutions (2 wt%) were also incubated with backbone-modifying enzymes (rhamnogalacturonan acetylesterase and pectin methylesterase). As an example, Fig. 1B shows the effect of rhamnogalacturonan acetylesterase on the viscosity of apple pectin (Sigma). A small decrease in viscosity was observed. Similar results were obtained for the other pectins used (Fig. 2). The flow curve changed

Table 1
Enzymatic modification of 2% solutions of different pectins (NA: not available)

Enzyme (10 mg)	Apple pectin (Fluka)[a]	Saponified apple pectin (Fluka)[a]	Apple pectin (Sigma)[a]	Saponified apple pectin (Sigma)[a]	Citrus pectin (Sigma)[a]	Saponified citrus pectin (Sigma)[a]
Arabinanase	4.4% of total weight	NA	3.0% of total weight	NA	2.7% of total weight	NA
Endoglucanase	0.60	NA	No reaction	NA	No reaction	NA
Galactanase	3.2% of total weight	NA	2.5% of total weight	NA	3.4% of total weight	NA
Pectin lyase	0.13	NA	0.13	NA	0.15	NA
Pectin methylesterase	8% of total methoxy	NA	10% of total methoxy	NA	10% of total methoxy	NA
Polygalacturonase	0.09	NA	No reaction	NA	0.05	NA
Rhamnogalacturonan acetylesterase	9.4 μg	NA	7.6 μg	NA	15.8 μg	NA
Rhamnogalacturonase A	0.50	0.92	No reaction	0.67	0.29	0.78
Rhamnogalacturonase B	No reaction	0.56	0.88	1.24	0.63	0.70
Xylanase	0.11	NA	0.13	NA	0.29	NA

[a] μmol reducing groups formed from 3.3 mg of pectin.

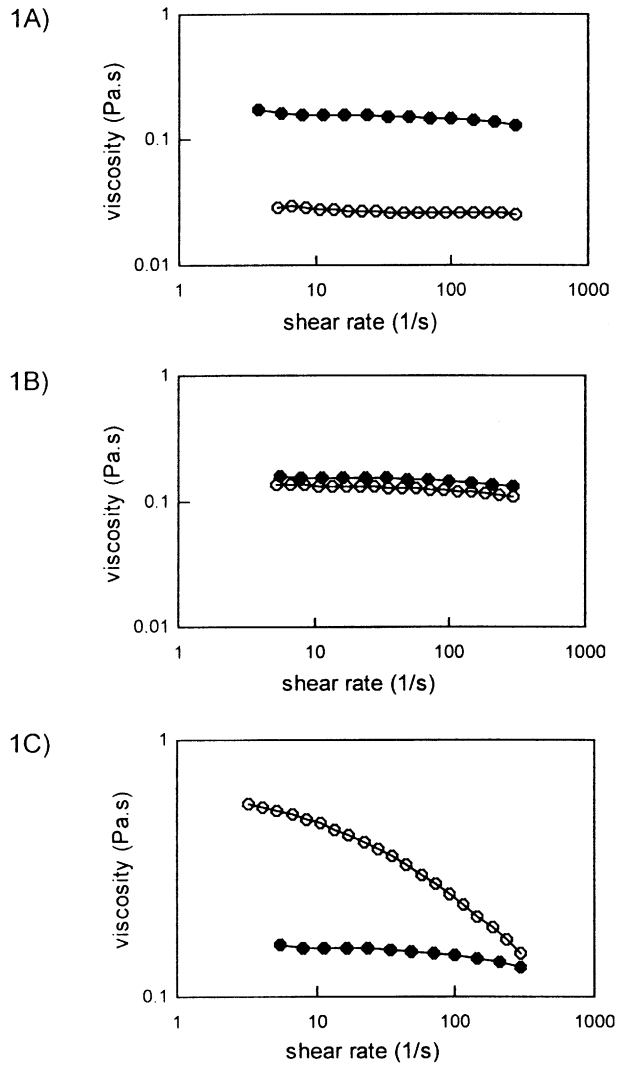

Fig. 1. Viscosity versus shear rate curves for apple pectin (Sigma) treated with (A) galactanase, (B) rhamnogalacturonan acetylesterase and (C) methylesterase (open circles) and the corresponding controls (closed circles). Pectin concentration: 2 wt%.

significantly after apple pectin (Sigma) was subjected to methylesterase activity (Fig. 1C). The zero-shear viscosity increased and the viscosity became strongly shear rate dependent. This increase of the zero-shear viscosity was particularly strong in the case of citrus pectin. Its zero-shear viscosity increased by more than a factor of 14 (due to the shear rate dependence, the zero-shear viscosity could only be roughly estimated), compared to a 7 or 4-fold increase measured for apple pectin from Fluka or Sigma, respectively. The corresponding zero-shear viscosities for apple pectin from Fluka were 0.4 Pa s (methylesterase-treated) and 0.06 Pa s (control) and for citrus pectin from Sigma 2 Pa s (methylesterase-treated) and 0.14 (control).

