ELSEVIER

Carbohydrate Research 289 (1996) 105–114

CARBOHYDRATE
RESEARCH

NMR spectroscopy studies of the action pattern of tomato pectinesterase: generation of block structure in pectin by a multiple-attack mechanism [1]

Hans Grasdalen *, Anne Kath Andersen, Bjørn Larsen

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

Received 25 July 1995; accepted in revised form 21 March 1996

Abstract

The enzymatic de-esterification of highly esterified poly(D-galacturonate) (degree of esterification, d.e. ≈ 0.92, $dp_n \approx 15$) was monitored by ^1H NMR spectroscopy. The enzymatic reaction resulted in a high content of homogeneous triads (GGG and EEE) demonstrating the production of a sequential structure. At high d.e. values, the enzymatic activity increased in proportion to the content of GE-diads. A multiple-attack mechanism was indicated by a relatively slow decrease of single G's (EGE) in the sequential structure. Production of EGG-triads in great preference to GGE-triads pointed to a degree of multiple-attack greater than the effective number average block length of E-residues, ≈ 7–8, and, interestingly, the residue at the reducing end was de-esterified faster than that at the non-reducing end. These findings support the assumption that the enzyme attacks in alternating sequences (-GE-) and de-esterifies linearly preferentially towards the reducing end. Simulation-computed results are reported to demonstrate the difference between multiple-attack and multiple-chain mechanisms. © 1996 Elsevier Science Ltd.

Keywords: Pectinesterase; Pectin; Sequential structure; Mode of action; Polygalacturonic acid

1. Introduction

The enzymatic de-esterification of esterified galacturonate residues in the main chain of pectin is a process of importance with respect to fruit and vegetables growing and

---

* Corresponding author.

[1] Presented in part at The First International Conference on Polysaccharide Engineering, Trondheim, Norway, June 1994.

PII S0008-6215(96)00079-1

ripening [1]. Several pectin pectyl-hydrolases exist with somewhat different action patterns [2]. However, most pectinesterases are known to produce a sequential block structure [2]. Possibly, the most conclusive proof of the mechanism of this type of pectinesterase was demonstrated by Solms and Deuel (1955) working on an enzyme preparation from orange peel [3]. They found that the enzymatic activity increased in proportion to free carboxyl groups introduced randomly by alkaline de-esterification down to d.e. ≈ 0.4. A dramatic reduction in the de-esterification rate was observed by using an enzymatically de-esterified sample as a substrate in which the number of block transitions were much lower. Evidently, the distribution of free carboxyl groups along the polymer chains affects the rate of enzymatic attack by pectinesterase. The active site on the polymer where the esterase could attack was considered to consist of a de-esterified unit (G) in the neighbourhood of an esterified one (E), i.e. all transitional sequences like GE.

Several methods to determine the distribution of free carboxyl groups in pectins have been used. However, the information has been qualitative. Recently, NMR spectroscopy was introduced as a useful method to obtain quantitative information on the composition and sequential structure of pectin in terms of molar fractions of monads, F_E and F_G, diads, F_{EE}, F_{EG}, F_{GE}, and F_{GG}, and triads, F_{GGG}, F_{EGG}, F_{EGE}, F_{GGE}, F_{EEE}, F_{GEE}, F_{GEG}, and F_{EEG}, respectively [4–6]. In alkali de-esterified pectin, these parameters corresponded to a Bernoullian sequential distribution [4], whereas in enzymatically de-esterified pectin, a block structure was produced as verified by a high content of homogeneous triads [7]. NMR Spectroscopy represents a direct approach to verify the sequential structure, and only a moderate degree of polymer degradation is needed, i.e. the molecules might be considered as intact.

Three main types of enzymatic action patterns leading to a copolymer block structure have been recognized: (i) *Multiple-chain* mechanism, where the enzyme–substrate complex dissociates after each reaction resulting in conversion (de-esterification) of just one residue for each attack [8], (ii) *Single-chain* mechanism, where an enzymic attack is followed by a contiguous conversion of all residues in an E-block starting with the E-residue in the attacked GE-diad [8], and (iii) *Multiple-attack* mechanism, where the enzyme copes with a limited average number of residues for every active enzyme–substrate encounter [9]. In the latter case, the average number of units de-esterified per productive enzyme–substrate association has been defined as the degree of multiple-attack [9]. As indicated by the present work, the latter mechanism is probably also responsible for the block structure in pectins after de-esterification by pectinesterase.

