ELSEVIER

Carbohydrate Research 273 (1995) 93–98

CARBOHYDRATE
RESEARCH

Note

Sequential structure by ^{1}H NMR as a direct assay for pectinesterase activity

Anne Kath Andersen, Bjørn Larsen, Hans Grasdalen *

Division of Biotechnology, Norwegian Institute of Technology University of Trondheim, N-7034 Trondheim-NTH, Norway

Received 21 October 1994; accepted 14 February 1995

Keywords: Pectinesterase; Sequential structure; NMR; Polygalacturonic acid; Enzyme assay

The polysaccharide pectin is composed primarily of linear polymers of partially esterified (1 → 4)-linked α-D-galactopyranosyluronic acid. This regular structure is interrupted with L-rhamnopyranosyl units with side chains containing neutral sugars [1]. The methyl ester is easily released in alkaline and acidic media resulting in a totally random sequential structure, whereas enzymic de-esterification is generally considered to generate a sequential block structure [2].

The degree of esterification (d.e.) can be determined by physico-chemical methods [3], whereas the distribution of esterified and free carboxyl groups along the polymer chain has been more difficult to characterize. Enzymic methods [4] and studies of calcium binding [5] have indirectly provided some knowledge about this distribution. In pectins containing free carboxyl groups arranged in blocks of a minimum lengths of ~ 10, the calcium binding is strongly cooperative. Furthermore, the use of electrophoresis to separate enzymic products should be mentioned [6]. In partly de-esterified pectin, a heterogeneous nature of the products typical for block de-esterification was observed.

NMR spectroscopy is a useful method for characterizing the composition and sequence of units in copolymers [7–9]. In this study, 600 MHz ^{1}H NMR spectra are shown which differentiate between diads, triads and some tetrads in partly esterified poly(D-galacturonic) acid, yielding unique information about the sequential structure. We have found that NMR spectroscopy can be used as a direct assay for pectinesterase activity, measuring and characterizing the block de-esterification. In particular, the NMR assay provides new possibilities to check if the enzyme activity is sequence dependent.

---

* Corresponding author.