Fig. 3 shows an example of the reduced viscosity, η_{red}, for a dilute solution of a xylanase treated citrus pectin. At low pectin levels ($c < 0.15$ wt%) η_{red} varies linearly with concentration. The intrinsic viscosity $[\eta]$ is determined from the intercept ($c \rightarrow 0$) and from the slope, the Huggins constant was found to be 0.6. In Table 2, the intrinsic viscosities for modified and unmodified citrus pectin are listed. For unmodified citrus pectin we found $[\eta] = 3.3\ \mathrm{dLg}^{-1}$. This value is comparable to the value of $3.13\ \mathrm{dLg}^{-1}$ reported previously for apple pectin (Hwang & Kokini, 1992). The intrinsic viscosity of modified citrus pectins remained unchanged when the experimental error was taken into account. This error was about 10%, mainly due to the inaccuracy in concentration. This unchanged intrinsic viscosity confirms that no backbone cleavage has taken place. An exception was endoglucanase-treated citrus pectin, whose intrinsic viscosity was reduced from 3.3 to $1.9\ \mathrm{dLg}^{-1}$. Here, degradation of the backbone seemed to have occurred.

The intrinsic viscosities of the two apple pectins were not determined since it can be assumed that their susceptibility to backbone cleavage is comparable to that of citrus pectin. The molecular weights of the modified pectins were calculated from the intrinsic viscosities by applying the Mark–Houwink equation (as described in the Experimental section). Results are given in Table 2. It should be noted that the modifications may effect the Mark–Houwink constants. Therefore the molecular weights of the modified pectins are only approximate values. However, since the hydrodynamic volume of these polymers does not decrease, the conclusion that no significant backbone cleavage has occurred seems justified.

3.4. Rheological properties of pectin gels

A rapid evaluation of the gel properties of modified pectins was performed by means of penetration tests. The gels for the penetration tests were prepared according to procedures A and B (see Experimental section). Under the conditions of procedure A, methylesterase action or both methylesterase and acetylesterase action were allowed to take place. All pectins gelled in the presence of Ca^{2+}. Stronger gels were formed with enzymatically de-methylesterified, or de-methylesterified and de-*O*-acetylated pectins. The strongest gel was obtained with methylesterase and acetylesterase-treated apple pectin (Sigma) and Ca^{2+}. The relative strength of the other gels in comparison to this gel is illustrated in Fig. 4. In all cases, the gel strength increased upon ageing.

Using procedure B (no calcium present), a gel was only obtained with citrus pectin (Stevens value: 12). Under these conditions the temperature of the test system was kept far below the temperature optimum of the methylesterase (50°C). Apparently, gel formation still took place even when only minimal enzyme activity was present.

More detailed rheological information was obtained from small oscillation measurements. In this study gel formation was followed in the rheometer and the storage modulus G' and loss modulus G'' were recorded as a function of time. As an example, Fig. 5A shows results for methylesterase-treated citrus pectin. A rapid initial increase in G' and G'' is