The present paper reports on the elucidation of the developing sequential distribution of free carboxyl groups in enzymatically de-esterified pectin in terms of G-centred triads by use of NMR spectroscopy. The results yield, in addition to the fine structure of the pectin, information about the mode of action of the enzyme.

2. Experimental

Materials and methods.—Poly(D-galactopyranosyluronic) acid (Fluka) was esterified to d.e. ≈ 0.92 with methanolic sulfuric acid as described [7] and used as a substrate in de-esterification reactions induced by tomato pectinesterase (E.C. 3.1.1.11) purchased from Sigma. Alkaline de-esterification was used to obtain pectins with a random sequential structure. Experimental procedures including 600-MHz ^{1}H NMR spectroscopy have been fully described previously [7].

Computational methods.—Monte Carlo simulation of enzymatic de-esterification processes was performed by using a linear array as a model for a very long linear chain containing n_0 monomer units. A polydisperse population of oligomers with a certain $\mathrm{dp_n}$ and d.e. having a random sequence was established by using the random number generator to generate random numbers n between 1 and n_0 to indicate the location of $n_0/\mathrm{dp_n}$ chain scissions and appropriate numbers of de-esterified units, respectively. For an enzymatic reaction, the generated numbers were tested against the sequential structure. If n was equal to a number of an esterified unit having a de-esterified neighbouring unit on the opposite side to the reducing end, a sequential de-esterification was simulated towards the reducing end until a de-esterified unit or the end of the oligomer chain was reached (single-chain mechanism). At suitable decrements in d.e., the numbers of de-esterified and esterified units within the chains were counted and divided into classes dependent on their nearest neighbours. The relevant sums were divided by the total number of counted units to give d.e. and the corresponding fractions of triads. Because the units at both molecular ends do not contribute to the triad frequencies, they were omitted. Their NMR lines are also in most cases separated from those originating from units within the chain.

3. Results and discussion

The spectral region of protons H-5 in G-residues and H-4 in G- and E-residues in the 600-MHz ^{1}H NMR spectra of partially alkali de-esterified poly(D-galacturonate) with d.e. ≈ 0.44 and ≈ 0.27, respectively, is shown in Fig. 1A. Chemical shifts are dependent on the nature of the neighbouring units, yielding four distinguishable lines for H-5, one for each of the G-centred triads denoted by GGG, EGG, GGE, and EGE, at 4.69, 4.66, 4.65, and 4.64 ppm, respectively, and two lines for H-4, one for G (4.45 ppm) and one for E (4.50 ppm). The relative line intensities in the spectra from alkali treated samples reflect the relative occurrence of triad frequencies in samples having a Bernoullian (random) distribution of free carboxyl groups along the polymer chains (Fig. 1A) and confirm our previous assignment [4]. The increased resolution achieved at high-field enabled all four G-centred triads to be measured directly. The corresponding region in spectra of samples with different d.e., having a random sequence at d.e. ≈ 0.92 and where the d.e. has been successively reduced by enzyme from ≈ 0.92 to ≈ 0.32, is shown in Fig. 1B. The fractional contents of G-centred triads are particularly well suited as structural reporter parameters as far as the mechanism of the de-esterification is concerned. Production of a block structure enhances the F_{GGG} fraction compared to the Bernoullian value as demonstrated in the spectra, where the intensity of the GGG-line dominates for the enzymatically de-esterified samples, whereas the EGE-line actually decreases monotonously with d.e.

