Carbohydrate Polymers 2 (1982) 193–203

EVALUATION OF A METHOD FOR DETERMINING THE FREE CARBOXYL GROUP DISTRIBUTION IN PECTINS

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(Received: 27 April, 1982)

ABSTRACT

The distribution of free carboxyl groups in pectins has been investigated by a method that involves blocking the free carboxyl groups by glycolation, and hydrolysis of the methylesterified regions with a mixture of pectic enzymes. The hydrolysis products are separated from the glycolated regions on an ion exchange column and after deglycolation the oligomer size distribution is obtained by Sephacryl S-200 chromatography.

The method was applied to five pectins with degrees of esterification in the range 5–70%. For two of the samples (an enzyme and an alkali de-esterified low methoxyl pectin) the degree of hydrolysis was significantly lower than would be predicted from the initial degree of esterification and thus for these materials the values obtained for the carboxyl group block sizes were considered to be a maximum rather than an accurate estimate.

All the samples investigated had a significant proportion of free carboxyl regions with a degree of polymerisation greater than 10. With the possible exception of the pectate (degree of esterification 5%) none of the samples had a random distribution of carboxyl groups. This was considered to be a reflection of the distribution in the native pectin rather than indicating that chemical de-esterification was non-random. The large free carboxyl group block sizes was consistent with the egg-box model for low methoxyl pectin gelation. Larger blocks were found in the enzyme de-esterified pectin compared with the alkali and acid de-esterified material.

Carbohydrate Polymers 0144-8617/82/0002-0193/$02.75

INTRODUCTION

It has been reported that low-methoxyl pectins with similar ester content prepared by different de-esterification procedures have different gelling properties (Hills *et al.*, 1942). This has been attributed to different distributions of free carboxyl groups along the polygalacturonic acid chain (Speiser *et al.*, 1947; Heri *et al.*, 1961; Kohn *et al.*, 1968). In particular, it has been generally accepted that pectins de-esterified by the action of pectin methyl esterase (PME) have a blockwise distribution of free carboxyl groups whereas chemically de-esterified pectins have a more random distribution pattern. The evidence for these differences has been recently reviewed by Taylor (1982). It is apparent that no quantitative data relating to this has yet been obtained although there is extensive qualitative evidence in support of different distributions.

Any quantitiative method for determining the size of free carboxyl groups must depend upon removing the methoxylated residues and analysing the remaining oligomers.

The methodology we have employed combines chemical and enzymic techniques, avoiding the use of highly purified enzymes and severe chemical reaction conditions. The basic steps involved in the scheme are (Tuerena *et al.*, 1981):

1. reaction of the free carboxyl groups with a chemical which will render this part of the chain immune from attack by pectin degrading enzymes;
2. degradation of the unblocked regions of the chain by a mixture of enzymes;
3. separation of the blocked oligomers from the hydrolysis products;
4. analysis of the molecular size distribution of the oligomers.

As a blocking reagent we chose ethylene oxide since Deuel (1947) has reported that complete blocking of the free carboxyls and facile removal of the blocking agent are possible with this compound. Pilnik *et al.* (1973) have shown that glycolated portions of the pectin chain are not degraded by pectic enzymes excepting low-methoxyl pectin lyase. Low methoxyl pectin lyase is absent from enzyme preparations from *Aspergillus niger* and, for this reason, a preparation of enzymes from this source is used. Following degradation of the unblocked residues the hydrolysed material is separated from the blocked oligomers by ion-exchange chromatography. The glycol groups are then removed by alkali treatment and the molecular weight distribution characterised by gel chromatography.

In this paper the application of the method to five pectins with widely varying degrees of esterification is reported.

MATERIALS AND METHODS

High-methoxyl pectin, polygalacturonic acid, pectin methyl esterase and 'Pectinase' (an enzyme preparation from *A. niger*) were obtained from the Sigma Chemical Co.

