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Improvement of the Gelation Properties of Sugarbeet Pectin following Treatment with an Enzyme Preparation Derived from *Aspergillus niger* — Comparison with a Chemical Modification

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ABSTRACT

Pectin derived from sugarbeet pulp is extracted and then subjected to either enzymic or chemical modification. The enzyme is derived from the fungus Aspergillus niger. *A partial purification of the enzyme yields a preparation which is devoid of polygalacturonase but which effects a number of changes in the pectin molecule; some deacetylation, some demethoxylation and a large reduction in the arabinose content. The resulting treated pectin possesses considerably enhanced gelling properties.*

Chemical modification of the pectin results in a greater degree of deacetylation and demethoxylation, but a much lower percentage removal of arabinosyl residues. Gels formed from the chemically modified pectin are firmer than those formed from the untreated material. However, they are more brittle and less like a conventional gel than those formed from the enzymatically treated pectin.

INTRODUCTION

The residual material following extraction of sugar from sugarbeet is a fibrous pulp which is commonly used for animal feed. On a dry weight basis approximately 10% of the pulp is a pectic material, and thus its potential as a commercial source of pectin is being investigated.

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During World War II, workers in Germany examined the possibility of using sugarbeet pectin as a substitute for citrus pectins (Kertesz, 1945, Hinton, 1945) but found that it had poor gelling qualities, which were later attributed to its high acetyl content (Pippen *et al.*, 1950). Indeed, it is now well known that even a low degree of acetylation of hydroxyl groups in alginate or pectin molecules, can significantly alter gelation and other physicochemical properties (Schweiger, 1962*a*, *b*, 1964; Kohn & Furoa, 1968). However, sugarbeet pectin is a complex molecule and other factors such as molecular weight, the non-uronide content and the degree of branching of the molecule may influence its gelation properties.

In this paper the enzymic modification of sugarbeet pectin which gives a marked improvement of its gelation properties is reported. The enzyme is derived from the fungus *Aspergillus niger*. Chemical modification of the sugarbeet pectin and gels subsequently formed from that pectin are also reported.

METHODS

Extraction of sugarbeet pectin

Sugarbeet pulp was supplied by the British Sugar Corporation's Cantley factory. Pectin was extracted from the pulp using an unpublished method of Bulmers Ltd (pers. comm., 1983). Pulp was heated with acid (HNO_3, 38 mM, pH 1·7) at 45°C for 16 h, macerated, then further heated at 70°C for 1 h. The macerate was filtered, the pH of the filtrate raised to 5·0, and the starch removed by centrifugation (12 000 rpm for 30 min), then the pectin was precipitated from solution using iso-propyl alcohol (final concentration 66% (v/v)). The precipitate was suspended in water, dialysed against water and subsequently freeze-dried. The lyophylised powder was stored dry at room temperature.

Enzyme preparation

Aspergillus niger CS180* was grown in a liquid medium with sugarbeet pectin as the sole carbon source. The crude culture filtrate was fractionated with solid ammonium sulphate. The fraction precipitated

**Aspergillus niger* strain CS180 has been deposited at the Commonwealth Mycological Institute Culture Collection, Ferry Lane, Kew, Richmond-upon-Thames, Surrey, UK. Under the accession number: CMICC 298302.

between 50–90% saturation was resuspended in 50 mM phosphate buffer at pH 6·3 and subjected to gel filtration on Sephadex G100 (Pharmacia LKB, Uppsala, Sweden). The column was eluted with 50 mM phosphate buffer as above.

Fractions were monitored for UV absorption and enzyme activity. Pectate hydrolase, pectate lyase and pectin lyase were detected by conventional cup plate assays. Deacetylase and arabinase activity were checked by monitoring the hydrolysis of, respectively, the acetate and the arabinose derivatives of *p*-nitrophenol. Demethoxylase was determined using a small scale version of the method described later. Fractions with the desired activity were pooled and stored at + 2°C for later use.

