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PII: S0144-8617(96)00132-4

Carbohydrate Polymers 32 (1997) 27–32

0144-8617/97/$17.00 + .00

Separation and quantification of pectins using capillary electrophoresis: a preliminary study

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(Received 1 February 1996; revised version received 14 July 1996; accepted 17 July 1996)

A rapid method for the analysis of the degree of esterification (DE) of pectin is presented. The migration time of pectin in capillary electrophoresis (CE) is a function of the DE, and separation of pectins with different DE values has been demonstrated. Using the average charge per residue, $z = -(100-DE)/100$, a linear relationship is found between pectin charge and mobility. For a fixed charge mobility increases with decreasing ionic strength, but does not depend significantly on whether the pectin charge distribution is blockwise or random. This CE method can be used for the quantitative detection of aqueous solutions of pectins in the concentration range 0.5–5 mg ml^{-1} using a 50 mM, pH 7.0 phosphate background electrolyte and u.v. detection at 192 nm. © 1997 Elsevier Science Ltd

INTRODUCTION

While capillary electrophoresis (CE) (Grossman & Colburn, 1992; Weinberger, 1993; Monnig & Kennedy, 1994) has been routinely used to perform efficient separations of proteins and nucleic acids for a number of years (Karger *et al.*, 1995; Barron & Blanch, 1995), applications to the analysis of carbohydrates have taken, by comparison, longer to develop owing in part to a lack of charge or a chromophore for many members of this class of biomolecule. A number of techniques that circumvent this difficulty have now been successfully applied including complexation, for example of sugars with anionic borate (El Rassi, 1994) and plant starches with iodine/iodide (Brewster & Fishman, 1995), derivatization with UV absorbing or fluorescent labels (Sudor & Novotny, 1993; Stefansson & Novotny, 1994) and the use of indirect UV detection at high pH (Richmond & Yeung, 1993). For large carbohydrate polymers derivatization methods are of limited scope as tagging can only be carried out at reducing sites, typically the end group, imparting only one label per molecule.

Pectin is a biopolymer found in the cell walls of many living plant tissues. The backbone of the pectin chain mainly consists of galacturonic acid, rhamnose, arabinose and galactose. Chains typically consist of 200–1000 galacturonic acid units linked together by 1–4 glucosidic bonds and occur with varying degree of esterification (DE). Unlike many other natural polysaccharides, pectins possess both charge and a UV chromophore, the carboxylate group, making CE an attractive analytical tool for the study of these carbohydrates. It is well-known that pectin can be used to form aqueous gels, and this property is commonly exploited by the food industry. In terms of gelling properties pectin can be divided into two broad categories, high DE and low DE. The widely-accepted model for the gelation mechanism in low DE pectins involves the formation of a rigid three-dimensional network, achieved by the co-ordination of available carboxyl groups to divalent metal ions, the presence of which is essential. A low DE pectin would usually be gelled with millimolar concentrations of Ca^{2+}. It has been proposed that Ca^{2+} bridges form an 'egg box' structure (Grant *et al.*, 1973; Morris *et al.*, 1978; Gidley *et al.*, 1979, 1980), although other researchers have argued against this (Axelos *et al.*, 1991; Renard *et al.*, 1993). For high DE pectins, gelation is achieved in systems of high solids content (typically containing 60% sucrose) and is thought to involve hydrophobic interactions and hydrogen bonding (Walkinshaw & Arnott, 1981; Oakenfull & Scott, 1984). The DE of a pectin sample is, thus, of great importance in understanding and controlling the nature of its gels.

At present the classical titration method (Hinton, 1939; Schultz, 1965), is still the official method used for the determination of DE (EEC, 1978; Food Chemicals Codex, 1981; FAO, 1992). Alternatives involve the

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liberation of methanol by de-esterifying enzymes or by acid or alkali treatments, and subsequent quantification of the methanol by chromatography (McFeeters & Armstrong, 1984; Voragen *et al.*, 1986). Recently, a method has been described which utilizes HPLC with a size-exclusion column and RI and CD detection (Plöger, 1992). The DE is calculated by comparison with calibration standards of known composition. The conversion from the number of carboxyl groups into DE, however, assumes that the pectins analysed have the same proportions of galacturonide and non-galacturonide as the pectins used for calibration.