SSDI 0008-6215(95)00100-X

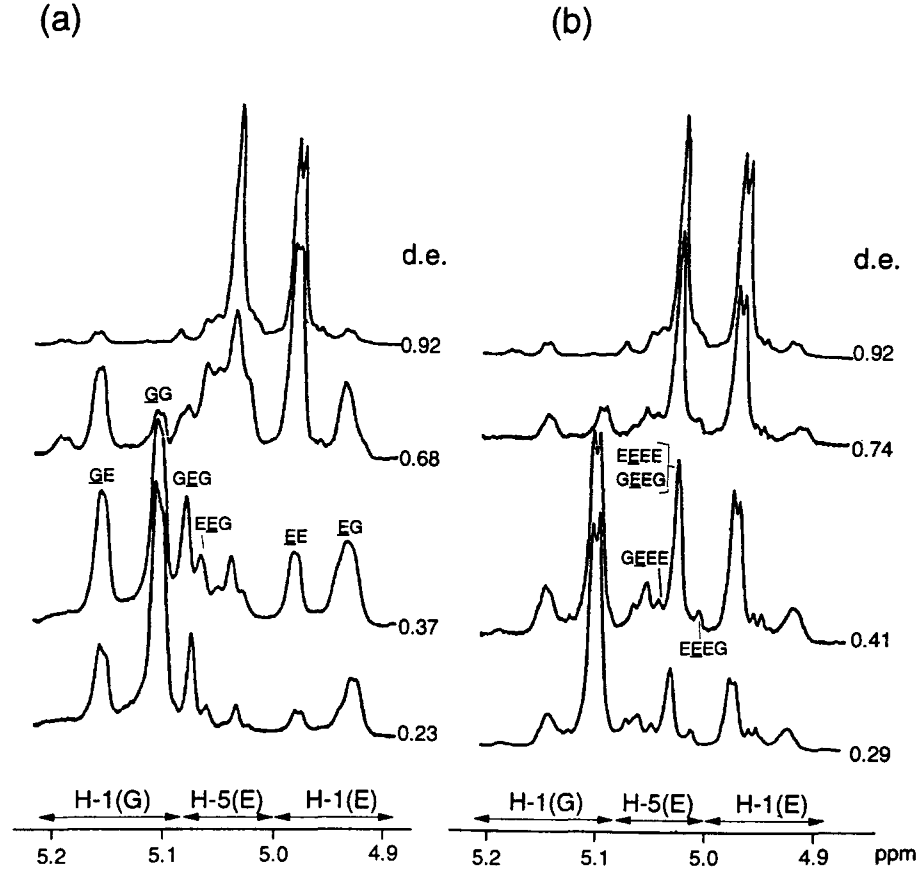

Fig. 1. The lower field region of H-1 in esterified (E) and de-esterified (G) units, and H-5 in E-units in the ^{1}H NMR spectra of pectin with various d.e. values resulting from: (a) Alkaline de-esterification; (b) Enzymic de-esterification. *E*G, G*E*G, and G*E*EE denote an E-unit followed by a G-unit (counting left to right), an E-unit having a G in both neighbouring positions, and an E-unit followed by two neighbouring Es and a G as its left-hand neighbour, respectively. Other diads, triads and tetrads are defined in an analogous manner. The interpretation of the different lines is indicated by underlining the units involved. The vertical scale is different for the spectra.

The lower field region of protons H-1 in esterified (E) and de-esterified (G) residues and H-5 in esterified residues in the 600 MHz ^{1}H NMR spectra of partly esterified poly(D-galacturonate) with various d.e. values is shown in Fig. 1. Chemical shifts are dependent on esterification and on the nature of the neighbouring units. Distinguishable line patterns arise in the NMR spectra caused by different d.e. values and sequential arrangements of free and methyl esterified carboxylic groups along the polymer chains.

The composition and the Bernoullian (random) distribution in alkali treated samples (Fig. 1a) provide keys to the assignments shown in the spectra. By using high-field NMR, the increased resolution allowed our previous sequential structure studies [7] to be extended to include all the triads and some tetrads. The assignments of the lines are

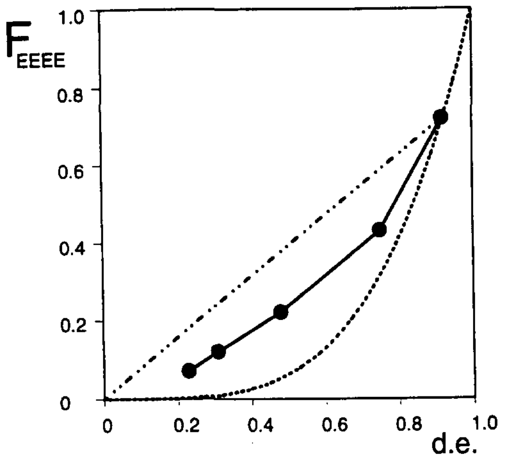

Fig. 2. Experimental probabilities (●) for the tetrad frequency F_{EEEE} in pectins, compared to calculated Bernouillian tetrad probabilities (dotted lines) and simulated ones for an ideal enzymic single chain mechanism (dashed-dot lines), respectively (see text).

those of Rees et al. [10] and Rinaudo et al. [11] for protons in contiguous residues containing no ester groups and those of Grasdalen et al. [7] and Westerlund et al. [12] for protons in partly esterified polymer. The spin–spin splitting, $J_{H1\text{-}H2}$ 3 Hz, of the resonances at 5.10 and 4.98 ppm, confirms that they arise from H-1 (Fig. 1) as pointed out by Andersen [13] and Westerlund et al. [12] based on a COSY spectrum. These lines were provisionally assigned to H-5 resonances in our previous study. However, the coupling $J_{H5\text{-}H4}$ is only 1.5 Hz and H-5 appears as a single line at 5.04 ppm in the esterified polymer.