Molecular weight determination

By reference to the calibration of the G100 column the molecular weight of the enzyme was found to be 67 000. Polyacrylamide gel electrophoresis of the enzyme preparation showed two bands (67 000 and 68 000 mol. wt).

Preparation of enzyme treated pectin

A 1 g sample of sugarbeet pectin dissolved in 188 ml of water was incubated with 12 ml of the enzyme preparation at room temperature overnight. The pectin was precipitated with two volumes of iso-propyl alcohol, taken up in 15 ml of water, and then dialysed against water and finally freeze-dried. The recovered, enzyme-treated pectin was stored dry at + 6°C.

Preparation of chemically modified pectin

A 1 g sample of sugarbeet pectin was dissolved in 50 ml of water and the pH adjusted to 1·0 using a few drops of concentrated hydrochloric acid. After acid treatment for 10 days the pH was brought to 5·0 and the pectin precipitated with two volumes of iso-propyl alcohol. Following dialysis the recovered pectin was freeze-dried and stored as a powder at + 6°C.

Assay of released free acetate

The amount of acetate removed from pectin by enzymic or chemical modification was determined in the supernatant following precipitation of pectin. Free acetate was adsorbed onto Dowex 1X-8 (Sigma Chemical

Co. Ltd., Poole, UK) and then extracted into diethyl ether from acid solution. Propionic acid was added to the supernatant fractions as an internal standard. Ether extracts were assayed by GLC on a Porapak QS column (J.&J. Chromatography, Kings Lynn, UK).

Determination of the methoxy content

The methoxy content of the pectins were determined by a method based on Hills and Speiser (1945). A 80 mg sample of pectin was dissolved in 10 ml (0·1 M) sodium hydroxide and left at room temperature overnight. Methanol released from the pectin was estimated by the method of Leatherhead Food Research Association (1982).

^{13}C-NMR spectroscopy

A Bruker CXP 300 spectrometer (Bruker Spectrospin, Coventry, UK) operating at 300·066 MHz for ^{1}H and 75·46 MHz for ^{13}C was used. A spectral width of 20 KHz was employed with a data table of 1 K points. Before Fourier transformation the data table was zero filled to 8 K and an exponential apodization function of equivalent 20 Hz line broadening was used. Data were acquired at 55°C under conditions of broad-band decoupling with a recycle time of 2 s and a flip angle of 60°; 30 000 transients were acquired. All the spectra were affected by severe base-line roll and required left shifting. In some cases only power spectra produced data of valuable quality.

Sugar analysis

Sugar content was determined using a modification of the Saeman hydrolysis (Selvendran *et al.*, 1979).

Gelation

Routinely, gels were formed by dissolving 180 mg freeze-dried pectin in 2·8 ml of citrate buffer at pH 5·0, placing the mix in a 3 cm diameter mould and dialysing against 1·0 M CaCl for 16 h. Gel strength was assessed using a Pulse Shearometer (Rank Brothers, Cambridge, UK), as described by Buscall *et al.* (1982). Visual assessment of the gels was made at intervals.

RESULTS

Acetyl content of pectins

Chemical deacetylation removed 18·3 mg of free acetate from 1 g of sugarbeet pectin and since the ^{13}C-NMR signal, normally between 20·5 and 21·5 ppm, indicative of O-CO$\underline{C}H_3$ found in sugarbeet pectin (Keenan *et al.*, 1985) as well as in other microbial polysaccharides (Wilkinson & Rex, 1983; Wilkinson *et al.*, 1983), is not discernible (Fig. 1(c)) it was concluded that the acid was sufficient to remove all acetate groups from the pectin. On this basis, only one in every thirteen glycosyl residues in unmodified sugarbeet pectin is acetylated (degree of acetylation 0·077).

Enzymic modification removed only 0·9 mg of free acetate from 1 g of sugarbeet pectin and a strong signal at 21·3 ppm was retained (Fig. 1(b)).

Methoxy content of pectin

Unmodified sugarbeet pectin had a methoxy content of 5·6% of total weight whereas enzymically and chemically modified material had 2·7% and 0·6% respectively.