A sample of acid de-esterified, low-methoxyl pectin was kindly supplied by Bulmers Ltd. Ion-exchange resins 'Dowex' 1-X8, 'Dowex'-50W and 'Amberlite' IRA-45 were also purchased through Sigma. Sephacryl S-200 was obtained from Pharmacia Fine Chemical Ltd. All other reagents used were of analytical grade.

Preparation of Low-Methoxyl Pectins

Two samples of low-methoxyl pectin were prepared from the Sigma high-methoxyl pectin, one by enzyme de-esterification and the second by alkali de-esterification.

Enzyme de-esterification. Pectin (5 g) was dissolved in 500 ml of water and the pH adjusted to 7·5 with 0·1 M sodium hydroxide. The temperature was kept constant at 30°C. A pectin methyl esterase suspension (0·5 ml) was added and the solution thoroughly mixed. The pH was maintained at 7·5 by continuous titration for 10 min. At the end of the reaction time the pH was adjusted to 1·5 with hydrochloric acid. The pectin was precipitated with two volumes of propan-2-ol, the solid precipitate washed twice with propan-2-ol–water (75/25 v/v) and washed once with 100% propan-2-ol. The sample was then dried under vacuum.

Alkali de-esterification. Pectin (5 g) was dissolved in 500 ml of water and the pH adjusted to 11·0 with 1 M sodium hydroxide. The temperature was maintained at 20°C. The solution was maintained at pH 11·0 by continuous titration with 0·1 M sodium hydroxide for 56 min. The sample was then treated as for the enzyme de-esterified sample.

Characterisation of Pectins

The galacturonic acid content (GA) and degree of esterification (DE) of the samples were determined titrimetrically using the method of Versteeg (1979). The number average degrees of polymerisation (DP) were determined by end-group analysis by the method of Milner & Avigad (1967). DPs were calculated as

$$DP = \frac{\text{Total galacturonic acid (GA)}}{\text{Galacturonic acid end-groups}}$$

Glycolation of Pectins

Glycolation was carried out as described by Deuel (1947). Pectic material (2 g) was suspended in 12 ml H_2O at ambient temperature. Twenty ml of ethylene oxide was added and the mixture was stirred for 24 h. Additional aliquots (20 ml) of ethylene oxide were added until a pale yellow solution was obtained. Normally a total of 40 ml of ethylene oxide was sufficient and the reaction time was 48 h. The glycolated material was precipitated with 2 volumes of propan-2-ol and then washed and dried as described for the preparation of the low methoxyl pectin samples (P2 and P4).

Enzymic Degradation

Degradation was carried out using a commercial enzyme preparation from *Aspergillus niger*.

Glycolated pectin solutions of approximately 0·5 mg/ml were prepared. Samples (25 ml) were treated, the pH of the solutions being adjusted to 4·0 prior to treatment. The 25 ml samples in 2·5 x 20 cm tubes were equilibrated at 30°C in a water bath. The enzyme preparation (1 ml, 113 units/ml) was added, the tubes left to stand for 1 h and then another 1 ml of enzyme preparation added. After a total of 3 h the reaction was stopped by immersing the tubes in boiling water for 5 min.

Separation of Blocked Oligomers

Separation of the blocked oligomers from the hydrolysis products was achieved by passing the hydrolysates through 'Dowex' 1-X8.

A 1·6 x 30 cm 'Dowex' 1-X8 column was prepared by running 20 ml of 1 M sodium hydroxide through the column and then eluting with water until the eluent pH was 8·0. Fractions (10 ml) were collected for each separation. The hydrolysate (10 ml) was added to the column and eluted with 40 ml of water. Elution was continued with 0·25 M sodium chloride. The fractions collected were assayed for total sugar by the method of Blumenkrantz & Asboe-Hansen (1973) and the degree of polymerisation of the hydrolysed fraction was obtained by end group analysis.

Deglycolation of Oligomers

This was carried out by alkaline saponification.