Present methods for obtaining the intermolecular DE distribution involve fixation of the pectin onto an ion exchange column, under conditions of low ionic strength and subsequent elution of the pectin with a buffer, the concentration of which is gradually increased. A useful overview of such techniques has been given (Kravtchenko, 1992). An HPLC version has been published by Schols *et al.* (1989). A weakness of this method is the irregular way in which pectins with blockwise intramolecular DE distribution eluted from the column. As a result, it was found that the determination of the DE of pectins with blockwise distributions was impossible, based on calibration with known pectins.

Rapid methods for determining the DE of pectins and in particular for the DE distribution are in great demand. The aim of this paper is to describe a CE method that can be used to provide a rapid, efficient characterization of pectin in terms of DE, with the potential also to provide information on the DE distribution.

EXPERIMENTAL

All samples were lemon peel pectins, supplied and characterised by Copenhagen Pectin. Three source pectins were used in the study, two with low calcium sensitivity, taken to imply a random charge distribution, and one with high calcium sensitivity, taken to imply a blockwise charge distribution. Pectins were de-esterified using the enzyme pectin esterase of *Aspergillus* to obtain fractions of varying DE. Characterisation was carried out using the titration method referred to in the introduction (Hinton, 1939; Schultz, 1965). Details of all the samples are given in Table 1. These were supplied as powder, and solutions were prepared by heating in deionized water at 60°C for 30 min. Phosphate buffers at pH 7.0 or 3.0, used as CE background electrolyte (BGE), were prepared by titrating aqueous NaH_2PO_4 (20, 50 or 100 mM) with 1 M NaOH or 0.2 M H_3PO_4, respectively.

Experiments were carried out on an automated CE system (Beckman P/ACE 5000), equipped with a diode array detector, thermostatted at 30°C. Electrophoresis

Table 1. Characteristics of the pectins used in this study

Source	Galacturonide / % w/w	DE / %
Batch 1 Random	85.1	70.3
Batch 1 Random	86.5	62.2
Batch 1 Random	89.0	55.8
Batch 1 Random	86.4	47.2
Batch 1 Random	85.2	31.1
Batch 2 Random	84.5	75.8
Batch 2 Random	81.5	64.4
Batch 2 Random	84.6	54.2
Batch 2 Random	83.3	45.2
Blockwise	86.6	69.4
Blockwise	83.9	62.0
Blockwise	84.2	51.7
Blockwise	85.7	42.6

was carried out in a fused silica capillary of internal diameter 100 μm and a total length of 57 cm (50 cm from inlet to detector). All new capillaries were conditioned by rinsing for 30 min with a 0.1 M NaOH solution. Between runs the capillary was washed for 2 min with 0.1 M NaOH, 1 min with water and finally 2 min with BGE solution. Detection was carried out using UV absorbance at 192 nm with a bandwidth of 5 nm. Samples were loaded hydrodynamically (10 s at 3450 Pa, giving an injection volume of 190 nl), and electrophoresed across a potential difference of 20 kV. All experiments were carried out at normal polarity (inlet anodic), unless otherwise stated. Electrophoretic mobilities, μ, were determined from migration times of the pectins and a neutral marker, t and t_o, respectively, using the equation:

$$\mu = \mu_{obs} - \mu_{eo} = \left(\frac{lL}{V}\right)\left(\frac{1}{t} - \frac{1}{t_o}\right) \quad (1)$$

where L is the total length of the capillary, l is the distance from the inlet to detector, V is the applied voltage, μ_{obs} is the observed mobility and μ_{eo} is the mobility of the electro-osmotic flow (EOF) (Grossman & Colburn, 1992). Ultraviolet/vis spectrophotometry was also carried out on pectin solutions of 1 mg ml^{-1} in water and phosphate BGE at pH 3.0 and 7.0. A Shimadzu UV spectrophotometer was used in all cases with cells of path length 1 cm.

RESULTS AND DISCUSSION

Preliminary experiments were carried out at pH 3.0 under reverse polarity (inlet cathodic); although analysis was sucessful for the lowest DE pectins, migration times for the highest DE samples were of the order of hours and peaks were extremely broad. A pH 7.0 phosphate BGE was found to give satisfactory electropherograms for all pectins. This pH is the centre of the buffer range for HPO_4^{2-}/ $H_2PO_4^-$, an electrolyte mixture which also has the advantage of low absorbance at 190 nm and

reproducible EOF. At pH 7.0 pectin ($pK_a = 3$–4) is fully charged. Although pectin is susceptible to base-catalysed β-elimination, no problems were encountered during run times of only minutes in the CE capillary at pH 7.0.