^{13}C-NMR spectra and sugar compositions of pectins

The sugar compositions of pectins modified enzymatically or chemically are almost identical (Table 1) but their ^{13}C-NMR spectra show marked differences (Figs 1(b) and 1(c)). Superimposition of either spectrum onto

TABLE 1
Sugar Composition on a Moles Percent Basis of Pectin and Residues Remaining following Enzyme and Chemical Treatments

Treatment	*Material*	*Galacturonic acid*	*Arabinose*	*Galactose*	*Glucose*	*Xylose*	*Mannose*
None	Pectin	69	19	7·4	1·7	0·2	0·5
Enzymic	Pectin	77·5	10	5·0	3·5	0·6	0·3
	Residue	0·5	96·5	1·5	0·7	0·4	0·3
Chemical	Pectin	76·7	12·8	6·9	0·6	0·2	0·2
	Residue	6·2	91	1·0	1·0	—	—

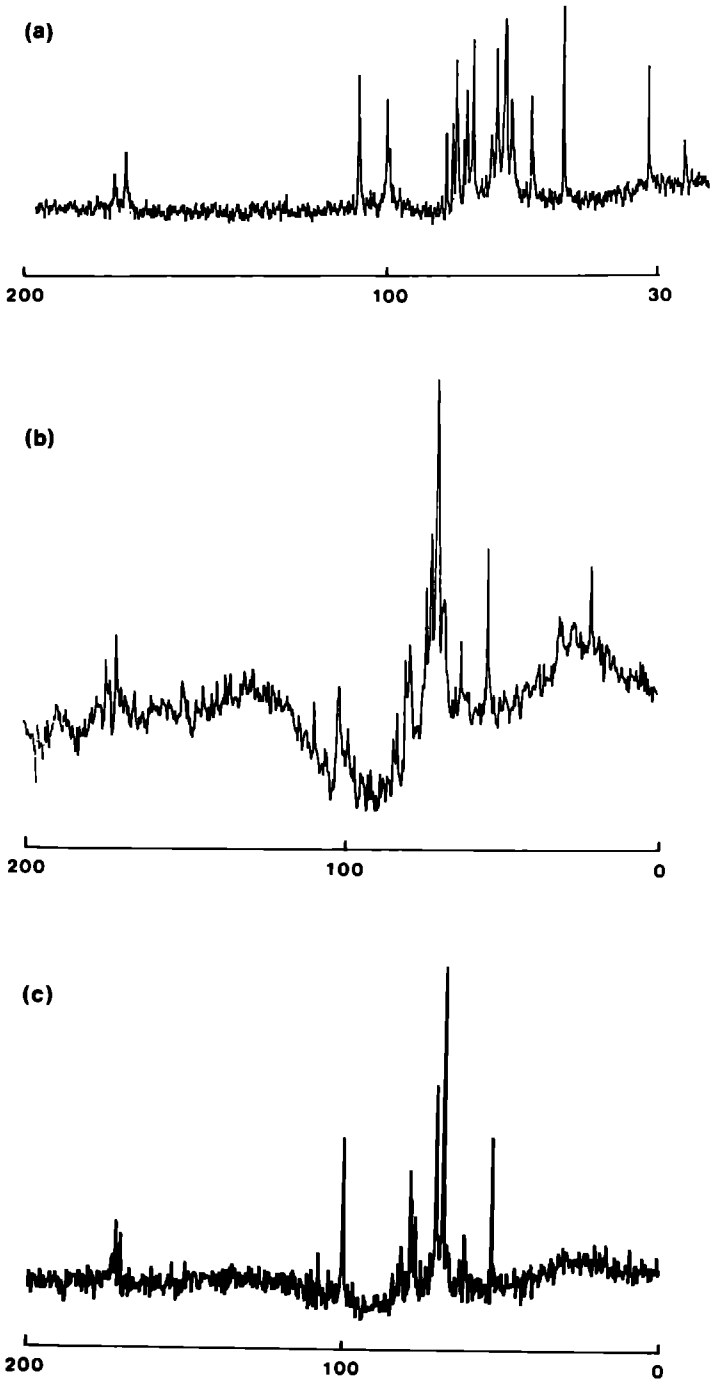


Fig. 1. ^{13}C-NMR spectra of sugarbeet pectin: (a) unmodified; (b) enzyme modified; (c) chemically deacetylated.