The fractions containing the glycolated oligomers were bulked and placed in glass vessels. To these, 10 ml 0·1 M sodium hydroxide was added, the vessels stoppered and left for 20 min at ambient temperature. The alkali was then neutralised with 10 ml 0·1 M sulphuric acid. The total GA and GA end-groups were determined as before, giving an estimate of the number average oligomer size.

Determination of Oligomer Size Distribution

Estimations of oligomer size distribution were made using gel filtration with Sephacryl S-200.

A 710 x 16 mm column was packed under pressure with Sephacryl S-200 prepared in 0·1 M acetate buffer, pH 3·6, containing 0·05% benzoic acid as a preservative. Two hundred to four hundred ml approximately 20 mg ml^{-1} samples were run at a flow rate of 1 ml min^{-1}. Fractions (4 ml) were collected and assayed for galacturonic acid content by the method of Blumenkrantz & Asboe-Hansen (1973) and, in the calibration runs, fractions were also analysed for reducing end-groups by the method of Milner & Avigad (1967). The column was calibrated for degree of polymerisation against elution volume using samples of sodium pectate hydrolysed to different extents with 'Pectinase'.

Computer Simulation of De-esterification Process

A program, written in BASIC and run on an Intertec Superbrain Microcomputer was developed allowing for a simulation of random and blockwise de-esterification.

In the case where random de-esterification alone was being simulated, every esterified residue selected by a pseudo-random number generator was converted to a free carboxyl group and the starting material had a DE of 100%.

The results briefly discussed in this paper were obtained by de-esterifying 100 molecules with a degree of polymerisation of 100.

RESULTS

Table 1 displays the galacturonic acid contents, degrees of esterification and degrees of polymerisation obtained for the five pectin samples.

The extent of glycolation and the degree of polymerisation of the samples after glycolation is shown in Table 2.

TABLE 1
Galacturonic Acid Content, Degree of Esterification and Degree of Polymerisation of Sample Pectins

Code	*Description*	*Galacturonic acid (%)*	*Degree of esterification (%)*	*Degree of polymerisation (number average)*
P1	Citrus high methoxyl pectin (Sigma)	73·7	71·2	103
P2	Prepared by treatment of P1 by pectin esterase	74·35	46·4	86
P3	Acid de-esterified low-methoxyl pectin. Source: lime peel (Bulmers)	86·4	33·2	420
P4	Prepared by treatment of P1 with alkali	76·8	25·4	65
P5	Citrus sodium pectate (Sigma)	88·3	5·0	56

TABLE 2
Extent of Glycolation and Degree of Polymerisation of Glycolated Pectins

Sample	*% free carboxyl group glycolated*	*% galacturonic acid residues not glycolated and not esterified*	*Degree of polymerisation of glycolate pectin*
P1	72·2	8·0	93
P2	87·5	6·7	85
P3	90·4	6·4	196
P4	90·5	7·1	70
P5	93·6	6·1	54

It is apparent that with the exception of the acid de-esterified pectin no significant depolymerisation occurs on glycolation. Although at this stage we would not wish to completely eliminate the possibility that the small number of carboxyl groups found after glycolation could be due to some systematic error in the method used to detect these groups, perhaps caused by the presence of acidic side-reaction products, we consider this to be unlikely. It therefore appears that in contrast to the reported data of Deuel (1947), a few carboxyl groups are not amenable to glycolation. The proportion of such groups on the chain is independent of the degree of esterification. For this reason it seems unlikely that the presence of methoxyl groups on adjacent galacturonic acid residues prevents glycolation. One possible explanation is that carboxyl groups in the vicinity of some of the neutral sugars in the chain cannot be glycolated.

Table 3 compares the extent of hydrolysis of the native and glycolated pectins. Also included in this table for comparison are the original pectin degrees of esterification.