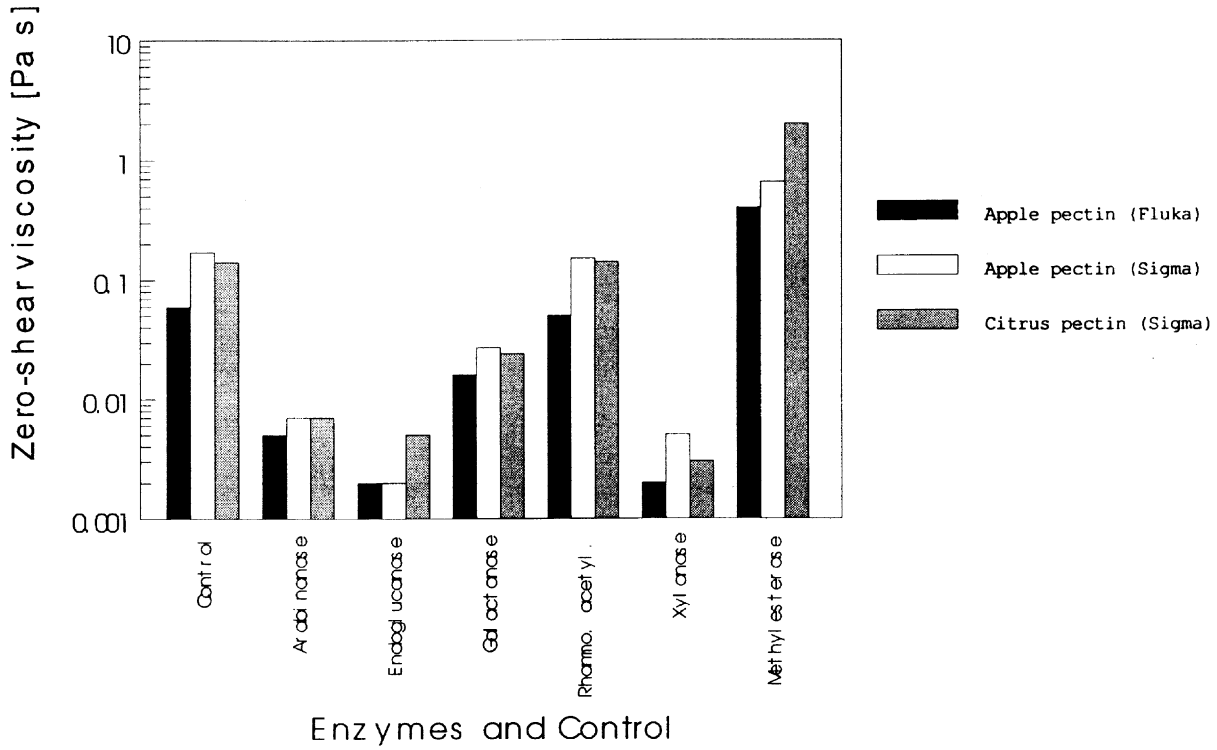

Fig. 2. Zero-shear viscosities of 2% solutions of unmodified pectins (control) and pectins that have been treated with pectin-modifying enzymes (see enzyme assays), in the absence of calcium.

observed followed by a much slower increase at later times. These results were compared to data obtained for unmodified citrus pectin that had been subjected to identical reaction conditions. In the latter case G' and G'' level off at much lower values, the maximum value for G' is ca. 35 times lower than that observed for the methylesterase-treated sample (measured at 1 Hz). Reaction with endoglucanase resulted in a 4-fold increase of G' and provided a stable gel. No gel formation took place when citrus pectin was subjected to either acetylesterase, arabinanase, galactanase or xylanase activity (Table 3). These systems had the characteristics of a purely viscous fluid. Simultaneous incubation of the pectin with acetylesterase, arabinanase, methylesterase and xylanase, followed by the addition of Ca^{2+}, also led to the formation of a strong gel (Table 3).

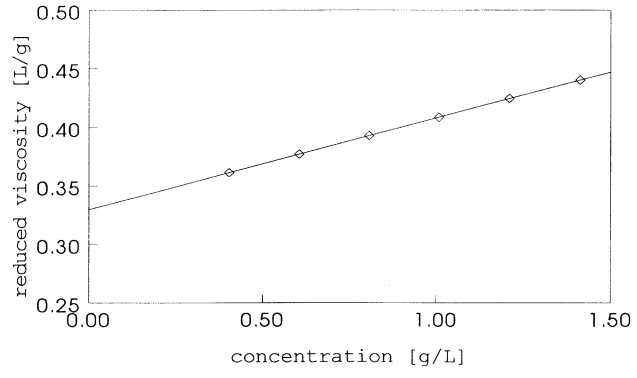

Fig. 3. Intrinsic viscosity of xylanase-treated citrus pectin.

Fig. 5B shows the frequency dependence of G' and G'' for methylesterase-treated citrus pectin. G' and G'' are almost independent of the frequency between 0.2 and 8 Hz, with G' being roughly 20 times higher than G'', indicating gel-like behaviour. The frequency dependence was also studied for the other enzyme-treated pectins. The results are summarised in Table 3.