Figure 1 shows typical electropherograms obtained for: (a) a 70.3% DE; and (b) a 31.1% DE pectin sample, in a 50 mM BGE solution at pH 7.0. The absorbance dip at ~4.2 min indicates the neutral marker position. This was confirmed by studies with mesityl oxide as a UV absorbing neutral marker.

Figure 1(a) and (b) shows that the migration time increases with increasing charge (decreasing DE). All these anionic polysaccharides migrate after the neutral marker. The observed mobility μ_{obs} is the vector sum of μ_{eo} and μ (see Equation 1) and since μ is negative and smaller in magnitude than μ_{eo} the anions having the most negative mobility have the smallest μ_{obs} and, thus, the longest migration times. Fig. 1(c) shows an electropherogram obtained following the injection of a mixture of pectins characterized by three DE values, 70.3, 47.2 and 31.1%. The three components are clearly resolved.

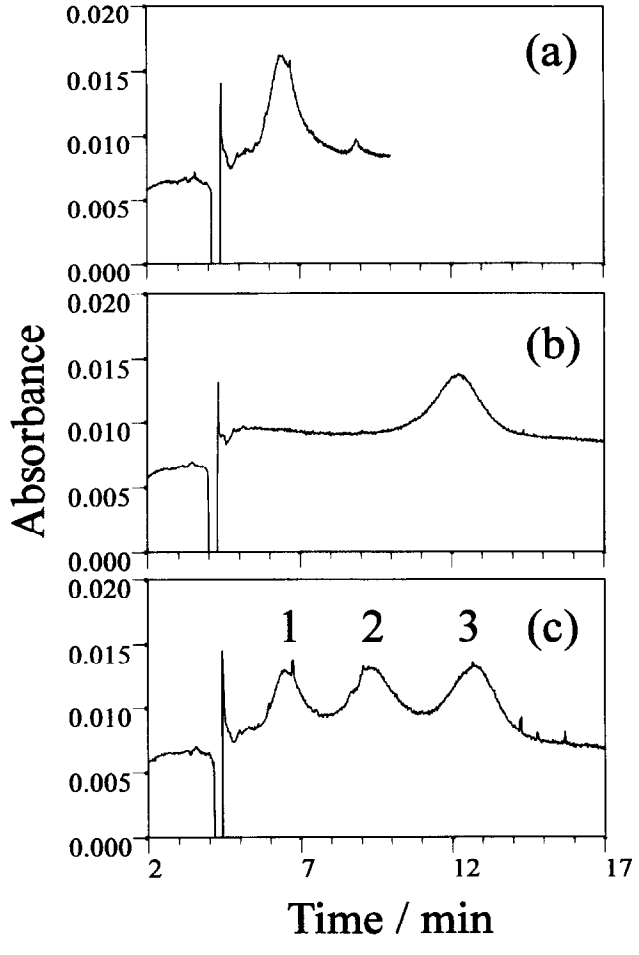

Fig. 1. Typical electropherograms for: (a) a 70.3% DE pectin; (b) a 31.1% DE pectin; and (c) a mixture of pectins characterized by three DE values, (1) 70.3%, (2) 47.2%, (3) 31.1%. Experimental conditions: BGE solution, 50 mM phosphate at pH 7.0; voltage 20 kV; capillary, 100 μm i.d., 57 cm length (50 cm to detector); capillary thermostat temperature, 30 °C; sample, pectin in deionized water, concentration 2.5 mg ml^{-1} in (a) and (b), 0.17 mg ml^{-1} each pectin in (c); injection, 10 s at 3450 Pa (190 nl).

The degree of resolution in DE that can be achieved is dependent on the peak widths. Peak widths are assumed to be determined by the polydispersity of the charge to size ratio within a particular pectin sample. Other contributions to the variance of the peaks, for example those arising from the length of the injected sample plug and time-dependent diffusion out of the sample zone (Grossman & Colburn, 1992; Weinberger, 1993), give far smaller contributions to the peak width than observed in Fig. 1. Peak widths were found to be the same when the injection time was reduced from 10 to 7 or 5 s.