The crude enzyme preparation employed is very effective in hydrolysing the native pectins down to monomeric galacturonic acid. It is also clear that, as reported by Pilnik *et al.* (1973), glycolation will protect the molecule from hydrolysis. Of the four known pectic enzymes – pectin esterase, polygalacturonase, pectin lyase and low methoxyl pectin lyase – only the latter can attack glycolated pectins and this is known to be absent in products based on cultures of *Aspergillus niger*.

Table 4 displays the total galacturonic acid recovered in the two fractions. Also displayed is the mean degree of polymerisation for the hydrolysed fraction and the degree of polymerisation of the oligomers obtained before and after deglycolation. As would be expected there is satisfactory agreement between the percentage galacturonic acid in the hydrolysed fraction from the column (Table 4) and the percentage hydrolysis of the glycolated fraction shown in Table 3.

Figure 1 displays the elution profile obtained from the Sephacryl S-200 column for native sample P5 and this sample after enzyme hydrolysis for three different times.

TABLE 3
Hydrolysis of Native and Glycolated Pectins

Sample	*Degree of esterification*	*% hydrolysis*[a]	
		Glycolated	*Native*
P1	71·2	70·8	95·3
P2	46·4	31·3	94·7
P3	33·2	31·2	95·7
P4	24·4	12·5	98·1
P5	5·0	7·1	99·7

[a] % hydrolysis = $\frac{\text{Number of reducing end groups}}{\text{Total number of galacturonic acid residues}} \times 100.$

TABLE 4
Separation of Glycolated and Non-Glycolated Hydrolysis Products: Degrees of Polymerisation of Fractions

% total galacturonic from column			*Degree of polymerisation (number average)*		
Sample	*Glycolated fraction*	*Hydrolysed fraction*	*Hydrolysed fraction*	*Glycolated fraction*	
				Before deglycolation	*After deglycolation*
P1	32·2	67·7	1·02	5·6	7·0
P2	66·7	33·3	1·1	10·3	9·6
P3	69·5	30·5	1·16	7·1	7·2
P4	89·3	10·7	1·19	13·6	10·4
P5	90·2	9·8	1·09	–	13·9

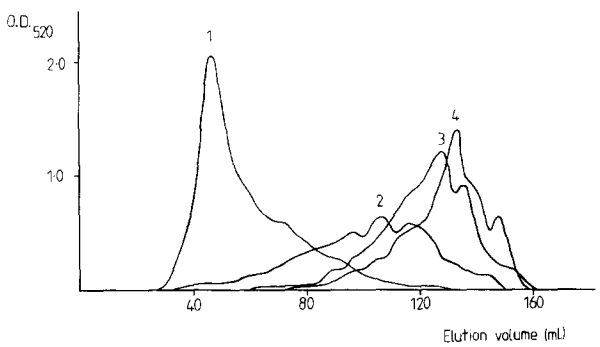

Fig. 1. Elution profiles of hydrolysed sodium pectates on Sephacryl S-200. Trace 1 was obtained from unhydrolysed sample P5. Traces 2–4 were obtained from P5 hydrolysed with pectinase for various times as described in the text. The ordinate represents the optical density obtained following the assay method of Blumenkrantz & Asboe-Hansen (1973).

A satisfactory linear relationship was obtained between the logarithm of the degree of polymerisation and the elution volume (Fig. 2). The data displayed in this figure was obtained using the elution profiles shown in Fig. 3 and evaluating DPs from the total galacturonic acid and number of reducing end groups as described previously.

Elution profiles for the deglycolated oligomers obtained from samples P1–P5 are displayed in Fig. 3. The differences between these profiles are brought out more clearly in Table 5 which displays the distribution of oligomer sizes obtained by combining the information in Figs 2 and 3.