4. Discussion

4.1. Modification of the side chains of pectin

At least one of the pectins was recognised by the pectin-modifying enzymes as a substrate (Table 1). By employing the Nelson–Somogyi assay, endoglucanase activity was only detected with apple pectin from Fluka. However, reacting all three non-saponified pectins with this enzyme resulted in a significant diminution of their viscosity (vide infra). This apparent contradiction can be accounted for by the fact, that the increase in reducing groups, formed by reaction of endoglucanase with the other two pectins, was below the detection limit of the Nelson–Somogyi assay. In this case however, only very few side chains could have been cleaved. Assuming that the observed reduction of the intrinsic viscosity by more than 30% was caused by a change of the polymer stiffness due to removal of side chains, a high endoglucanase activity would have been expected. Therefore the only explanation for the reduction

Table 2
Molecular weights and intrinsic viscosities of unmodified citrus pectin (Sigma) and citrus pectin (Sigma) treated with arabinanase, endoglucanase, galactanase, rhamnogalacturonan acetylesterase and xylanase

Citrus pectin	Unmodified	Arabinanase-treated	Endoglucanase-treated	Galactanase-treated	Rhamnogalact-uronan acetyl-esterase-treated	Xylanase-treated
Intrinsic viscosity (Lg^{-1})	0.33	0.28	0.19	0.32	0.33	0.32
Molecular weight (kDa)	197	162	98	190	197	190

of the intrinsic viscosity, for the endoglucanase treated pectin, is additional degradation of the backbone due to impurities in the enzyme preparation. However, the degradation product had a molecular weight of 97 kDa, which demonstrates that only limited cleavage took place.

The other side chain-modifying enzymes (arabinanase, galactanase and xylanase) formed only up to 1.24 μmol reducing groups from 3.3 mg of pectin (≈ 18 μmol monosaccharides), or released up to 4.4% (arabinanase) of the initial total weight of pectin. The difference in the activities of these enzymes with the pectins tested can, in general, be explained as follows: pectins contain side chains of varying length, number and monosaccharide composition (McNeil, Darvill & Albersheim, 1982; O'Neill, Albersheim & Darvill, 1990). They may consist of up to 15 monosaccharides (Bacic, Harris & Stone, 1988). As a consequence, the neutral sugars occur in varying amounts. This can even be the case for pectins from the same origin since the composition of the side chains also depends on the method of isolation and the purification of the pectin. Furthermore, if the potential reaction sites for the enzymes are located in the vicinity of the backbone, they might not be easily accessible.

Since these minimal enzymatic activities had a noticeable viscosity-decreasing effect, it seems likely that the degradation proceeded in an endo-fashion. Thus, cleavage of a single, glycosidic bond located in the inner part of the side chains resulted in the liberation of oligosaccharides. The diminution of viscosity due to backbone cleavage can be ruled out since the molecular weight of the modified pectins remained unchanged (taking into account the experimental error). Moreover, no galacturonic acid-containing degradation products were detectable with HPAEC analysis using PAD detection. Therefore, the observed effect on viscosity can be attributed rather to the degradation of side chains.

It has been documented that long branches can have a remarkable effect on the rheological properties of concentrated polymer solutions (Hwang & Kokini, 1992; Nielsen, 1977; Nordmeier, Lanver & Lechner, 1990). If the dimensions of the side chains are sufficient to cause a significant number of additional interchain entanglements, the viscosity can be increased at low shear rates compared to linear polymers of the same molecular weight (Fujimoto, Narukawa & Nagasawa, 1970; Graessley, 1977). However, if the branches do not have the length required for entanglements a decrease of viscosity is observed because the backbone of these chains is shorter than that of linear polymers (Miltz & Ram, 1973; Utracki & Roovers, 1973). Therefore, polymers containing short side chains are more compact than their corresponding linear counterparts. Consequently, their hydrodynamic volume is smaller, which in turn affects their viscosity. It is tentatively proposed that incubation of