Electrophoretic mobility is determined by the charge to size ratio in the free solution mode of CE used in these experiments. Figure 2 shows pectin mobility vs average charge per residue, z, (calculated as $z = -(100-DE)/100$), over the DE range 30–80% for 20, 50 and 100 mM phosphate BGE solutions. It should be noted that this definition neglects any difference in neutral sugar content. Each experiment was carried out in triplicate. Mobilities are lower at higher ionic strength, as would be predicted from polyelectrolyte theory (Manning, 1981). It is clear that within the range studied the electrophoretic mobility scales linearly with the average charge, and that capillary electrophoresis is capable of determining the DE of a pectin sample.

Linear regression analysis was carried out on all individual data sets and using all data taken at the same ionic strength. The results are summarized in Table 2. It is interesting to note that in this work there was no statistically-significant difference between the electrophoretic mobility of samples of the same DE irrespective of the distribution of esterified groups. A previous conductivity study (Thibault & Rinaudo, 1985) also showed little difference in the electrical transport properties of pectins with random and blockwise distributions. This would indicate that, within the distributions studied here, all factors affecting the mobility are very similar, including the three-dimensional geometry of the biopolymer and the topology of the average electrostatic interaction governing the counterion effects. Nevertheless, the migration times obtained for different DE samples of the same source pectin do exhibit a greater correlation within the source pectin set, compared with that observed between different pectins. It is possible that this is a result of a slightly different galacturonic acid composition. All the pectins studied here have a very similar proportion of galacturonic acid in the total mass, and this has not been taken into account in calculating z values. It is also worth commenting on the data sets obtained at different ionic strengths.

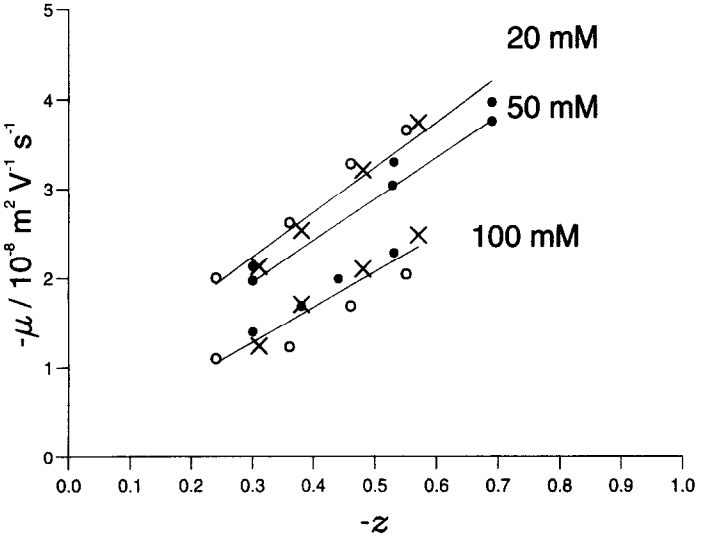

Fig. 2. Pectin mobility vs charge, z (calculated from $-(100-DE)/100$), over the DE range 30–80% for 20, 50 and 100 mM phosphate BGE solutions, (●) random distribution batch 1, (○) random distribution batch 2, (×) blockwise distribution.

Table 2. Linear regression analysis of pectin mobility as a function of charge, with gradient A, intercept C, and 95% confidence limits given

[Phosphate] / mM	Sample	A / $10^{-8}m^2V^{-1}s^{-1}$	C / $10^{-8}m^2V^{-1}s^{-1}$	Correlation Coeff.
20	Batch 1 Random	4.8±0.2	−0.72±0.13	0.998
20	Batch 2 Random	5.5±0.3	−0.68±0.11	0.997
20	Blockwiswe	6.3±0.5	−0.16±0.11	0.994
20	All Data	5.3±0.4	−0.64±0.19	0.979
50	Batch 1 Random	4.6±0.4	−0.56±0.20	0.998
100	Batch 1 Random	3.9±0.5	−0.22±0.08	0.997
100	Batch 2 Random	3.3±0.5	−0.20±0.23	0.974
100	Blockwise	4.5±0.3	0.07±0.15	0.996
100	All data	3.9±0.5	−0.10±0.21	0.940

Although, intuitively it may be expected that the linear fits to the data should pass through the origin, this is clearly not the case, and the apparent intercept of the linear fit varies with ionic strength. An apparent non-zero intercept has also been found in a study of pectin conductivity (Thibault & Rinaudo, 1985), which is directly proportional to mobility, when plotted as a function of z. This behaviour is due to the increasing importance of intramolecular hydrodynamic interactions as the inter-residue charge-spacing decreases below the Debye screening length (Manning, 1981). Under these conditions μ is not a simple linear function of z, and the dependence of μ on the Debye screening length gives rise to an ionic strength dependence.