that obtained for unmodified sugarbeet pectin (Fig. 1(a)) reveals that whilst some spectral features are maintained the intensities of many signals are diminished. All major signals for unmodified sugarbeet pectin have already been assigned (Keenan *et al.*, 1985). Spectra of both modified pectins are dominated by resonances at 174, 100·6, 79, 71 and 68·2 ppm and are almost identical to those obtained for (1→4)-α-D-galacturonan known to form the bulk of unmodified sugarbeet pectin (Keenan *et al.*, 1985). However, these resonances probably do not arise from the main backbone since these are too immobile to be observed under high resolution conditions (Keenan *et al.*, 1985) they are more likely to arise from side-chains or mobile ends. Since the intensity of these signals is not diminished to the same extent as other side chains on hydrolysis they are thus either particularly resistant to hydrolysis or arise from chain-ends or low molecular weight components. Similarly the combination of relatively weak resonances at 108·3, 84, 82 and 78 ppm (Fig. 1(c)) are consistent with the presence of α-(1→5)-linked arabinofuranose, the structure already described for arabinosyl residues present in unmodified sugarbeet pectin (Keenan *et al.*, 1985). A signal at 68·5 ppm which together with that at 108·3 ppm would confirm the structure (Wilkinson & Rex, 1983; Pressey & Himmelbach, 1984) is not discernible but is probably masked by the strong galacturonan signal also at that frequency. Nearly all arabinofuranosyl signals, which because of similar sugar compositions, would be expected in Fig. 1(b), are insufficiently greater than background noise to be recognised.

The change in prominence of arabinosyl derived signals in ^{13}C-NMR spectrum of unmodified sugarbeet pectin, in which arabinosyl and galacturonosyl signals were of equal intensity (Fig. 1(a)), to those of either modified pectin samples (Figs 1(b) and 1(c)), is not proportional to the change in arabinose composition of the pectins (Table 1). It appears that approximately half the arabinosyl content of sugarbeet pectin produces intense resonances, is mobile, and therefore probably occupies side-chain or chain-end positions within the molecule whilst the remainder, which does not produce clear signals, is non-mobile and therefore may form an integral part of the sugarbeet pectin structure or be held very close to the galacturonan backbone.

Residual material

^{13}C-NMR power spectra of the nonprecipitable material which is left following enzymic or chemical treatment of sugarbeet pectin are quite different (Figs 2(a) and 2(b)), even though their sugar compositions are almost identical (Table 1). The material released by the enzyme action