A block-type distribution in the enzyme treated samples is indicated by stronger lines in the spectra corresponding to contiguous arrangement of esterified and de-esterified units denoted by EE, EEEE, and GG, at 4.98, 5.03, and 5.10 ppm, respectively, and corresponding weaker lines from residues characterizing block transitions, EG at 4.92 ppm and and GE at 5.15 ppm, as shown in Fig. 1b.

Experimental probabilities for the tetrad frequency F_{EEEE} and the triad frequency F_{GGG} evaluated from the areas of the respective peaks in the NMR spectra, are shown in Figs 2 and 3, respectively. The contribution to the line at 5.03 ppm from the GEEG sequence was taken into account by evaluating its intensity from the lines EEG and EEEG by using the relationship $F_{GEEG} = F_{EEG} - F_{EEEG}$. ($F_{GGG}$ was obtained from H-5 resonances in de-esterified units, as shown in ref. [7].)

The dotted lines represent the frequencies given by Bernoullian triad and tetrade probabilities calculated by [7]:

$$F_{GGG} = (1 - \text{d.e.})^3 \qquad (1)$$

$$F_{EEEE} = (\text{d.e.})^4 \qquad (2)$$

The dashed-dot lines represent upper limits for the frequencies as simulated for an ideal enzymic single-chain mechanism assuming that the enzyme attacks in alternating

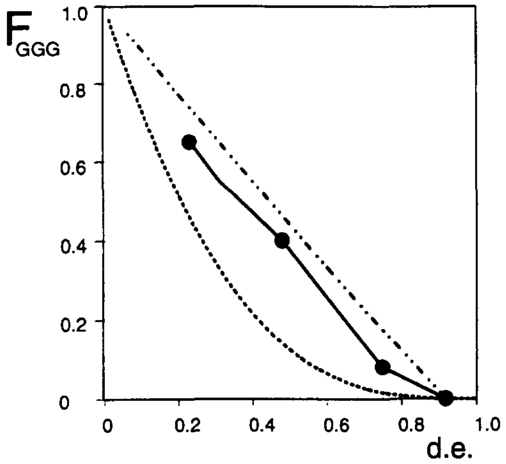

Fig. 3. Experimental probabilities (●) for the triad frequency F_{GGG} in pectins, compared to its calculated and simulated values for structural cases as explained in Fig. 2.

sequences and de-esterifies linearly until it reaches a de-esterified unit or the end of the chain [14]. Simulation for an ideal multiple-chain mechanism where the enzyme converts a single unit for each attack yields a similar linear dependence on d.e. [14].

The advantage of using NMR spectroscopy to study the mode of action of pectinesterase is obvious. The NMR assay provides new possibilities to measure and check if the enzymic activity is sequence dependent.

Evidently, at the present conditions, enzymic de-esterification of esterified poly(D-galacturonic) acid deviates somewhat from the idealized simulated block-producing mechanisms. The resulting sequential structure, averaged over the total molecular population, evidently contains some Bernoullian character. Several reasons for this can be suggested. It has been assumed that the enzyme reacts with a considerable lower rate, if at all, with oligomers smaller than ~ 10, and it might be possible that esterified blocks in the polymer chain also must have a certain minimum length for fully activating the enzyme. Another reason could be that the enzyme may attack within long esterified blocks, even if the probability for this has been demonstrated to be very low [15]. A slight saponification might also occur depending on the pH control, but we believe this to play a minor role in the present study.

1. Experimental

Materials and methods.—Poly(D-galactopyranosyluronic) acid (Fluka) was esterified to d.e. ~ 0.92 with methanolic sulfuric acid and used as a substrate in tomato pectinesterase (EC.3.1.1.11) induced de-esterification. The pectinesterase was purchased from Sigma Chemical Co. Alkaline de-esterification was used to obtain pectins with a random sequential structure.

The pectin was prepared for esterification by dissolving in water (5 mg/100 mL) and titrating with 0.1 M NaOH until dissolved and the pH value had stabilized around 7.5. After freeze-drying, this material (5 g) was mixed with methanolic 2 M H_2SO_4 (800 mL) at 4°C and stored at this temperature for about 20 days. During the esterification process, the methanolic sulfuric acid was replaced three times. Highly esterified pectin was collected by filtration and washed twice with cold methanolic sulfuric acid, twice with MeOH, then with 3:1 MeOH–(ion free)water. All solutions were kept cold. The residue was dissolved in ion-free water and pH was quickly adjusted to 4.0 and freeze-dried.