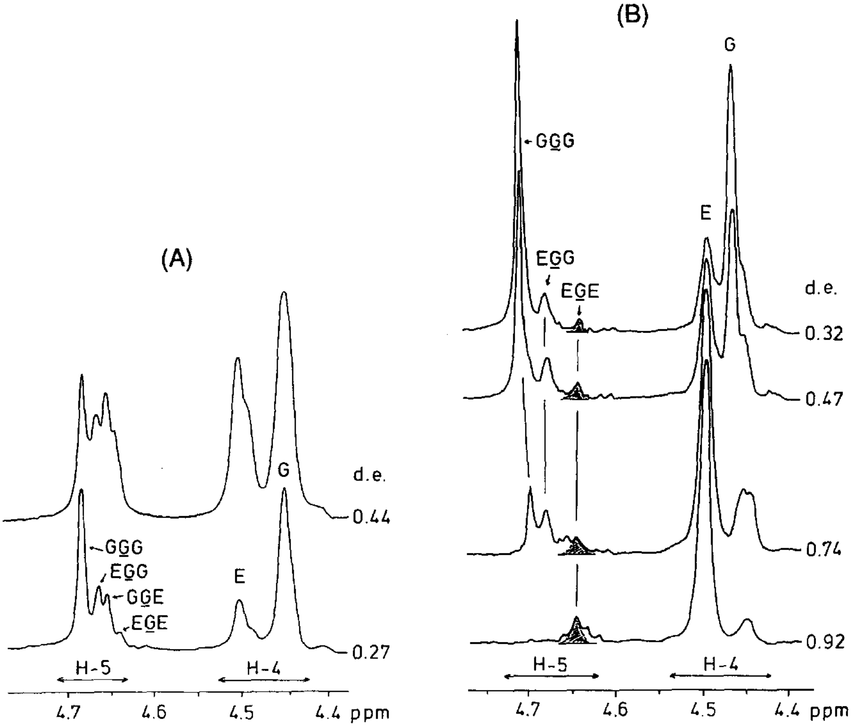

Fig. 1. The spectral region of H-5 in de-esterified (G) units, and H-4 in E and G units in the 600-MHz ^{1}H NMR spectra of poly(D-galacturonic) acid with various d.e. values resulting from: (A) alkaline de-esterification, (B) enzymatic de-esterification. $G\underline{G}G$ and $E\underline{G}E$ denote a G-unit in between two G's and two E's, respectively, and $G\underline{G}E$ and $E\underline{G}G$ denote a G-unit having an E-unit on its right hand and a G-unit on its left hand side, and vice versa, respectively. The interpretation of the different lines is indicated by underlining the units involved. The vertical scale is different for the spectra. The integrated area of the interesting EGE-line is hatched in (B).

Quantitatively, the values of d.e. and the triad fractions are related to the integrated intensities, I, of the spectral lines by the relationships:

$$\text{d.e.} = \frac{I_{\text{E(H-4)}}}{I_{\text{E(H-4)}} + I_{\text{G(H-4)}}}$$

$$F_{\text{GGG}} = \frac{I_{\text{GGG(H-5)}}}{I_{\text{E(H-4)}} + I_{\text{G(H-4)}}}$$

Experimental probabilities for F_{GGG} and F_{EGE} as a function of d.e. are shown in Fig. 2 and Fig. 3, respectively. Simulation-computed values are shown for comparison. This type of plot (progress curve of enzyme copolymer sequential structure), introduced recently [7], offers a great advantage in that it allows to characterize the enzymatic mode independently of enzyme kinetics. As demonstrated in Fig. 2, a single- and a multiple-chain mechanism yield almost similar values of F_{GGG}, and, as reported very recently [7],

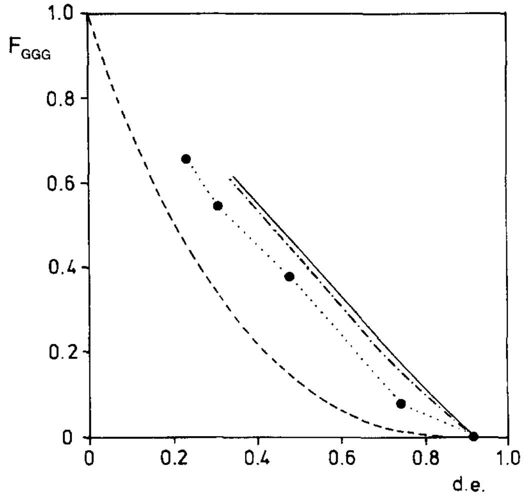

Fig. 2. Experimental probabilities (●) for the triad frequency F_{GGG} versus the degree of esterification, d.e., in partially esterified poly(D-galacturonic) acid de-esterified by pectinesterase, compared to calculated Bernoullian triad probabilities (dashed lines) and simulated ones for an ideal enzymatic single-chain (fully drawn lines) and multiple-chain mechanism (dash-dotted lines), respectively (see text). $dp_n = 15$.