Comparison between the results obtained in 20, 50 and 100 mM BGE solutions showed that the 50 mM BGE was the most suitable for the analysis of pectin. The 20 mM BGE gives the greatest electromigration dispersion, and has limited buffer capacity when injecting pectin solutions at concentrations of 5 mg ml^{-1}, equivalent to a residue concentration of ~20 mM. The current in the 100 mM produces heating effects that restrict the separation voltage to a maximum of 10 kV, leading to increased analysis times.

To investigate the use of the method in quantitative analysis, peak areas normalized for differences in migration times (Goodall *et al.*, 1991) were studied as a function of pectin concentration. Plots of normalised peak area vs concentration for all pectins studied were linear over the range of 0.5–5 mg ml^{-1} examined. Figure 3 is an example of such a plot, and gives a comparison between pectins of DE 31.1 and 70.3%. The concentration limit of detection (LOD) was found to be approximately 0.5 mg ml^{-1}, corresponding to ~50 ng injected on capillary. This relatively unfavourable LOD is a consequence of the low UV absorption coefficient and it is possible that it could be improved by derivatisation as discussed in the Introduction.

The lower normalised peak area for the higher DE pectin correlates with the higher UV transmission measured in separate experiments using direct UV spectrophotometry. This suggests that calibration standards of different DE pectins will be required when conducting quantitative analysis over a range of DE values.

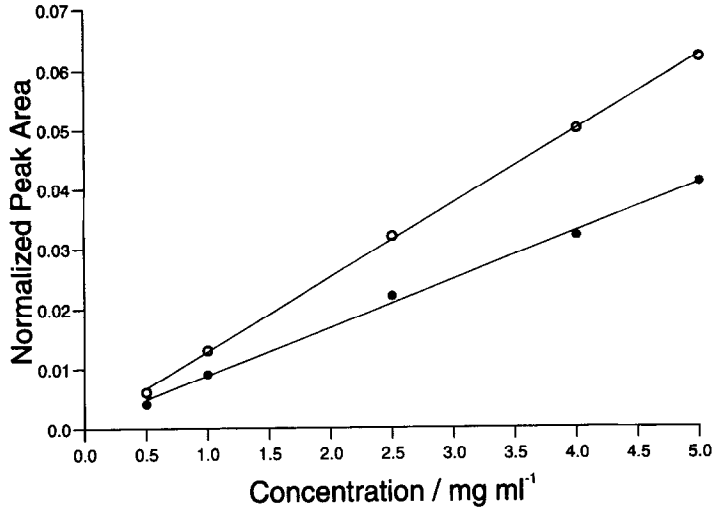

Fig. 3. The concentration dependence of the normalized peak areas obtained from 31.1% (○) and 70.3% (●) DE pectins.

CONCLUSIONS

It has been demonstrated that capillary electrophoresis provides a simple, rapid method for the quantitative detection and separation of pectins having different degrees of esterification. This technique also has the potential of quantifying the charge to size polydispersity directly. It is clear that the use of CE has a great deal of potential in the study of pectin. Further work is in progress modelling the electrophoretic mobility as a function of ionic strength and DE and carrying out more detailed experimental investigations of the effect of different charge distributions and neutral sugar contents. In addition separations are being optimized with particular regard to the development of separation strategies for use with mixtures of pectins and other food biopolymers, for example gelatin. CE could also be used to probe ion binding and intramolecular charge distribution in low DE pectins by investigating the effect of divalent metal ions on electrophoretic mobility.

ACKNOWLEDGEMENTS

This work was supported by a MAFF/DTI LINK programme, "The Behaviour Of Biopolymer Mixtures in Structuring Food Products". R.D.K. wishes to thank EPSRC and Nestlé for a CASE award.

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