Alkali de-esterified samples were obtained by dissolving esterified pectin (200 mg) in distilled water (20 mL). The selected value pH was adjusted in the range 8–12 by addition of 0.1 M NaOH and kept at this value by titration with 0.01 M NaOH at room temperature. Adjustment of pH to 4.0 by titration with 0.1 M HCl stopped the de-esterification. All samples were freeze-dried.

Enzymic de-esterification was carried out at room temperature in solutions containing highly esterified pectin (1 g) in 0.5 M phosphate buffer 0.05 M NaCl (100 mL) at pH 6.5. The reaction was initiated by adding pectinesterase (3 mg) to pectin-buffer solution (100 mL, corresponding to ~200 Units/100 mL according to specification and definition given in the product catalogue from Sigma Chemical Co. 1994, pp 775–776). Samples (10 mL) were consecutively collected every 5 min and transferred to tubes containing 0.5 M HCl (2 mL) to adjust pH to ~4. The enzyme reaction was stopped by boiling the samples for 5 min followed by cooling on ice. The samples were dialysed towards distilled water for 2 days and freeze-dried.

NMR samples were freeze-dried twice with D_2O (7–8 mg pectin in 2 mL and 1 mL D_2O (99.6%), respectively) to remove most of the protons. Then the samples were dissolved in 5 mm tubes at pD 5 (7–8 mg/0.7 mL 99.96% D_2O). Triethylene tetraaminehexaacetic acid was added (10 mM) to prevent traces of divalent ions from interacting with pectin. 600 MHz ^1H NMR spectra were recorded at 90°C on a Bruker AMX 600 spectrometer.

Acknowledgements

We thank SPECTROSPIN AG, and Dr D. Moskau for measurements of NMR spectra on a Bruker AMX 600 spectrometer. One of the authors (A.K.A.) acknowledges a research fellowship given by The Research Council of Norway.

References

[1] G.O. Aspinall, *Adv. Carbohydr. Chem. Biochem.*, 24 (1969) 333–380.

[2] L. Rexová-Benková and O. Markovic, *Adv. Carbohydr. Chem. Biochem.*, 33 (1976) 323–385.

[3] L.W. Doner, *ACS Symp. Ser.*, 310 (1986) 13–21 and refs therein.

[4] C.E. Tuerena, A.J. Taylor, and J.R. Mitchell, *Carbohydr. Polym.*, 2 (1982) 193–203.

[5] R. Kohn, G. Dongowski, and W. Bock, *Nahrung*, 29, (1981) 75–85.

[6] A.M. Henfrey, Ph.D. Thesis, University of Leeds, 1973.

[7] H. Grasdalen, O.E. Bakøy, and B. Larsen, *Carbohydr. Res.*, 184 (1988) 191.
[8] B. Casu, in E.D.T. Atkins (Ed.), *Polysaccharides: Topics in Structure and Morphology*, VCH Publishers, Deerfield Beach, 1985, pp 9–17.
[9] H. Grasdalen, *Carbohydr. Res.*, 118 (1983) 255–260.
[10] D.A. Rees and A.W. Wight, *J. Chem. Soc. B* (1971) 1366–1372.
[11] M. Rinaudo, G. Ravanat, and M. Vincnedon, *Makromol. Chem.*, 181 (1980) 1059–1070.
[12] E. Westerlund, P. Åman, R. Andersson, and R.E. Andersson, *Carbohydr.Polym.*, 15 (1991) 67–78.
[13] A.K. Andersen, M.Sc. Thesis, University of Trondheim, 1990, pp 46–54.
[14] C.T. Greenwood and E.A. Milne, *Adv. Carbohydr. Chem. Biochem.*, 23 (1968) 281–266.
[15] J. Solms and H. Deul, *Helv. Chim. Acta,* 7 (1955) 321–329.