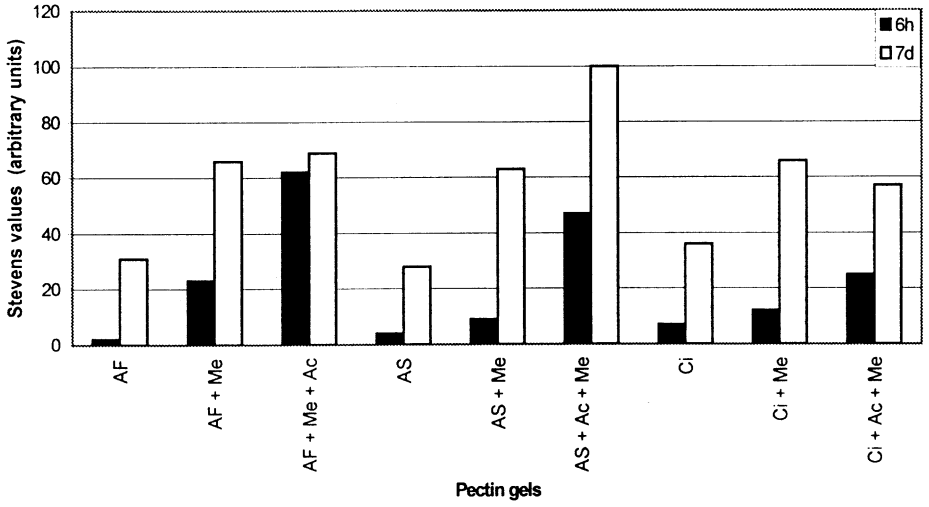

Fig. 4. Relative Stevens values in arbitrary units of different pectin gels formed in the presence of 50 mM Ca^{2+}. The Stevens values were determined after 6 h (■) and 7 d (□), respectively. Abbreviations: AF: apple pectin (Fluka); AS: apple pectin (Sigma); Ci: citrus pectin (Sigma); Ac: rhamnogalacturonan acetylesterase; Me: pectin methylesterase.

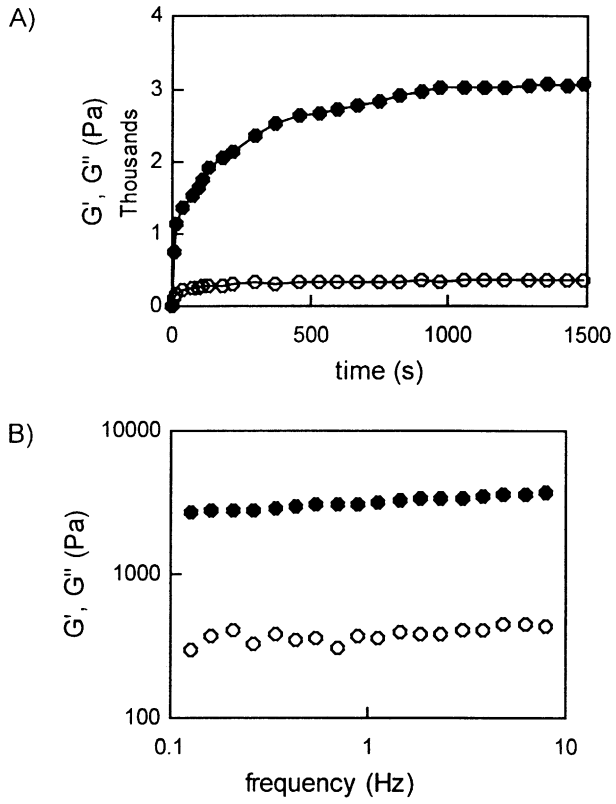

Fig. 5. G′ (●) and G″ (○) as a function of time (A) and frequency (B) for 2% citrus pectin (Sigma) treated with pectin methylesterase, in the presence of 0.8 mM Ca^{2+}.

pectins with the side chain-modifying enzymes leads to a reduction of the length of the branches below the critical dimension required for entanglements, which accounts for the reduction of viscosity observed here. For example, in the case of the xylanase-treated pectin we can predict a viscosity of only 2.6 mPa s due to hydrodynamic interactions using an intrinsic viscosity of 0.33 (Lg^{-1}) and a Huggins constant of 0.6 (see molecular weight estimation). The measured viscosity was 3 mPa s. Therefore the viscosity of the xylanase-treated pectin solution is solely caused by hydrodynamic interactions, indicating that the number of entanglements is strongly reduced. It should be mentioned that the occurrence of side chain entanglements in 2% solutions of unmodified pectins is not supported by the results in Fig. 1, which show that the viscosity is almost independent of shear rate. However a similar flow curve at a concentration of 2% was obtained by Hwang and Kokini (1992). They also measured a stronger shear rate dependence for higher concentrations and argued that by decreasing the concentration, the freedom of movement becomes less restricted due to the decreased number of entanglements. Therefore the time required to form new entanglements also decreases and, as a consequence, the shear rate at which Newtonian behaviour is no longer observed increases beyond the measurable shear rate range. Furthermore, when observing the intrinsic viscosity, we obtained a $c[\eta]$ value of 6.6. Since the overlap for pectin solutions normally starts to occur at a value 4 (Hwang & Kokini, 1992), this indicates that the 2% solution is in the entangled regime. An influence of conformational changes of the pectins due to the modifications of the side chains seems less likely. Hwang and Kokini (1992) have shown that the contribution of side branches to the conformation of pectins is negligible.