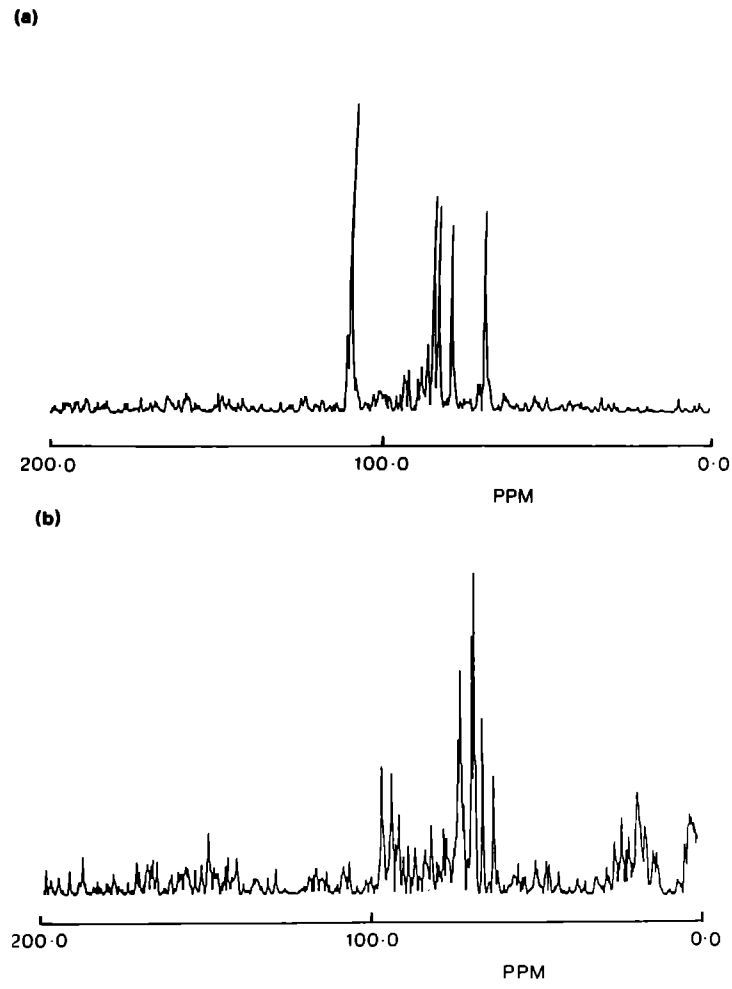


Fig. 2. ^{13}C-NMR power spectra of the nonprecipitable material which remains following: (a) enzymic modification; (b) chemical deacetylation.

contains mainly α-(1→5)-linked arabinofuranose, as demonstrated by the resonances at 108·3, 84, 82, 78 and 68·5 ppm (Wilkinson & Rex, 1983; Keenan *et al.*, 1985) (Fig. 2(a)). On the other hand, no such signals are present in Fig. 2(b). Indeed, as would be expected from an acid hydrolysis of an arabinofuranose polymer, the combination of shifts in this figure, namely 97·5, 94·6, 74·1, 73·8, 70, 69·4, 66·8 and 63·5 ppm are wholely consistent with a mixture of individual α- and β-arabinopyranose anomers (Breitmaier & Voelter, 1974). Thus although the nonprecipitable material in both cases probably has the same origin, chemical and enzymic treatments release it in different forms.

Sugar analysis

The sugar content of pectin before and after treatment and also that of the nonprecipitable material is shown in Table 1.

Gelation

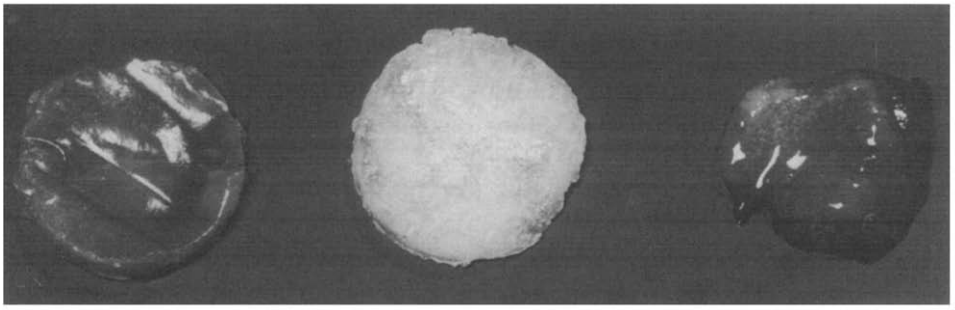
The results of gel-strength measurements are shown in Table 2. Both enzymic and chemical modification resulted in improved gels. However, whilst the strength of gels formed from different batches of sugarbeet pectin varied considerably (e.g. E1–E5), the strength of gels resulting from independent treatments of the same batch were similar (e.g. E1a and E1b).