the fractional content of GGG lies experimentally fairly close to the predicted results for block de-esterification. However, as shown in Fig. 3, the behaviour of the triad frequency F_{EGE} is very dependent on the mode of attack. Random attack induced by an alkaline treatment of a highly esterified sample results in a fairly steep initial increase of F_{EGE} due to a high probability for attack within long E-blocks. On the contrary, enzymatic attacks in alternating GE-sequences along the chain will always lead to a reduction in F_{EGE}. Interestingly, its initial decay versus d.e. is evidently much steeper for a multiple-chain compared to a single-chain mechanism. In the latter case, a single-attack in a GE-sequence, mostly represented by EGE-triads in poly(D-galacturonic) acid esterified randomly to d.e. ≈ 0.92, results in a decrease in d.e. equal to the fractional number of units in an averaged block length of E-residues, whereas a maximum of two EGE-sequences disappears, one at each end of the de-esterified block. On the other hand, for a multiple-chain attack, the initial decrease in F_{EGE} and d.e. at high d.e. values should not be very different since just one residue is converted per attack. The fully drawn line in Fig. 3, representing an ideal single-chain mechanism assuming that the enzyme attacks in alternating sequences, gives an almost perfect fit, whereas the dash-dotted line representing an ideal multiple-chain mechanism with a similar site of attack does not fit at all.

It should be noted that at a random initial d.e. of ≈ 0.92 and $dp_n \approx 15$, a significant fraction of substrate molecules is fully esterified and consequently completely protected from de-esterification by the mechanisms considered here. In addition, all units between the non-reducing end and the de-esterified unit nearest to this end become untouched due to the assumed polarity of the enzymic reaction towards the reducing end. This is the reason why the simulated curves stop at d.e. ≈ 0.4. Experimentally, the de-esterifica-

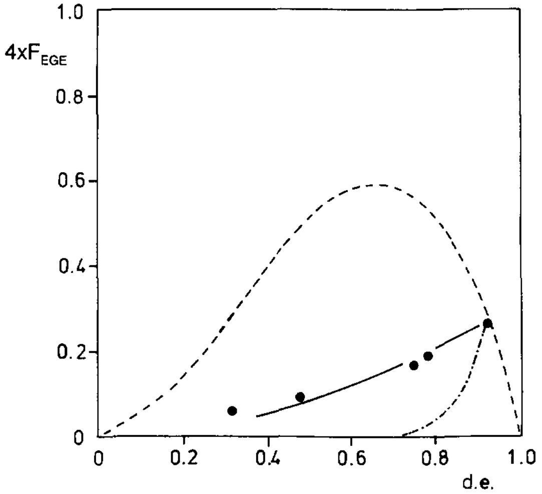

Fig. 3. Experimental probabilities (●) for the triad frequency F_{EGE} versus the degree of esterification, d.e., in partially esterified poly(D-galacturonic) acid de-esterified by pectinesterase. Calculation, simulation and symbols as in Fig. 2.

tion was recorded down to about 0.2–0.3. However, the reaction rate was slow at low d.e. values and was probably caused by side reactions as discussed below.

In the spectra from the enzymatic de-esterified samples, one of the unsymmetrical G-triads, EGG or GGE, is scarcely visible. This might be explained by assuming that the substrate molecules accessible to the enzyme consist of relatively short chains, $dp_n \approx 15$. They are highly esterified and contain on average one to three alternating sequences each, predominantly EGE-sequences randomly distributed along the molecule. It means that most of the E-blocks are initiated or terminated by the ends of the molecule. Therefore, in a single-chain or a multiple-attack mode with a degree of multiple-attack greater than the effective number average block length of E-residues, $\bar{N}_E$, once the enzyme forms an active enzyme–substrate complex, it catalyzes de-esterification in a zipper fashion and converts all residues toward the end of the molecule. An EGG- or a GGE-sequence is thereby preferably created, depending on whether the reaction occurs toward the reducing or the non-reducing end, respectively.