4.2. Modification of the backbone of pectin

It has been established that the degree of esterification has an impact on both, conformational and rheological properties of pectins (Anger & Berth, 1986; Fishman, Pfeffer, Barford & Doner, 1984). Therefore, the effect of pectin methylesterase incubation on the viscosity of pectins was investigated. Reaction of the pectins with this enzyme led to about 10% de-methoxylation. Addition of a second batch of enzyme at the pH optimum of the reaction did not improve the yield any further. A possible explanation is the well-known fact, that pectin methylesterases are inhibited by their end product, pectic acid, due to formation of inactive enzyme-product complexes (Lineweaver & Ballou, 1945). In order to screen the negative charges responsible for complex formation, the reaction was performed in presence of 100 mM sodium chloride. However, the degree of de-methoxylation achieved remained unchanged. Macdonald and Evans (1996) described an apple pectinesterase that was less active with higher methoxylated substrates. Accordingly, it is conceivable that the enzyme used in our study favours low methoxy pectins as a substrate, which could account for the results observed here.

Studies of the viscosity of the de-methylesterified pectins

Table 3
Rheological properties of 2% unmodified and 2% enzyme-treated citrus pectin solutions in the presence of 0.8 mM calcium. The loss and storage moduli compiled in the table were measured after 600 s. Abbreviations: Ci: citrus pectin; Ac: rhamnogalacturonan acetylesterase; Ara: arabinanase; Endo: endoglucanase; Gal: galactanase; Me: pectin methylesterase; Xyl: xylanase

Sample	G′ (Pa), 0.2 Hz	G′ (Pa), 8 Hz	G″ (Pa), 0.2 Hz	G″ (Pa), 8 Hz
Unmodified Ci	72	74	5	10.6
Ci + Ara	< 1.0	< 1.0	< 1.0	< 1.0
Ci + Endo	290	350	39	51
Ci + Gal	< 0.1	0.9	< 0.1	0.8
Ci + Me	2630	2720	120	138
Ci + Ac + Ara + Me + Xyl	2580	2610	115	124

revealed an increase which was especially remarkable in the case of citrus pectin (14-fold). It has been well established that the degree of methoxylation influences the conformational and rheological properties of pectins (Anger & Berth, 1986; Fishman et al., 1984). Thus the increase in viscosity of the less methoxylated pectins might be partly attributed to a conformational change which facilitates interactions between the polysaccharide chains. The effect of acetylesterase activity on the viscosity was insignificant. It is possible that the amount of acetyl groups released was insufficient to cause an alteration of the rheological properties of the pectins.

In this study less emphasis was placed on the investigation of backbone-degrading enzymes. The most promising enzymes were the rhamnogalacturonases A and B. HPAEC analysis revealed the formation of galacturonic acid-containing degradation products, which were not further characterised. The activity of both enzymes increased when the corresponding saponified pectins were used as substrates.

Mutter, Colquhoun, Schols, Beldman and Voragen (1996) described a native rhamnogalacturonase A and a recombinant rhamnogalacturonase B, both derived from *Aspergillus aculeatus*. The latter enzyme was provided by Novo. Their studies showed that the two enzymes operate in a different fashion. Enzyme A was found to be a hydrolase, whereas enzyme B was found to be a lyase. However, it is not known if these enzymes are identical to those applied here.

4.3. Pectin gels

The results obtained with procedures A and B (citrus pectin and pectin methylesterase) demonstrate that a 2% pectin solution gelled at pH 4.5. In the case of citrus pectin, a gel was formed with both procedures which suggests that methylesterase activity is not an absolute requirement. The gels obtained with de-methoxylated pectins or de-methoxylated and de-*O*-acetylated pectins were firmer (Fig. 4). This is in agreement with the study on the gelation properties of sugarbeet pectin (Williamson, Faulds, Matthew, Archer, Morris, Brownsey et al., 1990) and our own investigation of the elasticity and viscosity of pectins (vide infra). The authors reported that de-methoxylation and de-*O*-acetylation yielded a pectin of improved quality. They claimed that de-methoxylation facilitates the formation of calcium-linked junction zones.