Although both chemical and enzymic modification yielded pectins with enhanced gelling ability; visually the gels were distinct (Fig. 3). Gels formed from chemically modified pectins had a grainy texture and suffered shrinkage of total volume. Shrinkage continued over the 48 h

TABLE 2
Measurement of Rigidity Modulus of Gels Prepared from Pectins

Sample	*a* (Nm^{-2})	*b* (Nm^{-2})
E1[a]	13000	15500
E2	4800	5600
E3	1400	900
E4	971	1358
E5	6480	12280
C1[b]	20000	12000
C2	1900	683
C3	15000	34000[d]
C4	24300	19830
C5	28800	29490
U1[c]	Liquid	Liquid
U2	557	233
U3	970	800
U4	203	Liquid
U5	945	267
Polygalacturonic acid (Sigma)	11930	23380

[a]E series, enzyme treated sugarbeet pectin.
[b]C series, chemically modified sugarbeet pectin.
[c]U series, untreated sugarbeet pectin.
[d]Gel had been exposed to some drying before measurement.



(a) (b) (c)

Fig. 3. Test gels: (a) enzyme treated sugarbeet pectin; (b) chemically modified sugarbeet pectin; (c) untreated sugarbeet pectin.

observation period and these gels became hard and more brittle to handle. The enzymically modified pectins yielded a product with a jelly-like appearance and there was no shrinkage of volume.

DISCUSSIONS

Structure

Two lines of ^{13}C-NMR evidence point to the presence of two distinct arabinose populations in sugarbeet pectin. Firstly, only half the arabinosyl residues were removed from sugarbeet pectin by either enzymic or chemical treatment but in both cases almost all arabinosyl signals were lost from ^{13}C spectra. Secondly, the arabinose released by enzyme action was α-(1-5)-linked with no discernible signals corresponding to unbonded C-1 or C-5. In ^{13}C spectra of (1-4)-linked disaccharides, resonances assigned to bonded and unbonded C-1 and C-4 are distinct and of equal intensity (Joseleau *et al.*, 1977). Should that trend continue into oligo- and polysaccharides, it would be expected that non-bonded carbon atoms would be visible in ^{13}C spectra with our signal-to-noise ratios if they were present in chains of ten units or less. By inference, this defines a minimum chain length for the α-(1-5)-α-linked arabinofuranan released by enzyme action on sugarbeet pectin. Any similar chains of arabinosyl residues not released in enzyme-modified sugarbeet pectin would have been detected by ^{13}C-NMR spectroscopy of the pectin. Furthermore, neither did chemical treatment remove the non-mobile

arabinosyl content, again implying a form of arabinose not in α-(1→5)-linked chains. Hence, it is likely that sugarbeet pectin, already shown to have a highly branched galacturonan backbone with α-(1→5)-linked arabinofuranan side-chains (Keenan *et al.*, 1985) has in addition a population of closely bound arabinose units.

Gelation

Gels made using the enzymatically modified sugarbeet pectin resemble, visually, the jelly-like ones formed classically from commercial citrus pectin. Gels formed from sugarbeet pectin which has undergone chemical treatment are brittle and resemble, visually, those formed from commercial polygalacturonic acid, albeit that gels from polygalacturonic acid do not suffer the same shrinkage of total volume.

The major change effected by both treatments, was the removal of arabinosyl residues from the original pectin. This reduces the non-uronide content and probably accounts for some of the enhanced gelling characteristics of the pectins.

The enzymatically modified sugarbeet pectin was higher in acetyl content than was the chemically treated pectin. It is possible however, that the removal of small numbers of acetyl residues by enzymic action may have a significant effect on gel-strength, if they reside in positions in this complex polysaccharide where they sterically interfere with calcium bridge formation between adjacent pectin molecules.

Demethoxylation increases the acidic nature of pectin and renders it more likely to form a stable calcium gel. Thus the chemically modified pectin is likely to be easier to gel than the enzymatically modified form.

Enzyme action

The preparation contains three enzymes which when incubated with purified sugarbeet pectin effect some deacetylation, the removal of some arabinose and a degree of demethoxylation. As discussed above each of these effects plays a part in achieving an enhancement of gelling properties. In a preliminary study using conventional chromatographic techniques we prepared small amounts of the three enzymes, each one free of detectable amounts of the other two. We used these purified enzymes to treat sugarbeet pectin. The treated pectin was then used to make test gels. The results indicate that the enzyme mix is more efficacious than any of the enzymes alone, but treatment with demethoxylase has considerable gel enhancing effects.

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