Some quantitative information about the degree of multiple-attack might be obtained by estimating the effective number average block length of E-residues at conditions whereby the enzymatically produced fractions of the unsymmetrical triads EGG and GGE are significantly different. A prerequisite for this to happen is that a significant amount of E-blocks are terminated by the end of the molecule as mentioned above. In our case, where a very high fraction of substrate molecules contains just one acid residue per molecule randomly distributed along the molecular chain, the effective $\bar{N}_E$ should be reduced to a value of about $dp_n/2 \approx 7$–8. The corresponding value for a long chain is given by $\bar{N}_E = \text{d.e.}/F_{EG} \approx 13$. Hence, for degrees of multiple-attack lower than 7, it is reasonable to assume that both triads, EGG and GGE, should have shown up in the

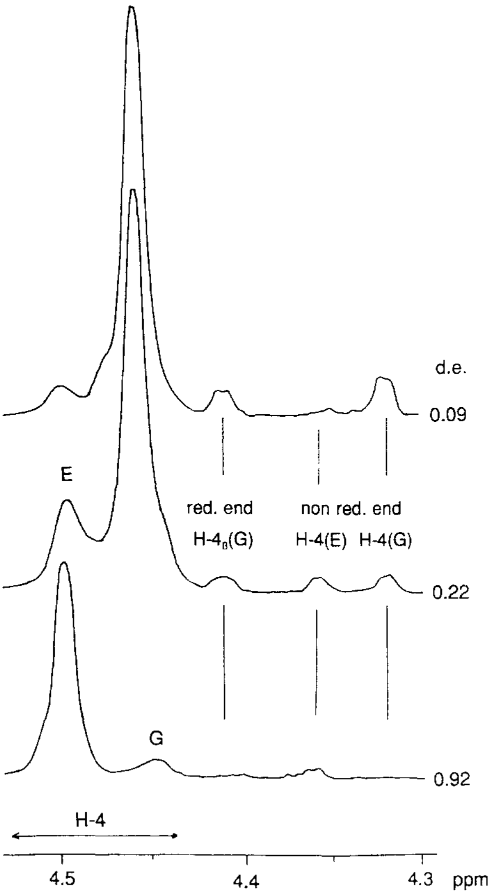

Fig. 4. The spectral region of H-4 protons in E- and G-units within the chain and at the non-reducing end, and of the H-4 proton in de-esterified β anomers at the reducing end in partially esterified poly(D-galacturonic) acid. Random esterification at d.e. ≈ 0.92 and subsequently enzymatic de-esterification to d.e. ≈ 0.22. Top spectrum: random esterification at d.e. ≈ 0.43 and subsequently enzymatic de-esterification to d.e. ≈ 0.09.

spectra. The presence of only one of the two triads in a significant amount indicated that the degree of multiple-attack may be greater than 7.

Although we intuitively assume a multiple-attack mechanism to be the most probable one for pectinesterase, a single-chain mechanism can not be ruled out by the present data. Higher values for d.e. and dp_n would help in this respect.

It is possible to prove the polarity of the pectinesterase by monitoring the de-esterification of the reducing and non-reducing end residues. In the spectral region of proton H-4 in Fig. 4, we see lines from H-4 in esterified (4.36 ppm) [10] and de-esterified units (4.32 ppm) [11] at the non-reducing end and from H-4 in de-esterified β anomers at the reducing end (4.41 ppm) in addition to H-4 protons within the polymer chain. In the bottom spectrum from the sample with a randomized sequence at d.e. ≈ 0.92, both end residues are highly esterified and just one of their resonances is visible at 4.36 ppm in