In order to obtain further data concerning the stability and quality of gels, the gelling of unmodified and modified citrus pectins was monitored in the rheometer (Table 3). The results clearly show that the release of only methoxyl, or the simultaneous release of acetyl, arabinose, methoxyl and xylose residues from citrus pectin yielded pectins with significantly improved gelling properties. The elastic modulus G' of the corresponding gels was about 35 times higher than G' of a gel obtained with unmodified pectin (Table 3). A similar enhancement of gelation properties was observed with sugarbeet pectin when the degree of acetylation and methoxylation and the amount of arabinose were decreased (Matthew et al., 1990). The authors attributed this effect to steric considerations, a decrease in the neutral sugar content and an increased acidic nature of the modified pectins. In principle, we assume that the same arguments explain the results obtained in our study. However, our data suggest that the gel-improving effect was mainly due to methylesterase activity. The notion that pectins with shorter side chains should have better gelation properties was solidified by our results with endoglucanase-treated pectin (Table 3). The modification led to a 4-fold increase of G' of the corresponding gel when compared with the control. Moreover, measurements of the mechanical spectrum between 0.2 and 8 Hz indicated enhanced gel-like properties. The reduction of G' due to incubation with acetylesterase, arabinanase or galactanase (Table 3) does not contradict the previous interpretations. It is conceivable that these enzymes (or impurities in the enzyme preparations) complexed Ca^{2+}, thereby reducing the Ca^{2+} concentration below the critical value required for gel formation. Pectins which are less esterified are more susceptible to Ca^{2+}-induced gelation. This could explain the lack of inhibition of gel formation in the presence of methylesterase activity.

5. Conclusions

Background of the described research was the mapping of various hydrolytic enzymes as to their ability to modify pectins. If this treatment leaves the backbone untouched and exclusively modifies the branching it should open a way to alter and enhance the rheological behaviour of such polymers.

The results presented here demonstrate that highly specific modifications of pectins are feasible. Enzymatic alterations of the side chain regions provided polymers having a considerably lower viscosity, in the absence of calcium. In contrast, the viscosity of calcium-free pectin samples was increased after de-esterification of the backbone with pectin methylesterase. If the linear viscoelastic rheological properties of de-methoxylated pectin were studied in the presence of calcium, G'-values were increased 35-fold and the gel-like properties were markedly enhanced.

These preliminary experiments emphasise the high application potential of enzyme-treated pectins. However, further investigations are required to custom-tailor pectins for utilisation in various food or other products.

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References

Anger, H., & Berth, G. (1986). Gel permeation chromatography and the Mark–Houwink relation for pectins with different degrees of esterification. *Carbohydrate Polymers*, *6*, 193–202.

Axelos, M. A. V., & Thibault, J. F. (1991). The chemistry of low methoxyl pectin. In R. H. Walter, *The chemistry and technology of pectin* (pp. 109–117). New York: Academic Press.

Bacic, A., Harris, P. J., & Stone, B. A. (1988). *The biochemistry of plants*, *Carbohydrates*, Vol. 14. London: Academic Press (pp. 297–369).

Berth, G., Anger, H., & Linow, F. (1977). Light scattering and viscosimetric studies for molecular weight determination of pectins in aqueous solutions. *Die Nahrung*, *21*, 939–946.

Collmer, A., Ried, J. L., & Mount, M. S. (1988). Assay methods for pectic enzymes. *Methods in Enzymology*, Vol. 161 Academic Press, London, pp. 329–335.

DeVries, J. A., Rombouts, F. M., Voragen, A. G. J., & Pilnik, W. (1982). Enzymic degradation of apple pectins. *Carbohydrate Polymers*, *2*, 25–33.

DeVries, J. A., Den Vijl, C. H., Voragen, A. G. J., Rombouts, F. M., & Pilnik, W. (1983). Structural features of the neutral sugar side chains of apple pectic substances. *Carbohydrate Polymers*, *3*, 193–205.