Also shown in Table 5 is the distribution obtained from the computer simulation assuming random de-esterification. It can be seen that whereas for samples P1–P4 the

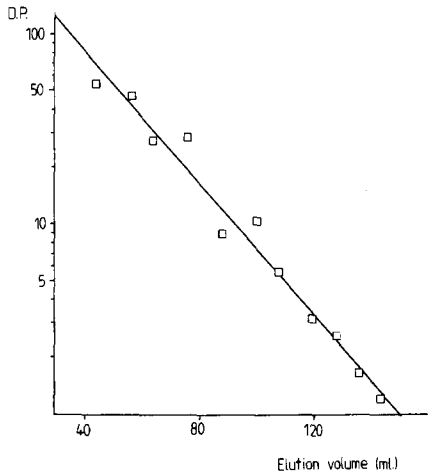

Fig. 2. Calibration plot for Sephacryl S-200 column.

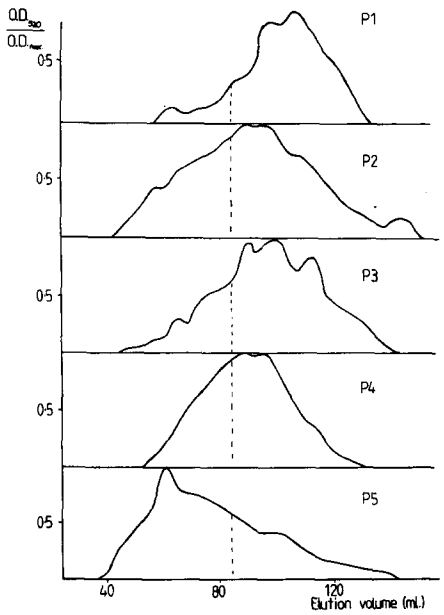

Fig. 3. Elution profiles of deglycolated oligomers obtained from samples P1–P5.

TABLE 5
Relative Proportion of the Oligomers in Different Size Ranges[a]

Oligomer size	*Sample*				
	P1 (%)	*P2 (%)*	*P3 (%)*	*P4 (%)*	*P5 (%)*
>30	– (–)	7·2 (–)	1·8 (–)	0·9 (–)	16·9 (39·4, 22·4)
20–30	3·3 (–)	12·2 (–)	6·0 (–)	10·0 (4·6)	24·5 (22·3, 20·2)
10–20	14·0 (–)	30·1 (–)	28·0 (3·5)	42·6 (18·5)	31·2 (23·8, 31·7)
5–10	34·5 (0·4)	25·7 (14·9)	33·1 (27·6)	34·2 (33·9)	15·7 (10·5, 15·5)
1–5	48·2 (99·6)	24·8 (85·1)	31·1 (68·9)	12·3 (43·0)	11·7 (4·0, 10·2)

[a] The figures in parentheses give the distribution calculated by the computer for random de-esterification to the appropriate DE. The final column of figures shows the distribution obtained for random de-esterification to a DE of 8·1.

observed distribution shows far larger block sizes than would be predicted for a completely random mechanism from a starting degree of esterification of 100, for sample P5 the predicted block sizes are somewhat larger than is actually observed. For this sample there is reasonably good agreement between the actual distribution and that obtained by random de-esterification to a DE of 8·0.

DISCUSSION

The major uncertainty in this procedure is associated with the incomplete hydrolysis of the esterified regions of the glycolated pectins. If it is assumed that a glycolated region of the chain is completely resistant to attack but all the other residues are available to the enzymes then the extent to which the glycolated molecule will be hydrolysed should be approximately equal to the initial degree of esterification plus the percentage of carboxyl groups that have resisted glycolation. If, as in this case, the extent of hydrolysis is obtained by end group analysis then the measured degree of hydrolysis might be expected to be somewhat higher than this because if a chain of n non-glycolated residues situated between glycolated regions is completely hydrolysed then $(n + 1)$ additional end groups will be detected.

In practice it is found that for three of the samples the degree of hydrolysis is close to the initial degree of esterification whereas for the enzyme and alkali de-esterified pectins the degree of hydrolysis is significantly lower than this.