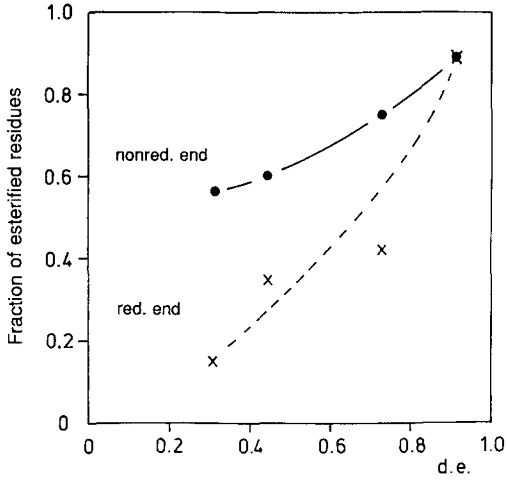

Fig. 5. Fractional contents of residues at the non-reducing (●) and reducing end (×) that are esterified versus the degree of polymer de-esterification, d.e., by pectinesterase.

Fig. 4. The increase of the de-esterified end signals and the corresponding decrease of the esterified ones during the enzymatic de-esterification (spectrum at d.e. ≈ 0.22), prove that the enzyme is able to react with both end residues. In the top spectrum from a sample de-esterified randomly to d.e. ≈ 0.43 and subsequently by enzyme to d.e. ≈ 0.09, the resonances show that both end residues are almost fully de-esterified. The $\alpha:\beta$-anomeric ratio was measured to be ≈ 0.56 using intensities of H-1 in de-esterified units at the reducing end. Therefore, the intensities of H-4 protons (Fig. 4) made it possible to evaluate that the de-esterification of residues at the reducing end occurred about three times faster than of those at the non-reducing end as shown in Fig. 5. The intermediate line in Fig. 1B could therefore be assigned to the triad EGG.

To check the enzymatic reaction further, a poly(D-galacturonic) acid, $\mathrm{dp}_n \approx 15$, de-esterified randomly to d.e. ≈ 0.43, was used as a substrate. All the G-triads were clearly visible by well-defined distinguishable spectral lines (see Fig. 1A). As shown in Table 1, the sequential structure obtained by alkaline de-esterification to d.e. ≈ 0.43 corresponded closely to a Bernoullian distribution with $F_{\mathrm{EGG}} = F_{\mathrm{GGE}}$. However, after de-esterification of this sample by pectinesterase down to d.e. ≈ 0.23, a significant difference between the fractions of F_{EGG} and F_{GGE}, 0.16 and 0.10, respectively, was observed. It is worth noting that the listed triad fractions simulated according to a single-chain mechanism gave a slightly better fit than those simulated for a multiple-chain mechanism, indicating the former as the operating mechanism. The latter predicted lower F_{EGE} values and a somewhat smaller difference between F_{EGG} and F_{GGE}. However, the predicted differences between the two modes of action are small, and the most conclusive proof for the mode of operation is obtained from the sequential de-esterification pattern at high d.e. values where the behaviour of the EGE-sequence offers a crucial test (Fig. 3).

As pointed out previously, the enzymatic de-esterification deviated somewhat from

Table 1
Degree of esterification, d.e., and the G-centred triad frequencies in partially esterified poly(D-galacturonic) acid obtained by alkaline and enzymatic de-esterification. Simulated triads are included for comparison

Sample treatment	F_E [a]	F_G	F_{GGG}	F_{EGG}	F_{GGE}	F_{EGE}
Alkaline de-esterification	0.43	0.57	0.18	0.14	0.14	0.11
Simulated Bernoullian distribution			0.19	0.14	0.14	0.11
Subsequent enzymatic de-esterification	0.23	0.77	0.47	0.16	0.10	0.05
Simulated single-chain attack ($dp_n = \infty$)			0.54	0.10	0.10	0.03
Simulated single-chain attack ($dp_n = 15$)			0.54	0.11	0.09	0.03
Simulated multiple-chain attack ($dp_n = \infty$)			0.53	0.11	0.11	0.02
Simulated multiple-chain attack ($dp_n = 15$)			0.53	0.11	0.10	0.01

[a] F_E = fraction of esterified residues = d.e.