Fishman, M. L., Pfeffer, P. E., Barford, R. A., & Doner, L. W. (1984). Studies of pectin solution properties by high-performance size exclusion chromatography. *Journal of Agricultural Food Chemistry*, *32*, 372–378.

Fujimoto, T., Narukawa, H., & Nagasawa, M. (1970). Viscoelastic properties of comb-shaped polystyrenes. *Macromolecules*, *3*, 57–64.

Graessley, W. W. (1977). Effect of long branches on the flow properties of polymers. *Accounts of Chemical Research*, *10*, 332–339.

Hwang, J., & Kokini, J. L. (1991). Structure and rheological function of side branches of carbohydrate polymers. *Journal of Texture Studies*, *22*, 123–167.

Hwang, J., & Kokini, J. L. (1992). Contribution of the side branches to rheological properties of pectins. *Carbohydrate Polymers*, *19*, 41–50.

Lapasin, R., & Pricl, S. (1995). *Rheology of industrial polysaccharides, theory and applications*, London: Blackie Academic and Professional.

Lineweaver, A., & Ballou, G. A. (1945). The effects of cations on the activity of alfalfa pectinesterase (pectase). *Archive Biochemistry*, *6*, 373–387.

Macdonald, H. M., & Evans, R. (1996). Purification and properties of apple pectinesterase. *Journal of Science and Food Agriculture*, *70*, 321–326.

Matthew, J. A., Howson, S. J., Keenan, M. H. J., & Belton, P. S. (1990). Improvement of the gelation properties of sugarbeet pectin following treatment with an enzyme preparation derived from aspergillus niger — comparison with a chemical modification. *Carbohydrate Polymers*, *12*, 295–306.

McNeil, M., Darvill, A. G., & Albersheim, P. (1982). Structure of plant cell walls. XII. Identification of seven differently linked glycosyl residues attached to O-4 of the 2,4 linked L-rhamnosyl residues of rhamnogalacturonan. *Plant Physiology*, *70*, 1586–1591.

Miltz, J., & Ram, A. (1973). Flow behaviour of well characterised polyethylene melts. *Polymer Engineering Science*, *13*, 273–279.

Mutter, M., Colquhoun, I. J., Schols, H. A., Beldman, G., & Voragen, A. G. J. (1996). Rhamnogalacturonase B from aspergillus aculeatus is a rhamnogalacturonan (-L-rhamnopyranosyl-(1 → 4)-(-D-galactopyranosyluronide lyase. *Plant Physiology*, *110*, 73–77.

Nelson, D. B., Smit, C. J. B., & Wiles, R. R. (1977). Commercially important pectic substances. In H. D. Graham, *Food colloids* (pp. 418–437). Westport, Connecticut, USA: Avi Publishing.

Nielsen, L. E. (1977). *Polymer rheology* (pp. 69–72). New York: Marcel Dekker (pp. 80-82).

Nordmeier, E., Lanver, U., & Lechner, M. D. (1990). The molecular structure of low-density polyethylene. 1. Long-chain branching and solution properties. *Macromolecules*, *23*, 1072–1076.

O'Neill, M., Albersheim, P., & Darvill, A. G. (1990). Methods in plant biochemistry. In P. M. Dey, *Carbohydrates* (pp. 415–441). , Vol. 2. London: Academic Press.

Powell, D. A., Morris, E. R., Gidley, M. J., & Rees, D. A. (1982). Conformation and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. *Journal of Molecular Biology*, *155*, 517–531.

Schols, H. A., & Voragen, A. G. J. (1994). Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. *Carbohydrate Research*, *256*, 83–95.

Utracki, L. A., & Roovers, J. E. L. (1973). Viscosity and normal stress of linear and star branched polystyrene solutions. II. Shear-dependent properties. *Macromolecules*, *6*, 373–377.

Wijesundera, R. L. C., Bailey, J. A., & Byrde, R. J. W. (1984). Production of pectin lyase by *Colletotrichum lindemuthianum* in culture and in infected bean (Phaseolus vulgaris) tissue. *Journal of General Microbiology*, *130*, 285–292.

Williamson, G., Faulds, C. B., Matthew, J. A., Archer, D. B., Morris, V. J., Brownsey, G. J., & Ridout, M. J. (1990). Gelation of sugarbeet and citrus pectins using enzymes extracted from orange peel. *Carbohydrate Polymers*, *13*, 387–397.

Wood, P. J., & Siddiqui, I. R. (1971). Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. *Analytical Biochemistry*, *39*, 418–428.