From the data in Table 4, it is apparent that the hydrolysed fraction consists almost entirely of galacturonic acid monomers. Therefore the fraction that comes first off the ion-exchange column will most probably be glycolated oligomers to which some non-glycolated residues are attached. In view of the high degree of hydrolysis of the

native pectins it seems unlikely that esterified galacturonic acid oligomers alone will be present. It seems reasonable that the galacturonic acid residues which are resistant to glycolation for steric reasons will also be resistant to enzyme attack. We would also take the view that not all the linkages between esterified galacturonic acid residues and glycolated residues will be split by the enzyme. This is because a combination of pectin esterase and exopolygalacturonase will not be effective since the former attacks from the reducing end of the chain whilst the latter works from the non-reducing end. Thus, although a single esterified galacturonic acid residue attached to a glycolated oligomer may be de-esterified by pectinesterase, if the linkage involves the non-reducing end of the non-glycolated residues, a further attack by exo-polygalacturonase will not occur. However, further galacturonic acid residues at the non-reducing end are still open to attack by endo-polygalacturonase.

The other possibility is attack by pectin lyase. It seems unlikely that this will occur for linkages involving both the non-reducing and reducing ends of the esterified galacturonic acid residue since the substrate will be very different in the two cases.

If the galacturonic acid residues which are resistant to glycolation are immune to enzyme attack and if a single galacturonic acid residue is attached to each glycolated oligomer, then the degree of hydrolysis determined by the end group method would be expected to equal the degree of esterification. This is approximately true for samples P1, P3 and P5 and for these preparations we consider the value found for the degree of polymerisation of the oligomers following separation to be a reasonable estimate of the mean length of the non-esterified regions in the original pectins. For samples P2 and P4 the values obtained should be regarded as the maximum chain length because of the incomplete hydrolysis.

It is not clear why these samples proved more difficult to hydrolyse after glycolation than the others. One possibility is that aggregation in some samples protects parts of the chain from hydrolysis.

The most notable feature of the results is that for all the samples there appears to be a significant percentage of blocks of carboxyl groups with a DP >10. Only for the pectate sample (P5) is there any correspondence between the observed and random distributions. This is to be expected because as the DE approaches zero all models for the de-esterification process will predict similar distributions.

The smaller block sizes obtained for this sample than would be calculated for random de-esterification of a homogeneous pectin with a degree of polymerisation of 100 could be explained by the presence of some very short chains in the original preparation or a small degree of degradation on deglycolation or even the presence of neutral sugars interrupting the free carboxyl regions. However, because of the experimental errors involved in both the DE determination and the calibration of the column it is probably not necessary to invoke these interpretations.

In view of the relatively large block sizes found in the high methoxyl pectin (P1) it would seem that the non-random distribution found after acid de-esterification and perhaps also alkali de-esterification could well be a reflection on the original distribu-

tion in the molecule rather than indicating that chemical de-esterification does not remove methoxyl groups at random.

The large blocks of free carboxyl groups suggested by this work are consistent with the 'egg box' model for the junction zones in low methoxyl pectin and alginate gels (Grant *et al.*, 1973; Morris *et al.*, 1978). This model implies that several contiguous free carboxyl groups on adjacent chains are necessary for a stable crosslink to form. It is not clear how many contiguous carboxyl groups are necessary to give a linkage with a significant lifetime but the work of Kohn & Luknar (1977) would suggest that for maximum junction zone strength $\gtrsim 15$ carboxyl groups on each of the two participating chains need to be involved.

The uncertainty over the results obtained for the alkali and enzyme de-esterified pectins due to incomplete hydrolysis makes it dangerous to be too dogmatic regarding the effect of different methods of de-esterification on carboxyl group distribution. There is, however, a strong suggestion from the data displayed in Table 5 that there are more large blocks in the enzyme de-esterified material than in the other two low methoxyl pectin samples despite the fact that the former has a higher degree of esterification. This is consistent with the sequential de-esterification mechanism of pectin esterase (Pilnik & Rombouts, 1979).

ACKNOWLEDGEMENT

The authors gratefully acknowledge valuable discussions with Dr Graeme Blackwood and Mr Peter Cheney.

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