an idealized simulated block-producing mechanism in the way that some GGG-triads were missing [7]. This is even more clearly demonstrated by the production of EGG-triad frequencies shown in Fig. 6. Its experimental fraction increased to a level of about three to four times the simulated value for a single-chain attack by a relatively small enzymatic de-esterification from d.e. ≈ 0.92 to ≈ 0.75, and remained approximately constant during further de-esterification. A multiple-chain attack predicted no difference between the fractions of EGG and GGE produced initially (simulated data not shown). Some experiments were therefore carried out with substrate molecules de-esterified randomly to different degrees to test if the pectinesterase from tomato, like that from orange peel, attacks in alternating sequences. The results demonstrated an

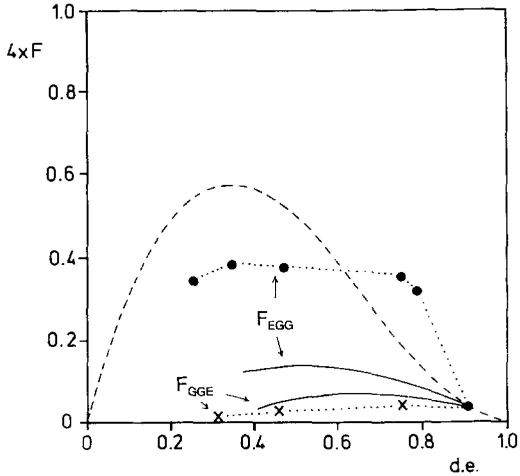

Fig. 6. Experimental probabilities for the triad frequencies F_{EGG} (●) and F_{GGE} (×) versus the degree of esterification, d.e., in partially esterified poly(D-galacturonic) acid de-esterified by pectinesterase compared to calculated Bernoullian triad probabilities (dashed lines) and simulated ones for an ideal single-chain mechanism (fully drawn lines).

increasing initial enzymatic reaction rate in proportion to 1 − d.e. (data not shown) and confirmed the similarity between the two pectinesterases.

However, according to the model of single-chain attack in transitional sequences, the produced fraction of EGG-triads should never exceed the initial value of the F_{EGE} frequency. The resulting fractional content of EGG, about 40% higher than the initial F_{EGE}, means that some new GE-transitions are created initially due to either or both of two secondary mechanisms: (i) alkaline de-esterification, or (ii) a low but significant activity for the enzyme to de-esterify fully esterified polymer chains.

Even if some deviations from an idealized mode of attack exist and can not be fully explained yet, the present results provide new, direct experimental evidence, supporting earlier suggestions that the multiple-attack mechanism operates with pectinesterase and is responsible for the block structure in partly esterified pectinic acids.

Acknowledgements

We thank SPECTROSPINN 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] W. Pilnik, F. Voragen, H. Neukom, and E. Nittner, *Polysaccharide*, in E. Bartolomé, E. Biekert, H. Hellmann, H. Ley, W.M. Weigert, and E. Weise (Eds.), *Ullmann's Encyclopädie der Technischen Chemie*, Chemie Verlag GmbH, Weinheim, 1980, Vol. 19, pp 233–263.

[2] A.J. Taylor, *Carbohydr. Polym.*, 2 (1982) 9–17.

[3] J. Solms and H. Deuel, *Helv. Chim. Acta*, 37 (1955) 321–329.

[4] H. Grasdalen, O.E. Bakøy, and B. Larsen, *Carbohydr. Res.*, 184 (1988) 183–191.

[5] A.K. Andersen, M.Sc. Thesis, University of Trondheim, 1990.

[6] E. Westerlund, P. Åman, R. Andersson, and R.E. Andersson, *Carbohydr. Polym.*, 15 (1991) 67–78.

[7] A.K. Andersen, B. Larsen, and H. Grasdalen, *Carbohydr. Res.*, 273 (1995) 93–98.

[8] C.T. Greenwood and E.A. Milne, *Adv. Carbohydr. Chem. Biochem.*, 23 (1968) 281–366.

[9] J. Robyt and D. French, *Arch. Biochem. Biophys.*, 122 (1967) 8–16.

[10] S. Cros, C. Hervé du Penhoat, N. Bouchemal, H. Ohassan, A. Imberty, and S. Pérez, *Int. J. Biol. Macromol.*, 14 (1992) 313–320.

[11] V.-M. Ló, M.G. Hahn, and H. van Halbeek, *Carbohydr. Res.*, 255 (1994) 271–284.